Betulinic Acid Induced Tumor Killing

Franziska Müllauer

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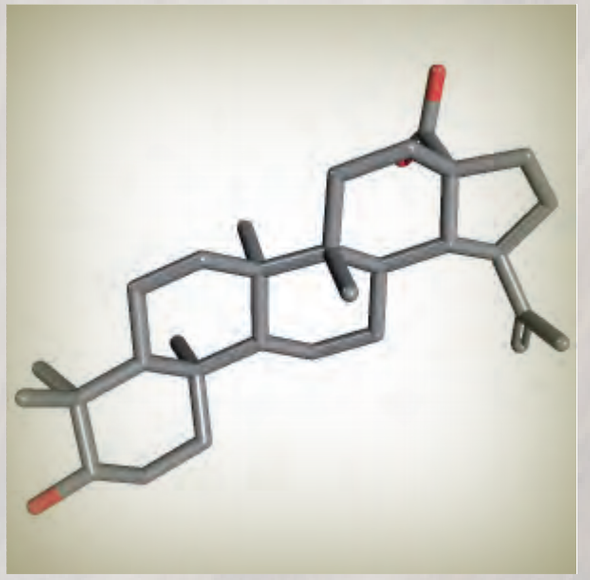

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Betulinic Acid
Induced Tumor Killing

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Contents

Chapter 1

Betulinic Acid, a Natural Compound with Potent Anti-Cancer Effects

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Abstract

New therapies employing novel mechanisms to induce tumor cell death are needed with plants playing a crucial role as a source for potential anti-cancer compounds. One highly promising class of natural compounds are the triterpenoids with betulinic acid (BetA) as the most prominent representative. *In vitro* studies have identified this agent as potently effective against a wide variety of cancer cells, also those derived from therapy resistant and refractory tumors, whereas it has been found relatively non-toxic for healthy cells. *In vivo* preclinically applied BetA showed some remarkable anti-cancer effects and a complete absence of systemic toxicity in rodents. BetA also cooperated with other therapies to induce tumor cell death and several potent derivatives have been discovered. Its anti-tumor activity has been related to its direct effects on the mitochondria.

Chemotherapies based on compounds from nature

Two prominent classes of natural compounds are the vinca alkaloids and the taxanes. Already in the late 1950s the vinca alkaloids vinblastine (Velban®) and vincristine (Oncovin®) were introduced into the clinic, later on semi-synthetic derivatives such as vindesine (Eldisine®), vinorelbine (Navelbine®) and vinflunine followed [1]. In 1963, four vinca alkaloid members isolated from *Vinca rosea* (vinblastine, vinleurosine, vincristine and vinrosidine) were reported for their anti-tumor activity [2]. Detailed investigations revealed the disappearance of microtubules and appearance of crystal structures upon vinca alkaloid treatment [3,4]. By now, the molecular anti-cancer mechanism of these compounds has been identified to be the destabilization of microtubules, which leads to G2/M arrest (by blocking mitotic spindle formation) and apoptosis [5].

The taxanes belong to the diterpenes (terpenoids) and are another class of natural compounds successfully used in the clinic. Taxol was originally discovered and obtained from the *Taxus* (pacific yew tree) in 1964 [6], and was shown in 1979 by Susan Band Horwitz to promote microtubule assembly [7]. It was approved by the FDA in 1992 for the treatment of ovarian cancer [8]. Today taxol is also approved for the treatment of various other cancer types, including lung and breast cancer. Other natural products or their analogs used as anti-cancer drugs include camptothecin, a topoisomerase I inhibitor originally obtained from *Camptotheca* [9], and the DNA-intercalating anthracyclines, which are derived from *Streptomyces* bacteria. The most prominent member of the latter one is doxorubicin, a daunorubicin derivative [10].

Many other natural compounds are under investigation as anti-cancer treatments, amongst which the triterpenoids gained much attention lately because of their highly promising results in pre-clinical studies.

Triterpenoids

Triterpenoids belong to the terpenoids (also known as isoprenoids), the largest group of natural products [11] to which the taxanes also belong (see above). These compounds consist of six isoprene units and can be isolated from many different plant sources. They occur in countless variations and can be sub-classified into several groups including squalenes, lanostanes, dammaranes, lupanes, oleananes, ursanes, hopanes, cycloartanes, friedelanes, cucurbitacins and miscellaneous compounds [12,13]. Many of them or their synthetic derivatives are currently investigated as medicinal products for various diseases, including cancer. For example 3beta,25-epoxy-3alpha-hydroxylup-20(29)-en-28-oic acid, a lupane-type triterpenoid, showed remarkable inhibitory effects in a two-stage mouse skin carcinogenesis model that was initiated with ultraviolet-B (UVB) and promoted using 12-O-tetradecanoyl-phorbol-13-acetate (TPA). The number of mice bearing papillomas was significantly reduced as was the number of papillomas per mouse in the treated group. Overall, oral administration of 3beta,25-epoxy-3alpha-

hydroxylup-20(29)-en-28-oic acid resulted in almost 50% inhibition of papilloma incidence [14]. In a different *in vivo* study similar anti-tumor effects of lupeol (Fig. 1), another lupine type triterpenoid, were observed. Pre-application of lupeol in TPA- treated mice inhibited skin tumorigenesis, and resulted in a decrease in skin edema, hyperplasia and markers of inflammation and tumor promotion. On account of its presence in many vegetables and fruits including olives, strawberries and mangos it was proposed to have potential as a dietary anti-tumor agent [15]. *In vitro* studies showed that lupeol possesses anti-tumor effects against cell lines derived from lung, prostate and pancreatic cancer, leukemia and hepatocellular carcinomas through the induction of apoptosis [16].

Also oleananes (Fig. 1) are investigated for their anti-tumor properties. Synthetic oleanane triterpenoids, for example, were shown to selectively induce apoptosis in cancer cells that are resistant to conventional chemotherapeutics, to suppress tumor cell growth and to induce differentiation of cancer cells [17]. Two of these potent synthetic oleananes, 2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oic acid (CDDO) and its methyl ester (CDDO-Me) are currently tested in phase I clinical trials [17].

From the subfamily of ursanes, ursolic acid (Fig. 1) was found to have anti-tumor effects by inhibiting expression of TNF-induced and NF-κB-regulated genes cyclin D1, COX-2 (cyclo-oxygenase) and MMP-9 (matrix metalloproteinase-9). Suppression of NF-κB activation induced by different carcinogens, inflammatory and tumor promoting agents by ursolic acid was observed in a broad range of cells [18]. Furthermore, ursolic acid inhibited STAT3 (signal transducers and activators of transcription 3) activation in multiple myeloma cells and subsequently expression of STAT3 regulated gene products, such as cyclin D1, Bcl-2, Bcl-xL, Mcl-1 and survivin. Finally, this non-toxic triterpenoid inhibited proliferation and induced apoptosis in tumor cells. Because of its presence in apples, basil, prunes and cranberries, it was suggested to have potential, not only for treatment, but also for prevention of different cancer types including multiple myeloma [19]. Another representative of the triterpenoids with anti-cancer activity is cucurbitacin B (Fig. 1) which can be found in many cucurbitacaea species [20], for example also in the stems of *Cucumis melo* (melon) [21]. Cucurbitacin B was found to have anti-proliferative activity on glioblastoma multiforme (GBM) cells [22], breast cancer [23], myeloid leukemia [24], pancreatic cancer [25], laryngeal squamous cell carcinoma, and other tumor cells [26]. It was reported to exert its anti-cancer effects via inhibition of the JAK/STAT signaling pathway [21,25].

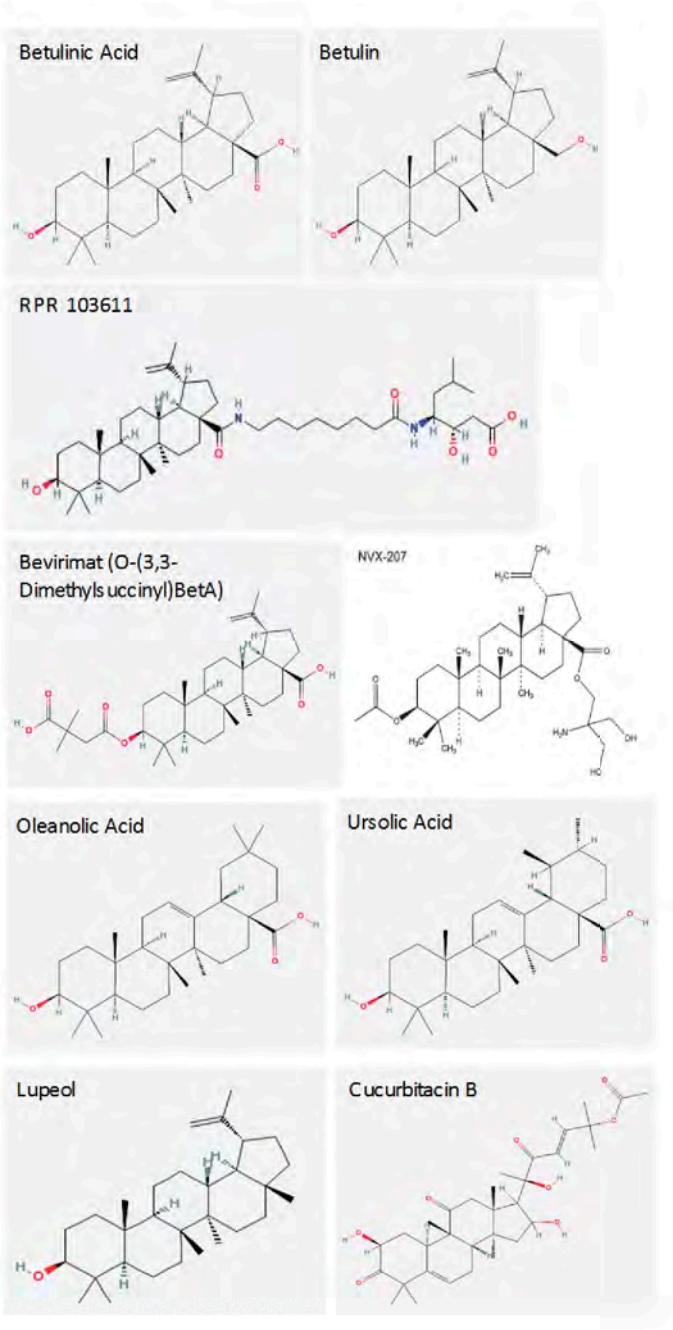

Fig. 1 Structures of various triterpenoids as published on PubChem, NVX-207as published by Willmann et al [91].

Finally, betulinic acid, which is a lupane-type triterpenoid, was selected from an extensive screen of 2500 plant extracts executed by the NCI (National Cancer Institute). The extract was prepared from the bark of *Ziziphus mauritania* Lam. and displayed remarkable cytotoxic effects against human melanoma cells in this screen. Subsequently, the active constituent was discovered to be betulinic acid [27].

Betulinic Acid

Discovery and sources

Betulinic acid (BetA, Fig. 1) is, as mentioned, a plant derived pentacyclic lupane-type triterpenoid. Betulin (Fig. 1), the reduced form of BetA was first isolated from plants in 1788 by Johann Tobias Lowitz and found to be a prominent constituent of the outer-bark of white-barked birch trees [28,29]. Both BetA and betulin are widely distributed throughout the plant kingdom. BetA has been extracted from a wide range of diverse plants, ranging from meat-eating plants like *Sarracenia flava* (Sarraceniaceae) [30] to trees and shrubs like *Diospyros* spp (Ebenaceae), *Inga punctata* (Fabaceae) [31], *Ziziphus* spp (Rhamnaceae), *Vauquelinia corymbosa* (Rosaceae) [32] and *Syzygium* spp (Myrtaceae) [29,33,34]. Owing to the high betulin content (up to 22%) in the bark of the white birch tree (*Betula alba*) the most convenient source for BetA is via a simple oxidation process from betulin isolated from this tree [33,35]. Interestingly, the white birch bark has a long tradition in folk medicine for treatment of stomach and intestinal problems used for example by native Americans and in Russia [34]. Moreover, Inonotus obliquus (Chaga mushroom), which is a parasitic fungus on Birch trees that is applied in folk medicine against cancer has been shown to contain high levels of BetA and betulin and is active against cancer cells [36]. The chemical structures of betulin and BetA differ at the C-28 position and are shown in Fig. 1.

Effects of betulinic acid against infectious diseases

Before its discovery as an anti-cancer agent BetA had already been shown to be effective against HIV via inhibition of replication [37]. A derivative of BetA (RPR 103611, Fig. 1) showed even more potency as an anti-HIV-1 agent, although at the same time it was inactive against HIV-2 [38]. A very promising BetA derivative is PA457 (bevirimat, Fig. 1), which prevents HIV-1 virus maturation and virus release from infected cells [39]. It was well tolerated in a phase I/II clinical trial as single dose administration and importantly no bevirimat resistance mutations were detected in this study [40]. Other studies, however, showed mutations in a certain region of the viral protein gag causing resistance to bevirimat [41,42]. Nevertheless, it is a highly promising candidate and currently under further investigation in HIV-1 patients in two phase II clinical trials (clinicaltrials.gov: study NCT00511368, drug: Bevirimat; study NCT00967187, drug: Bevirimat dimeglumine). Recently, other derivatives of BetA were also shown to possess anti- HIV-2 activity. Interestingly, this was achieved by a shorter C-28 side

chain and carboxylic acid terminus and it was hypothesized that optimal pharmacophores for HIV-1 and HIV-2 targeting are different [43].
BetA has also been shown to possess anti-bacterial activities, although the results are conflicting [29]. A recent study that analyzed the antibacterial effects of BetA, ursolic acid and oleanolic acid, showed that BetA was, in contrast to the other two molecules, inactive against gram-positive bacteria [44]. When it was first tested as an anti-malarial drug in a murine malaria model, it was ineffective in reducing parasitaemia [45]. Recent *in vivo* results in mice infected with Plasmodium berghei and treated with BetA-acetate suggest an anti-plasmodial activity by analysis of parasitaemia [46]. Other biological activities of BetA include anthelmintic and anti-inflammatory effects [47,48].

Anti tumor effects
In a systematic screening of 2500 plant extracts tested by the NCI, BetA was re-discovered in 1995 as a potent anti-melanoma compound. It showed *in vitro* cytotoxic activity against melanoma cell lines MEL-1 (derived from lymph node), MEL-2 (derived from pleural fluid) and MEL-4 (derived from a primary skin tumor) with IC_{50} values ranging from 0.5 -4.8 µg/ml whereas tumor cell lines from other tumor types were found to be relatively resistant in this study. The observed shrinking of cells and membrane blebbing together with the detected sub-G1 peak by flow cytometry analysis in MEL-2 cells suggested that BetA induced apoptosis. Most importantly, this study also demonstrated the *in vivo* efficacy of BetA in nude mice injected subcutaneously with the melanoma cell line MEL-2. Highly effective tumor growth inhibition was achieved by intra-peritoneal application of 50, 250 or 500 mg per kg bodyweight BetA with no signs of toxicity to the host cells. In a different setting, using MEL-1 cells, a dramatic decrease in tumor size was achieved by applying 50 mg per kg bodyweight BetA [27]. Based on these results it was selected for the RAID (Rapid Access to Intervention Development) program by the NCI [49].
Initially described to be specific against melanoma cells [27], it was subsequently established that BetA is also effective against cancer cells derived from other tumor types. The sensitivity of neuroectodermal tumor cells to BetA was established (IC_{50} for human neuroblastoma cell lines: 14 - 17 µg/ml) and for the first time the underlying molecular apoptotic pathways were studied [50,51]. It was shown that other brain tumors such as glioma cells [52], medulloblastoma and glioblastoma cell lines as well as primary medulloblastoma (IC_{50} 3 - 13.5 µg/ml) and glioblastoma (IC_{50} 2 - 17 µg/ml) cells were sensitive to BetA whereas no cytotoxic signs in murine non-malignant neuronal cells were observed [53]. In 2001, BetA was demonstrated to induce anti-proliferative effects in ovarian carcinoma (IC_{50} 1.8 - 4.5 µg/ml) , non-small and small cell lung carcinoma (IC_{50} 1.5 - 4.2 µg/ml), cervix carcinoma (IC_{50} 1.8 µg/ml) and melanoma cell lines (IC_{50} 1.5 – 1.6 µg/ml) independently of the p53 status [54]. In contrast, normal cells (human normal dermal fibroblasts and peripheral blood lymphocytes) were unaffected at the same concentrations, suggesting a tumor specific effect of BetA. The anti-neoplastic

effects of BetA were confirmed in an *in vivo* ovarian carcinoma xenograft mouse model [54]. Later on, head and neck squamous cellular carcinoma cells were also discovered to be sensitive to BetA [55]. On top of this it also has potential for treatment of hematological malignancies. Already, in 1997 it was shown that the murine leukemia cell line L1210 was sensitive to BetA in a pH and exposure-time dependent manner [56]. Importantly, further studies on acute leukemia confirmed the activity of BetA on primary hematologic malignancies. The apoptosis inducing effects of BetA were independent of patient age and sex, leukemia type and risk stratification [57]. BetA also induced apoptosis in the anti-leukemic therapy resistant human chronic myelogenous leukemia (CML) cell line K-562 (derived from the blast crisis stage) without affecting the levels of Bcr-Abl [58].
However, if BetA would also be effective against solid, prevalent tumor types including colon-, lung-, prostate- and breast cancer was not clear when the work for this thesis was started. To clarify the effect of BetA on cancer cells derived from these tumors was therefore one of the first goals for this project.

Effects of betulinic acid on healthy cells
One of the most striking features of BetA is its differential effect on cancer cells and healthy cells *in vitro*. In general, BetA is concluded to be less toxic to cells from healthy tissues. Melanoma cells were shown to be much more sensitive to BetA as compared to normal melanocytes as measured by growth analysis [59] and apoptosis [60]. Interestingly, normal human keratinocytes differentiated into corneocytes whereas the immortalized keratinocyte cell line HaCaT underwent apoptosis [60]. In addition, peripheral blood lymphocytes and human skin fibroblasts were reported to be highly resistant toward BetA [54,61]. The molecular mechanisms underlying this remarkable phenomenon remain to be elucidated. Most importantly, BetA's non toxicity toward healthy cells is conferrable to *in vivo* systems as discussed in a later section.

Betulinic acid, mechanism of action

Even though the direct molecular target(s) of BetA remain largely to be clarified it is clear that its toxic effects on cancer cells are manifold. The investigation of the exact mechanisms underlying the remarkable anti-cancer potential of BetA is still a challenge for researchers. A lot of effort has been put in the investigation of BetA-induced apoptosis, but has resulted in some conflicting results, especially with regards to the role of Bcl-2. The apoptosis inducing effects of BetA have been studied quite extensively and are discussed below. But also other pathways and targets have been suggested to be involved in BetA induced cytotoxic effects. To unravel these pathways as well as their role in BetA-induced cell death will be an extremely challenging task because of the numerous reported targets of BetA. These include enzymes (kinases, aminopeptidase N, Acetyl-CoA acetyltransferase, topoisomerase I/II), the transcription factor NF-κB as well as cell cycle regulation and the proteasome. Because of the broad anti-cancer effects of BetA it is likely that even more molecular targets of this compound will be discovered in the future. However, it is doubtful that all these molecules are specific and/or direct BetA targets. Moreover, how these interactions would all contribute to BetA-induced cell death remains to be elucidated.

Introduction Apoptosis

Apoptosis is an intrinsic program of stressed or damaged cells resulting in ’organized’ cell death. Two main pathways are distinguishable, the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway (Fig. 2). The death receptor pathway is activated by binding of a ’death ligand’ to its death receptor (e.g. CD95/APO-1/Fas-ligand binding to CD95/APO-1/Fas) belonging to the tumor necrosis factor (TNF) receptor super-family [62-64]. This leads through the adaptor molecule FADD (Fas associated death domain) to cleavage of caspase-8 and 10 [65,66]. The mitochondrial pathway is regulated by the Bcl-2 family proteins consisting of pro-survival (e.g. Bcl-2, Bcl-XL or Mcl-1) and pro-apoptotic members (Bax/Bak; BH3-only proteins). BH3-only proteins are activated by diverse signals such as cellular stress, DNA damage, death receptor activation or cytokine withdrawal. Once activated, these BH3 molecules modulate the delicate balance between the pro-apoptotic (Bax and Bak) and anti-apoptotic (Bcl-2, Bcl-XL or Mcl-1) Bcl-2 family members. This results in mitochondrial membrane permeabilization and release of cytochrome-c from the mitochondria [67]. Also p53 plays an important role in this pathway as activation of p53 can lead to the expression of BH3-only molecules Puma and Noxa [68] or it can directly transcriptionally or functionally activate Bax [69,70].

Induction of apoptosis with subsequent cell death is the goal of many anti-cancer therapies. The pathways involved, however, are complex and cancer cells often become resistant to conventional therapies through developing escape-mechanisms in the signaling cascade. These therapies usually target apoptosis either indirectly, by inducing cellular stress leading to the intrinsic activation of apoptosis for

example via p53, or otherwise upstream of the mitochondria. The latter one includes CD95L, TRAIL and other death ligands, which bind to their respective death receptors, thereby triggering apoptosis. For example the natural occurring and widely used chemotherapeutic compound taxol binds to microtubule polymers, which results in formation of depolymerization resistant microtubules. Resistance of cancer cells towards taxol involves next to alterations in tubulin [71] also changes in the apoptosis pathway. The BH3-only molecule Bim is normally bound to the LC8 dynein light chain, thereby being sequestered to the microtubule-associated dynein motor complex. Treatment with taxol (and also other apoptotic stimuli) results in translocation of Bim and neutralization of Bcl-2, thereby inducing apoptosis [72]. Deletion of Bim or over-expression of Bcl-2 in tumor cells causes resistance to taxol [73]. Other examples are DNA-damaging treatments (including gamma irradiation), causing an accumulation of the tumor suppressor protein p53. This transcription factor with additional functions in the cytosol is a central key player for numerous pathways including DNA repair, cell cycle and apoptosis. Examples of p53 targets in the apoptotic pathway are Puma, Noxa, Bax, Bak, Bcl-XL and Bcl-2. It is estimated that about half of all human tumors have acquired p53 mutations and most of the remaining ones have deactivated the p53 pathway by other means such as increasing its inhibitors or decreasing its activators [74]. As such, treatment modalities that depend on p53-dependent apoptosis are not likely to function. Therefore a new class of anti-cancer agents directly targeting mitochondria and not depending on p53 - such as BetA - holds great promise in overcoming drug-resistance in tumor cells (Fig. 2) [75-77].

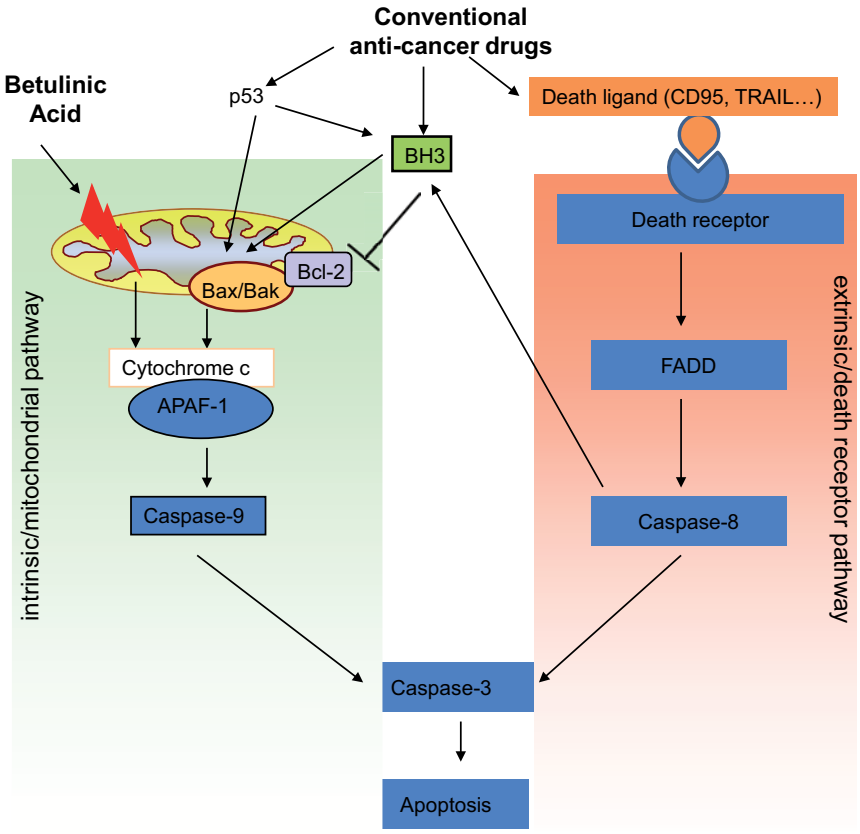

Fig. 2 Induction of apoptosis by conventional anti-cancer drugs and BetA:
Commonly used anti-cancer agents either trigger the death receptor pathway of apoptosis or induce cellular stress such as cytokine withdrawal or DNA damage. This results in activation of the apoptotic signaling cascade via p53 and/or BH3-only proteins. In contrast, BetA directly induces mitochondrial damage - leading to Bax/Bak independent release of cytochrome c - thereby overcoming resistance that a tumor cell may have acquired upstream of the mitochondria.

Betulinic acid and the mitochondria

Role of p53: In neuroectodermal tumor cells BetA-induced apoptosis was independent of p53; however an induction of p53 was reported by another group in melanoma cells [78]. No change of p53 expression levels was found in LN-229 and LN-18 cells transfected with a temperature sensitive p53 mutant. In addition, no difference in these cells was observed on BetA sensitivity compared with the control transfected cells [52]. In ME20 melanoma cells, induction of p53 expression was not detected [59] and other studies exploring the effects of BetA on various p53 wildtype and mutant cell lines found no difference in sensitivity [54,79,80]. Taken together these results suggest that BetA-induced apoptosis does not involve p53.

Role of the Bcl-2 family and reactive oxygen species (ROS): In SHEP neuroblastoma cells over-expression of Bcl-2 and Bcl-XL blocked BetA-induced loss of mitochondrial membrane potential, ROS hyper-production, caspase processing and PARP cleavage. The expression of the pro-apoptotic molecules Bax and Bcl-Xs was induced in BetA treated cells [50]. BetA also triggered permeability transition and cytochrome c release in isolated mitochondria suggesting a direct effect on mitochondria. Mitochondria isolated from SHEP cells over-expressing Bcl-2 or Bcl-XL were resistant to BetA-induced effects [81]. Interestingly it was found that in contrast to doxorubicin, BetA caused caspase-8 cleavage downstream of the mitochondria. In addition, this effect was inhibited by Bcl-2 or Bcl-XL over-expression [82]. Consistently, in glioma cells BetA induced ROS generation, which was blocked by Bcl-2 or the antioxidants N-*tert*-butyl-a-phenylnitrone (PBN) and *N*-acetyl-cysteine (NAC). Expression levels of both, Bcl-2 and Bax were increased after BetA application whereas the levels of Bcl-Xs and Bcl-XL were not altered. Furthermore, ROS formation was dependent on new protein synthesis and was crucial for caspase activation [52]. In contrast, in human melanoma cells no up-regulation of Bax and Bcl-Xs was observed; however, the pro-survival molecule Mcl-1 was clearly induced under the same conditions [59]. Expression of Mcl-1 can change the balance in pro- and anti-apoptotic molecules and thus be crucial for BetA-induced apoptosis at least in melanoma cells. Interestingly, Bcl-2 over-expression provided only partial protection in Jurkat cells [57], and melanoma cells treated with BetA [80]. These results suggest that the protective effects of pro-survival members of the Bcl-2 family are possibly cell type specific. In addition, the differential up-regulation of pro-survival and pro-apoptotic Bcl-2 family members in different cell types adds weight to this notion. Gene expression levels of Bcl-2 and Bax were further analyzed in a series of cell lines derived from several different cancer types. In this particular study BetA treatment induced a consistent down-regulation of Bcl-2 whereas Bax levels were increased, resulting in a significant change in the Bax/Bcl-2 ratio [61]. In clear contrast, two head and neck squamous cellular carcinoma cell lines, however, treated with BetA displayed decreased Bax expression and no change in expression levels of Bcl-2 and Mcl-1 was observed [55]. Taken together the results in the literature were not conclusive and could not provide an explanation for a general

role for the Bcl-2 family of proteins in BetA induced apoptosis. We aimed to shed light on this phenomenon as to why such differential results were obtained previously. For other typical apoptosis inducing drugs including e.g. taxol the effects of the Bcl-2 family of proteins are broadly applicable among different cell types and test systems [67] and results are not as controversial as is the case for BetA.

Betulinic acid and other cell death pathways

After the discovery of BetA as an anti-cancer agent, it was immediately established that it exerts its cytotoxic activity via induction of apoptosis [27,51]. This was independent of the death receptor CD95 (APO-1/FAS), but was dependent on caspase activation because apoptosis was inhibited in the presence of zVAD.fmk, a pan-caspase inhibitor [50]. Thus, BetA-induced apoptosis was suggested to be independent of the death receptor pathway in neuroblastoma, glioma and melanoma cells [29], although the role of other death receptors, such as TNFR1 or DR5 (TRAIL-R2/KILLER), was not addressed. As BetA and TRAIL, however, cooperated to induce apoptosis in cancer cells [83], it is highly unlikely that BetA would exert its cytotoxic effects through this pathway.

Other targets of Betulinic acid

Aminopeptidase N (CD13)

Aminopeptidase N is a transmembrane peptidase which is expressed in neovessels in developing tumors whilst normal endothelial cells do not express it. As aminopeptidase N is a potent angiogenic regulator and related to tumorigenesis [84] the potential of BetA as an inhibitor of angiogenesis was investigated. One study suggested that the anti-melanoma effects of BetA are because of the inhibition of aminopeptidase N activity [85]. The results of another study, however, showed that the anti-angiogenic activity of BetA was not due to effects on aminopeptidase N but rather through an effect on the mitochondria of endothelial cells [86]. It is therefore unclear what the significance of BetA-induced inhibition of aminopeptidase N is for tumor cell death.

Acetyl-CoA acyltransferase (ACAT), diacylglycerol acyltransferase (DGAT)

ACAT exists in mammalians in two isoforms and catalyzes the acylation of cholesterol to cholesteryl ester. Therefore, ACAT inhibitors are investigated for treatment of hypercholesterolemia and atherosclerosis [87]. BetA was found to be a potent inhibitor of human ACAT1 (mitochondrial acetyl-CoA acetyltransferase) and ACAT2 (cytosolic acetoacetyl-CoA thiolase) [87]. Because the anticancer effects of BetA are strongly linked to the mitochondria, it is interesting to study whether ACAT inhibition is associated with BetA-induced anti-cancer effects.

Diacylglycerol acyltransferase (DGAT), a microsomal enzyme linked to obesity, catalyzes the terminal step in triacylgycerol synthesis and plays an important role in lipid metabolism [88]. It is inhibited by BetA [89] and in this context BetA has been also suggested to be a potential lead compound for treatment of obesity [90].

Its link to BetA-induced cancer cell death remains unexplored but because of the differential metabolism of cancer cells and healthy cells it is feasible that BetA-induced DCAT inhibition contributes to its anti-cancer effects. Of note, a BetA derivative (NVX-207, Fig 1) was found to bind to apolipoprotein A-I which plays an important role in lipid metabolism and cholesterol transport [91].

Kinases

BetA treatment was shown to cause activation of p38 and other pro-apoptotic MAP (mitogen-activated protein) kinases whereas anti-apoptotic MAP kinases remained unaffected [92]. The authors concluded that reactive oxygen species (ROS) induced by BetA, act upstream of the MAP kinases. The same study also confirmed the depolarization of the mitochondrial membrane potential that was reported earlier [92]. Another study described the antagonizing effects of U0126, a MEK (MAP kinase kinase) inhibitor, on BetA-induced apoptosis [93]. Interestingly, BetA was also reported to transiently activate the EGFR/AKT survival pathway and to enhance survivin expression, resulting in decreased sensitivity of melanoma cells [94]. Others, however, did not detect significant changes in ERK1/2 and AKT kinase activity [61] and survivin expression was decreased in the prostate cancer line LNCaP [95]. It is important to note, though, that all these kinase activation/inhibition events could be indirect and a consequence of BetA-induced stress/cell death.

Topoisomerases

Anti-cancer agents etoposide and camptothecin depend for their action on topoisomerase inhibition [96]. BetA has also been reported to be a catalytic inhibitor of topoisomerase I and II activity. The mechanism of its inhibitory effects on topoisomerase I was discovered to be the prevention of binding of the enzyme to the DNA, the first of the three topoisomerase - mediated steps being binding, strand breakage and re-ligation [97,98]. In a different study the role of BetA-induced topoisomerase inhibition on cell death was investigated. Silencing of topoisomerase I did not substantially affect BetA-induced cell death, pointing to the fact that this inhibition is not involved in the process of cell death [99]. However, it is possible that one or more of the numerous other cytotoxic effects reported for BetA might simply 'override' the effects of topoisomerase inhibition, making it difficult to assess the role of topoisomerase inhibition on cell death. Recently, semi-synthetic BetA analogues were discovered to possess strong topoisomerase I and IIα inhibitory effects and also exhibited stronger cytotoxic effects on cancer cells as compared with BetA itself [100]. Although whether cell death depends on topoisomerase was not studied, the authors concluded that BetA is a useful platform for designing potent new topoisomerase inhibitors [100].

NF-κB

The role of NF-κB in BetA-induced cell death was examined with contradictory results. It was found that BetA inhibited NF-κB. This involved both decreased IKK

(IκB kinase) activity and suppressed NF-κB activation, which was induced by different stimuli including tumor necrosis factor (TNF), thereby enhancing TNF-induced apoptosis. In addition, NF-κB-regulated growth factors such as COX-2 (cyclooxigenase 2) and MMP-9 (matrix metalloproteinase 9) were suppressed [101,102]. In contrast, another group showed the activation of NF-κB by BetA in tumor cell lines resulting in apoptosis. BetA-induced apoptosis was reduced in the presence of chemical inhibitors of NF-κB [103]. One explanation for these seemingly contradictory results might be the use of tumor cell lines originating from different tumor types. The studies observing inhibition of NF- κB used colon cancer [101] and prostate cancer [102] cell lines whereas the activation of NF- κB by BetA was found in the neuroblastoma cell line SHEP [103]. It was also suggested that the role of NF- κB in BetA-induced apoptosis is context specific [75-77]. Furthermore it is important to note that SHEP cells gave different results as compared to cell lines derived from other tumor types when the effect of Bcl-2 over-expression in BetA-induced cell death was examined.

Cell cycle

Cell lines derived from different tumor types showed decreased cyclin D1 expression (on mRNA and protein level) upon BetA treatment [61,95]. Cyclin D3 was found to be sharply decreased in Jurkat cells treated with BetA and the same study also found that BetA regulates the cell cycle via induction of a G0/G1 arrest, thereby inhibiting proliferation [104]. Another group found accumulation of p21 on BetA exposure in glioma cells. This, however, did not result in cell cycle arrest [52]. Similarly, BetA did not affect cell cycle distribution in an ovarian cancer line [54]. In melanoma cells, BetA induced cell cycle arrest in the G1 phase [105] and selectively caused a decrease of cdk4 protein, but had no effect on other cell cycle proteins such as cdc2, cdk2, cdk7 and cyclin A [93]. Again, the effects of BetA on the cell cycle appear to be highly cell type specific. If or how they relate to BetA's cytotoxicity requires further investigation.

Proteasome

It was hypothesized that the anti-cancer effects of BetA might be partly because of the degradation of the transcription factors specificity proteins 1, 3 and 4 (Sp1, Sp3 and Sp4). Cycloheximide, a protein synthesis inhibitor had no effect on Sp protein levels in BetA treated cells whereas the proteasome inhibitor MG132 reversed BetA-induced effects, suggesting that BetA induced proteasome dependent degradation of Sp proteins (and also cyclin D1) [95]. Another study discovered that BetA directly interacts with purified proteasome and activates primarily the chymotrypsin-like proteasome activity. Interestingly, modifications on the C-3 position resulted in a derivative with proteasome-inhibitory effects [106]. The effects of BetA on the proteasome are of special interest because the ubiqutin-proteasome pathway is the target of an entire new class of drugs. The concept of treating cancer by inhibiting the proteasome with agents such as bortezomib is highly promising and already applied in the clinic for multiple myeloma [107].

Whether proteasome activation by BetA is a general feature of all cells treated with BetA remains to be determined. In addition, it is unclear whether the proteosome plays a role in BetA-induced cell death.
When combined the plethora of targets affected by BetA suggest that BetA has a very complex mode of action that may allow circumvention of blocks in cell death activation that normally interfere with chemotherapy. This could explain the broad effectiveness of this compound against a wide range of tumors.

Betulinic acid in vivo

The first study reporting on the very successful *in vivo* application of BetA was published in 1995 [27]. It is surprising that only a few studies addressing the *in vivo* efficacy of BetA have followed since. This is likely due to the very lipophilic characteristics of BetA and its consequently poor solubility, which makes *in vivo* application difficult. This is often a hampering step during drug development. Nevertheless, the limited data that are available on *in vivo* treatment with BetA all point to a significant anti-cancer effect. The initial report described a method for enhancing the solubility by co-precipitating BetA with polyvinylpyrrolidone (PVP). After reconstitution, PVP-complexed BetA was injected intraperitoneally (i.p.) into nude mice bearing subcutaneous human melanoma (Mel-1 and Mel-2, see before) xenografts. A dose of 50 mg per kg body weight injected every four days was enough to prevent tumor outgrowth and six injections of the same dose induced tumor regression. Complete lack of toxicity was observed up to 500 mg per kg body weight (as judged by body weight) [27]. Together this indicates a broad therapeutic window. Pharmacokinetic studies using the same BetA formulation revealed that BetA is rapidly absorbed with a slow, biphasic disappearance from the serum. High tissue concentrations were found in peritoneal fat, ovary, spleen, mammary gland, uterus and bladder, low tissue concentrations were found in the heart and the brain [49]. BetA showed anti-metastatic activity by itself and in combination with vincristine in a B16F10 melanoma mouse model. The treatment dose of BetA was 10 mg per kg bodyweight per day and again, no signs of toxicity were detected [105]. In an ovarian cancer xenograft model BetA-treated mice (100 mg per kg bodyweight every 3-4 days in a 10% ethanol, 10% Tween-80 and 80% water formulation) had a clear survival advantage compared with the control group [54]. In all these studies BetA was applied intraperitoneally (i.p.). Importantly, one report describes the inhibition of outgrowth of a subcutaneously injected prostate cancer cell line upon oral treatment. Mice received 10 or 20 mg BetA per kg bodyweight orally every other day with corn oil serving as vehicle [95]. This indicates that BetA retains its activity even after oral application. Similarly, activity is observed when the route of application is via intravenous (i.v.) injection in a human adenocarcinoma xenograft mouse. Even though BetA induced significant tumor growth inhibition under these conditions, a derivative was found to be even more effective [108]. Importantly, all these in vivo studies showed complete absence of systemic signs of toxicity.

Combination treatments

Chemotherapies are usually applied as combination treatments in the clinic with many benefits compared with single treatments. A higher percentage of tumor cells can be killed by targeting different pathways simultaneously, avoiding tumor cell survival due to drug resistance towards one of the compounds, and resulting in either additive or synergistic anti-tumor effects. Moreover, such protocols generally can suffice with lower concentrations of the single compounds and toxic effects for the patient can therefore be lesser. The anti-cancer effects of BetA have been studied in combination with several other cancer treatments. Sensitizing effects of BetA were demonstrated *in vitro* for hyperthermia applied on human melanoma cells that were first adapted to low pH [109]. Treatment with BetA in combination with irradiation resulted in additive growth inhibition of melanoma cells. The authors concluded that the additive effects were because of the targeting of either different pathways or different tumor cell populations [59]. In another murine melanoma cell model the combination effects of BetA and vincristine were explored *in vitro* and *in vivo.* The effect of the combination treatment on cell growth inhibition *in vitro* was synergistic and in an *in vivo* metastasis model fewer lung nodules were observed compared with the respective single treatments [105]. In addition, the combination of BetA with the epithelial growth factor receptor (EGFR) inhibitor PD153035 was found to enhance cell death of melanoma cells *in vitro* [94]. Furthermore, BetA cooperated with anti-cancer drugs doxorubicin and etoposide to induce apoptosis and to inhibit clonogenic survival in SHEP neuroblastoma cells [110]. It also cooperated with TRAIL (tumor necrosis factor related apoptosis inducing ligand) to induce apoptosis in tumor cell lines and primary tumor cells, but not in normal human fibroblasts [83]. Although these reports would suggest BetA cooperates with many different pathways, other studies did not find such an effect. For instance, the combination of BetA and cisplatin was tested *in vitro* in two head and neck cancer cell lines but the results were not encouraging. Treatment for longer periods (72 hours) even showed antagonistic and sub-additive effects [111]. Combined treatment of BetA and *NF-κB* inhibitors was concluded to have no therapeutic benefit and could in certain tumors even be contra-productive [103]. These results indicate that the combination of BetA with other therapies needs to be carefully evaluated for each treatment and tumor type. Another study investigated the potential of BetA to sensitize drug-resistant colon cancer cells and results indicate that the chemosensitizing effects of BetA enhance the efficacy of 5-Fluorouracil, irinotecan and oxaliplatin [112]. A beneficial effect of combining triterpenoids including BetA with 5-Fluorouracil was indeed also found when applied on esophageal squameous cell carcinoma cell lines *in vitro* [113]. Generally, it can be concluded that BetA is a promising candidate to be used in combination treatments, especially because of its low cytotoxicity on normal cells.

Betulin

Betulin, as an abundantly available product of the bark of the white birch tree, has been mostly regarded as the pre-cursor molecule of BetA. Initially it was described as being inactive or less active against cancer cells compared with other triterpenoids [114-116]. The results of recent reports, however, suggest that also betulin might have potential as an anti-cancer drug [117,118].

Betulinic acid derivatives

BetA holds great promise as an anti-tumor agent, but as mentioned has a severe drawback in its poor solubility in aqueous solutions and thus its application in vivo. Another non-scientific fact is that as a broadly available product from nature, BetA is difficult to patent. For these reasons, and of course in search for even more potent anti-cancer drugs, a lot of effort has been put into developing and testing BetA derivatives, of which several examples are discussed here.

Modifications of BetA are possible at numerous positions, such as C-3, C-20 or C-28 [115]. Modifications at C-20 did not enhance cytotoxicity in several cancer cell lines [119], but derivatives at the C-3 and C-28 position were found to be promising. Amino acid conjugates at the C-28 position enhanced water solubility as well as cytotoxicity [120]. Hydroxylation at the C-3 position gave promising results when tested on murine melanoma cells [121] and another chemical modification at the C-3 position (dimethylsuccinyl BetA) turned BetA from a proteasome activator into a proteasome inhibitor [106]. Yet another C-3 modification gave better anti-tumor results in a colon cancer xenograft mouse model when compared with BetA [108]. The ring skeleton of BetA is the platform for many other interesting modifications [97,122,123]. One novel, well tolerated BetA derivative is NVX-207, which showed significant anti-tumor activity in clinical studies in canine cancer patients with treatment-resistant malignancies [91].

Scope of this thesis

The scope of this thesis was to investigate the potential of BetA as an anti-tumor agent in more detail. When the studies for this thesis were started in 2005 several aspects of BetA as anti-cancer compound needed clarification. As it was not precisely clear whether cytotoxicity exerted by BetA was tumor cell type specific, we first aimed to chart its anti-tumor potential by subjecting broad cell line panels of different tumor types to BetA treatment. We analyzed the cytotoxic effects via different readouts for cell death, clonogenic survival and metabolic enzymatic activity, thereby increasing reliability and relevance of the results. These results are described in chapter 2. As only few *in vivo* studies with BetA were available, we were interested to study the efficacy of BetA *in vivo*. BetA's poor solubility has prevented its wide applicability for *in vivo* use, which prompted us to improve the formulation of BetA. Especially for a potential future use in the clinic it is important to find an allowed non-toxic formulation that enables a sufficiently high dose to be reached *in vivo*. We therefore tested the feasibility of BetA containing liposomes for *in vivo* application. The results are laid out in chapter 3.

The mechanisms underlying the broad efficacy and tumor-selectivity of BetA was the second main focus of this thesis. The central questions addressed were firstly how BetA, in contrast to other chemotherapies, is able to induce cell death in multidrug-resistant tumor cells. Secondly, to elucidate the exact role of the Bcl-2 family proteins as partially conflicting results had been previously published on this topic. Chapter 4 describes our findings on how BetA is able to bypass the Bcl-2 family of proteins which are also often responsible for drug-resistance of cancer cells. Chapter 5 goes deeper into the mechanisms of mitochondrial damage and subsequent cell responses. Among others the appearance of autophagosomes, a previously unnoticed phenomenon in BetA-treated cells is reported. In this chapter, the role of these autophagosomes for survival/cell death is discussed as well as the caspase-independent component in BetA-induced cell death.

Finally, we were also interested if betulin, the precursor molecule and main natural source of BetA, has a potential as an anti-tumor agent itself. This question has been largely neglected in literature. Chapter 6 describes the tumor cytotoxic effects of betulin by itself and in combination with cholesterol.

Finally, chapter 7 discusses the data of this thesis in relation to the literature.

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Chapter 2

Broad In Vitro Efficacy of Plant-Derived Betulinic Acid Against Cell Lines Derived from the Most Prevalent Human Cancer Types

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Abstract

Betulinic acid (BA) is a widely available plant-derived triterpene with reported activity against cancer cells of neuroectodermal origin and leukaemia's. Treatment with BA was shown to protect mice against transplanted human melanoma and led to tumor regression. In contrast, cells from healthy tissues were resistant to BA and toxic side-effects in animals were absent. These findings have raised interest in the chemotherapeutical anti-cancer potential of BA.

A comprehensive assessment of the efficacy of BA against the clinically most important cancer types is currently lacking. Therefore, we tested the in vitro sensitivity of broad cell line panels derived from lung, colorectal, breast, prostate and cervical cancer, which are the prevalent cancer types characterized with highest mortalities in woman and men. Multiple assays were used in order to allow a reliable assessment of anti-cancer efficacy of BA. After 48 hr of treatment with BA, cell viability as assessed with MTT and cell death as measured with propidium iodide exclusion showed clear differences in sensitivity between cell lines. However, in all cell lines tested colony formation was completely halted at remarkably equal BA concentrations that are likely attainable in vivo. Our results substantiate the possible application of BA as a chemotherapeutic agent for the most prevalent human cancer types.

Introduction

Betulinic acid (BA) is a plant derived pentacyclic lupine-type triterpene, which was discovered in a National Cancer Institute drug screening program of natural plant extracts, and has been recognized to possess potent pharmacological properties [1]. BA and derivatives thereof have been shown to exert anti-inflammatory [2], anti human immunodeficiency virus (HIV) [3, 4] and, most notably, anti-cancer activities. BA can be isolated from numerous botanical sources [5, 6], and its structurally related precursor, betuline, which can be readily converted into BA [7], is contained in higher quantities in widespread plant sources [8]; e.g. betuline constitutes up to 22% of the bark of the white birch tree (betula alba) [9].

Initially, BA was reported to induce melanoma specific cytotoxicity [1]. In athymic mice that were challenged with human melanoma xenografts and treated after one day with BA, tumor development was strongly impeded. In addition, when treatment was initiated 41 days after tumor challenge, the established tumors regressed for more than 80% [1]. Although in this study the toxicity of BA towards tumor cell lines from non melanoma origin appeared to be limited [1], in subsequent studies it was revealed that BA induced potent cytotoxicity in various other tumor types of neuroectodermal origin next to melanoma [10-13].

Fulda and coworkers demonstrated that BA induces apoptosis in neuroblastoma, medulloblastoma and Ewing's sarcoma cell lines [10], which are the most common solid tumors in childhood. Sensitivity of neuroblastoma cell lines for BA-induced apoptosis was simultaneously observed by Schmidt et al. [11]. Primary tumor cells cultured from medulloblastoma and glioblastoma [12], glioma cell lines [13] and head and neck squamous cellular carcinoma cell lines [14] were also sensitive to BA-induced cytotoxicity. However, more recent studies disproved the selectivity of BA for neuroectodermal-derived tumors. Zuco et al. reported anti-proliferative capacity of BA in vitro in tumor cell lines originating from different tissues [15]. This study also addressed the in vivo activity of BA by showing enhanced survival times in mice grafted with a human ovarian carcinoma when treated with BA [15]. Subsequently, BA was also shown to induce apoptosis in haematological malignancies where 65% of primary pediatric acute leukaemia cells and all cell lines of this type were sensitive for BA in vitro [16].

Next to the broad specificity of BA for multiple tumor types, BA was reported to be devoid of cytotoxic effects against healthy cells. Normal human fibroblasts [15], peripheral blood lymphoblasts [15], melanocytes [17] and astrocytes [13] were shown to be resistant against BA treatment in vitro. Also, systemic in vivo toxicity was not apparent in mice treated with BA up to 500 mg/kg bodyweight [1, 15] and an earlier study did not detect BA-induced toxicity in rats as monitored with the so-called Hipocratic screening test [18].

Although the precise mechanisms contributing to BA-induced cell death have still to be unravelled in detail, several studies have provided considerable insight in BA-induced cytotoxicity. BA was shown to induce apoptosis in a p53 independent

manner [10, 13, 15, 17] by a direct effect on mitochondria [19]. In neuroectodermal cells BA induced mitochondrial membrane permeabilization [10, 20, 21] facilitating the release of cytochrome C, apoptosis-inducing factor (AIF) [19] and Smac [16]. Formation of reactive oxygen species and protein neosynthesis have been reported to be required for BA-induced cell death [10, 13, 21] and proapoptotic mitogen-activated protein kinases (MAPKs) were found to be involved [21]. Bcl-2 or Bcl-XL overexpression or treatment with bongkrekic acid, a reported stabilizer of the permeability transition pore complex, inhibited cytochrome C release and BA-induced apoptosis [10, 13, 20]. Finally, reports have also described that inhibition of topoisomerases may be involved in BA-induced cell death as an additional mechanism [22, 23].

The specific cytotoxicity induced by BA in a diversity of cancer types in conjunction with its lack of cytotoxicity for healthy cells has raised optimism that this reagent can be used as a non-toxic anti-cancer drug. The objective of the current study is to investigate the anti-tumor efficacy of BA against prevalent cancer types that are characterized by the highest mortalities [24].

BA treatment of broad cell line panels was monitored by three different assays to allow a reliable and representative assessment of BA-induced anti-cancer effects in these cancer types. We report that BA induced cytotoxic and anti-proliferative effects in cell lines derived from lung, colorectal, breast, prostate and cervix cancer, thus confirming the broad specificity of this reagent and substantiating its possible application in a non-toxic chemotherapy for these cancer types.

Material and Methods

Reagents

Betulinic acid (A.G. Scientific, San Diego, CA) was dissolved in dimethyl sulfoxide **(**DMSO) at 4 mg/ml and aliquots were stored prior to use at -80 C. ZVAD-fmk (benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone) was obtained from Sigma.

Cell lines, healthy cells and cell culture

All cell lines were cultured in IMDM medium (Cambrex) containing 8% fetal calf serum (FCS), 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (Life Technologies, Täby, Sweden) and maintained in logarithmic growth phase in 75 or 175 cm^2 culture flasks (Costar) prior to BA treatment.

Lung cancer cell lines H460, H322, H187, N417 were kindly provided by dr. F. Kruyt (dept. of Medical Oncology, VU University Medical Center, Amsterdam, The Netherlands), and non-small cell lung cancer cell lines GLC-2, GLC-4 and GLC-36 were kindly provided by dr. L. de Leij (dept. of Pathology and Laboratory Medicine, University of Groningen, The Netherlands). Colon cancer cell lines CO115, SW480, T84, HCT81 and LS180, lung cancer cell line MBA9812 and prostate cancer cell lines DU145 and PC3 were kindly provided by J. van Eendenburg and dr. A. Gorter (dept. Pathology, Leiden University Medical Center, The Netherlands). Breast cancer cell lines MCF7, SKBR3, MDA-231, MDL13E, BT474 and T47D were kindly provided by dr. E. Verdegaal (dept. Clinical Oncology, Leiden University Medical Center, The Netherlands). Breast cancer cell lines BT483, BT549 and ZR-75-1, and prostate cancer cell line 22Rv1 were obtained from the American Type Culture Collection. All other cell lines are from our laboratory.

Peripheral blood mononuclear cells (PBMC), cytoxic T lymphocytes (CTL) clones and activated B cells were generated from blood obtained from healthy donors and cultured with cytokines to maintain viability as described [25].

Analysis of cell death and cell viability

Adherent cells were seeded in 6 well plates (300.000 cells/well) 24 hr prior to BA application. Suspension cells were seeded in 12 well plates (300.000 cells/well) directly before addition of BA.

Cells were treated with BA at indicated concentrations. To exclude differential effects of DMSO, for all concentrations as well as the control, DMSO was compensated to an equal concentration of 0.5%. Cells were harvested at indicated time points, resuspended in 1 ml IMDM complete medium and subsequently divided over the 3 different assays, which guaranteed equal treatment levels.

Cell death was determined by propidium iodide (PI; Molecular Probes) exclusion as previously described [26]. In short, treated cell lines were harvested and cell samples of 300 µl were stained with PI at 1 µg/ml for 15 min. Samples were measured by flow cytometry using a FACSCalibur system (Becton Dickinson, San Jose, CA, USA) and analysed using CellQuest software.

For quantification of apoptotic DNA fragmentation (Nicoletti assay) cells were resuspended in Nicoletti buffer containing 50 µg/ml PI for at least 24 hr as described [27], subsequently flow cytometric measurement of PI stained nuclei was performed.

For the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) viability assay[28], which measures metabolic activity, 100 µl of the treated cells were tested in triplicates in 96 well plates, subsequently 20 µl MTT (2.5 mg/ml) was added and 2 hr later 100 µl solubilisation buffer (75% DMSO, 5% SDS) was added. Optical density (OD) was measured after 24 hr at 560 nm (reference wavelength 655 nm) using a spectrophotometer. For data analysis, the background OD (100 µl medium without cells, 20 µl MTT, 100 µl solubilisation buffer) was subtracted from each sample value. In experiments where caspase activity was blocked with the pan-caspase inhibitor zVAD.fmk, 20 µM was applied 2 hours prior to BA-treatment and every 12 hr additional zVAD.fmk (10 µM) was added.

Clonogenic assay

Depending on the plating efficiency of each cancer cell line, 100-1000 cells were seeded in duplicates in 6 well plates. BA was applied after 24 hr at indicated concentrations and cells were cultured without change of medium until macroscopic colonies were detected in the untreated control (usually about 6-8 days). Colonies were counted after fixation with 6% glutaraldehyde and staining with crystal violet.

Immunoblot analysis of cleaved poly-ADP ribose polymerase (PARP)

PARP western analysis was performed essentially as described before [29]. In short, cells were lysed for 20 min at 4 C in Triton X-100 buffer (1x10^6 cells per 40 µl) and centrifuged at high speed for 8 min. Samples of 500.000 cells were loaded per lane. Proteins were separated by 8% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene fluoride (PVDF) transfer membrane (Amersham Biosciences, Piscataway, NJ). The blot was blocked with 5% low fat milk powder (in phosphate buffered saline (PBS), 0.2% Tween-20) and probed with primary anti-PARP polyclonal antibody (1:2500, Cell Signaling) overnight. The membranes were washed in PBS with 0.1% Tween, incubated with horseradish peroxidase (HRP) conjugated anti-rabbit IgG (1:10000) as secondary antibody (Southern Biotechnology Associates, Birmingham, AL) and visualized by chemiluminescence (ECL; Amersham Biosciences). Experiments were repeated two times.

Results

BA has been reported to be active against cancer cell lines from neuroectodermal origin [10-13]. However, since additional data also suggested activity of BA against various different cancer types, we set out to investigate its cytotoxic potential in a panel of cell lines of those prevalent cancer types that are characterized with the highest mortality rates.

In the U.S., breast cancer and prostate cancer are the most commonly diagnosed cancer types for women and men respectively. However, mortality rates show that lung cancer is the most common fatal cancer in men (31%), followed by prostate cancer (10%), and colorectal cancer (10%). In women, lung (27%), breast (15%), and colorectal cancer (10%) are the leading causes of cancer death [24]. In contrast, in the developing countries cervical cancer is most frequently resulting in cancer death among woman [30, 31].

A comprehensive survey of BA-induced cell death for these cancer types has not yet been conducted. Only fragmentary and sometimes conflicting reports revealed that cell lines derived from lung cancer [15, 32, 33], colon cancer [34], prostate cancer [34] and cervical cancer [15] can be sensitive to BA-treatment. Therefore, in the current study, broad cell line panels derived from these tumor types were collected and tested for BA sensitivity in multiple assays monitoring overall cell death, metabolic activity as a measure for cell viability and clonogenic survival. First, the T cell leukemia cell line Jurkat was tested as a positive control to validate our assays and compared to healthy cell types to confirm the reported relative non-toxicity of BA against non-cancer cells under our experimental conditions.

Sensitivity to BA treatment of the Jurkat leukemia cell line and healthy cells

Because BA has been reported to act via different pathways [5], overall BA-induced cell death was our primary focus. In addition, cell metabolism and viability were assessed by the MTT dye assay. Moreover, the percentage of cells showing DNA fragmentation, which is one of the typical characteristics of apoptosis, was measured. The Jurkat T cell leukemia cell line (variant J16 [35]) was chosen as positive control because it was proven sensitive for BA treatment before[16]. Analysis of flowcytometric measurements (Fig 1A) showed that BA applied at 10 µg/ml induced cell death that was dependent upon length of incubation, starting with approximately 30% cell death after 24 hr and leading to more than 80% cell death after 3 days of incubation (Fig 1B). This coincided with a comparable level of cells displaying DNA fragmentation at these time points (Fig 1B). Remarkably, cell metabolism, as measured in the MTT assay, was affected much more rapidly when J16 was treated with 10 µg/ml BA. In this assay, inhibition was already observed after 6 hr. This difference between the sensitivity of J16 for BA observed in the MTT assay and the cell death assay was also observed in dose response curves. That is, a sharp decline in MTT conversion was observed already with 2.5 µg/ml BA treatment, whereas BA concentrations of 7.5 µg/ml and higher were

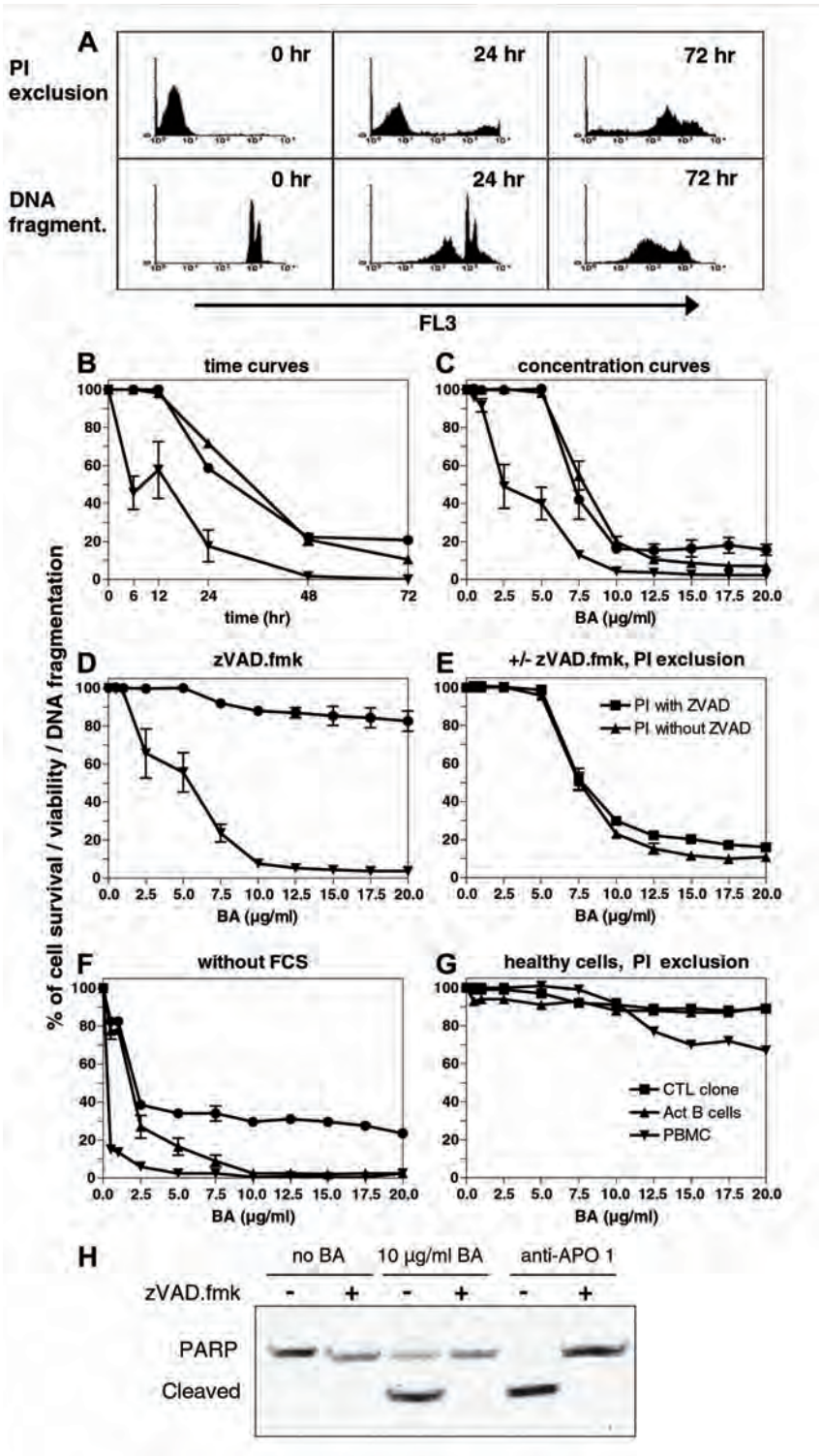
needed to induce cell death and DNA fragmentation at the same time point (48 hr) (Fig 1C). Nevertheless, concentrations of 10 µg/ml and higher resulted in comparable toxicity when measured by MTT conversion, PI exclusion and DNA fragmentation (Fig 1C). This suggests that BA has a stronger effect on MTT conversion and affects membrane integrity (PI) and apoptosis (Nicoletti) only at higher concentrations.

To which extend the cell death induced by BA is fully caspase-dependent is still a matter of debate [5]. We therefore treated J16 with BA under conditions where caspase activity was abrogated by co-treatment with the pan-caspase inhibitor zVAD-fmk. This resulted in a complete inhibition of DNA fragmentation (Fig 1D) and (caspase-mediated) cleavage of Poly (ADP-ribose) polymerase (PARP) (Fig 1H). However, the overall induction of cell death was not reduced at all by zVAD.fmk co-treatment (Fig 1E), indicating that BA-mediated cytotoxicity ensues as efficiently when caspases are blocked. Such caspase-independent cell death has been described for other chemotherapeuticals as well [36].

Because BA-induced cytotoxicity is often measured in serum free or low serum conditions in the literature[16], we compared the results of BA treatment of J16 in the presence and absence of fetal calf serum (FCS). Without FCS, J16 was more sensitive for BA (Fig 1F), which may reflect the additional effect of growth factor deprivation or may be due to the fact that BA is sequestered by serum proteins.

Finally, to ascertain that BA is non-toxic to healthy cells as was reported before [15], we tested human blood-derived PBMC, cytotoxic T lymphocyte clones and activated B cells. These cell types were highly resistant for BA-induced cytotoxic effects as measured with PI exclusion after 48 hr (Fig 1G), which is in line with the literature [15].

The primary interest of the current study is to test BA-induced anti-cancer effects irrespective from the precise mechanisms attributing to it. However, as BA may induce cell death by multiple, likely intertwined, and possibly cell type dependent mechanisms, we decided to test for multiple read-outs. In the first place, the cancer cell lines were screened for overall cell lethality by PI exclusion after 48 hr incubation with different concentrations of BA. Additionally, effects on cell survival and proliferative capacity were monitored by a clonogenic growth assay, which is time point independent. Finally, the MTT assay was included in the screening because it was found to sensitively monitor an early inhibition of cell metabolism at low BA concentrations that is missed by measuring PI exclusion alone (Fig 1B). The assays were performed in the presence of FCS because this more physiologically mimics in vivo circumstances than serum free conditions.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Act B cells
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anti-APO 1
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PARP
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Fig 1. BA sensitivity of T cell leukemia cell line Jurkat and healthy cells

PI exclusion was used to measure cell death (▲), DNA fragmentation to measure apoptosis (●), and the MTT dye assay to measure cell viability (▼) (symbols used in panels B, C, D, G). (A) Flowcytometric FL3 histograms of Jurkat cells (J16) treated with BA (10 µg/ml) after cellular staining with PI (to measure cell death) or nuclear staining with PI (to measure DNA fragmentation). (B) Kinetics of BA-induced effects in Jurkat cell line when treated with 10 µg/ml BA. (C) Dose response curves of BA treatment measured at 48 hr. (D) Dose response curves of DNA fragmentation and MTT conversion at 48 hr in the presence of pan-caspase inhibitor zVAD.fmk. (E) Dose response curves of BA treatment in the cell death assay at 48 hr with and without pan-caspase inhibitor zVAD.fmk. (F) Dose response curves of BA treatment at 48 hr in conditions of serum free medium. (G) Dose response curves in the cell death assay of healthy cell types (PBMC, CTL clones and activated B cells) after 48 hr of BA treatment. (H) BA (10 µg/ml) induced PARP cleavage measured at 48 hr in the presence and absence of zVAD.fmk. Anti-APO1 induced PARP cleavage was included as control. Mean values and error bars (SEM) are derived from at least three experiments performed.

Sensitivity to BA treatment of lung cancer cell lines

A panel of 10 lung cancer cell lines was collected. This panel was chosen to consist primarily of cell lines derived from non-small cell lung cancer (NSCLC) because this is the most common form of lung cancer, accounting for 80% of all lung cancer cases (in the US). Non-small cell lung cancer cell lines SW1573, H460, A549, H322, GLC-2, GLC-4, GLC-36 and small cell lung cancer lines H187, N417 and MBA9812 were tested in the three assays afore mentioned (Fig 2). Overall cell death, as measured by PI exclusion, showed that 4 cell lines (A549, H187, N417, MBA9812) were efficiently killed with BA treatment at 20 µg/ml, because less than 10% of the cells were viable after 48 hr incubation (Fig 2, Table 1). At the other end of the spectrum, half maximal cell death at 48 hr was not reached for SW1573. The remaining 5 cell lines displayed intermediate BA sensitivity. The BA concentration needed for half maximal cell death (50% effective concentration; EC_{50}) at 48 hr for the lung cancer cell lines (SW1573 excluded) ranged from 6.1 to 12.3 µg/ml (Table 1). In contrast, all lines that could be tested in the clonogenic assay (8 out of 10) were halted in their growth and mostly at concentrations that were lower than the EC_{50} derived from the PI exclusion assay (Fig 2, Table 1).

Measurement of MTT conversion at 48 hr showed a biphasic character for most cell lines, i.e. a sharp decrease was observed at low BA concentrations (up to 2.5 µg/ml), followed by a more gradual decline at higher BA concentrations. Overall, in the MTT assay, 50% reduction in MTT conversion was reached for all 10 lung cancer cell lines and ranged from 1 to 8 µg/ml (Table 1). The BA-induced effects in the MTT assay were more intense than the cytotoxic effects measured with PI exclusion for all these cell lines (Fig 2, Table 1).

Table 1
BA sensitivity of lung, colorectal, breast, prostate and cervical cancer cell lines

Cancer type	Clonogenic growth halted at[a] (μg/ml)	Cell death[b]		Viability[c] EC_{50} (SEM) (μg/ml)
		EC_{50} (μg/ml)	Max % at 20 μg/ml	
Lung cancer				
SW1573	10	NR[d]	47	2.2 (0.3)
H460	7.5	6.1	75	3.8 (0.4)
A549	7.5	8.3	90	2.6 (0.1)
H322	7.5	12.3	55	7.3 (0.3)
GLC-2	7.5	8.8	65	6.8 (0.1)
GLC-4	7.5	10.0	70	5.9 (0.2)
GLC-36	5	9.6	75	8.0 (0.1)
H187	NT[d]	8.7	92	2.1 (0.1)
N417	NT	6.2	85	1.0 (0.1)
MBA9812	7.5	7.6	99	2.3 (0.4)
Colorectal cancer				
SW1463	10	3.8	69	2.8 (0.3)
SW837	7.5	11.3	75	8.6 (0.3)
RKO	7.5	9.5	95	8.6 (0.3)
CO115	10	12.2	60	5.8 (1.7)
SW480	5	15.1	61	2.5 (0.1)
T84	10	11.3	61	11.6 (0.3)
HCT81	12.5	16.4	54	6.7 (1.7)
DLD1	10	NR	45	2.5 (0.2)
HT29	10	NR	40	5.2 (0.2)
LS180	10	11.7	62	7.7 (1.1)
Breast cancer				
MCF7	10	NR	22	8.3 (1.2)
SKBR3	7.5	16.2	46	5.7 (0.1)
MDA231	7.5	10.4	63	6.4 (0.1)
MDL13E	7.5	11.5	71	2.4 (0.1)
BT483	NT	12.8	67	11.6 (0.1)
BT474	NT	12.1	79	5.8 (1.2)
T47D	7.5	13.0	56	2.5 (0.1)
BT549	7.5	5.5	95	3.3 (0.1)
ZR-75-1	NT	NR	11	NR
Prostate cancer				
DU145	7.5	11.6	63	5.3 (0.3)
PC3	7.5	12.3	58	2.3 (0.3)
22Rv1	5	10.1	73	8.2 (0.1)
LNCaP	7.5	11.9	65	2.8 (0.5)
Cervical cancer				
CaSki	7.5	9.6	63	6.7 (0.2)
HeLa	7.5	14.3	60	1.8 (0.1)
SiHa	7.5	11.8	67	5.0 (0.8)

[a] BA concentration at which clonogenic growth stopped was determined in duplicate with equal results.
[b] Cell death as measured with PI exclusion at 48 h. Both EC_{50} and % cell death with 20 μg/ml BA are provided.
[c] Cell viability as measured in triplicate by the MTT assay at 48 h. EC_{50} and SEM values are provided.
[d] NR, not reached; NT, not tested (no colony formation in the untreated control).

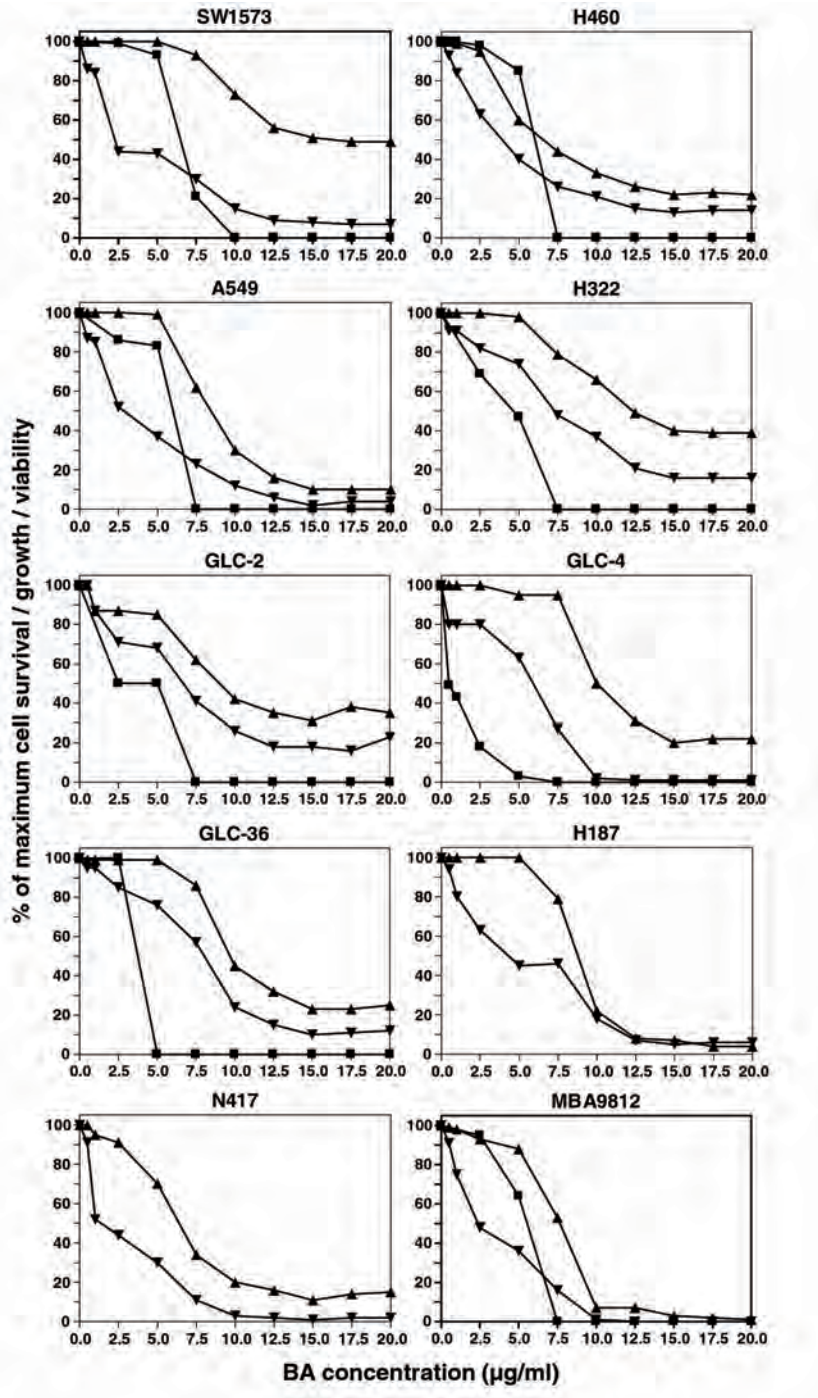

Fig 2. BA sensitivity of 10 lung cancer cell lines.
Cytotoxic effects measured with PI exclusion at 48 hr (▲), cell viability measured at 48 hr with MTT conversion (▼) and clonogenic growth (■) were tested for the indicated concentrations of BA.

Sensitivity to BA treatment of colorectal cancer cell lines

A panel of 10 colorectal cell lines, consisting of rectal cancer cell lines SW1463 and SW837 and colon cancer cell lines RKO, CO115, SW480, T84, HCT81, DLD1, HT29 and LS180 was tested for BA-induced cytotoxic effects. Marked differences between the cell lines were observed for overall cell death at 48 hr as measured by PI exclusion. This ranged from sensitive cell lines, e.g. for the most sensitive cell line RKO only 5% viable cells were left after treatment with 20 μg/ml BA, to relatively insensitive cell lines, e.g. half maximal lysis (EC_{50}) was not reached for DLD1 and HT29 at the highest concentration tested (Fig 3). EC_{50} values in the cell death assay for the colorectal cell lines, excluding DLD1 and HT29, ranged from 3.8 to 16.4 μg/ml BA (Table 1).

Despite this incomplete cytotoxic effect at 48 hr, all cell lines were completely inhibited in their clonogenic growth by BA treatment. Clonogenic proliferation of 9 out of the 10 cell lines was halted at 10 μg/ml BA or below, while only HCT81 was slightly less sensitive (colony formation was inhibited at 12.5 μg/ml) (Fig 3, Table 1). Thus, long term incubation with BA resulted in a complete anti-proliferative and/or cytotoxic effect for all colon cell lines tested. Similar to the lung cancer cell lines, viability of all colon cancer cell lines in the MTT assay was affected by treatment with BA concentrations that were lower (EC_{50} range 2.5 - 11.6 μg/ml) than the BA concentrations needed for half maximal cell death at 48 hr (Fig 3, Table 1).

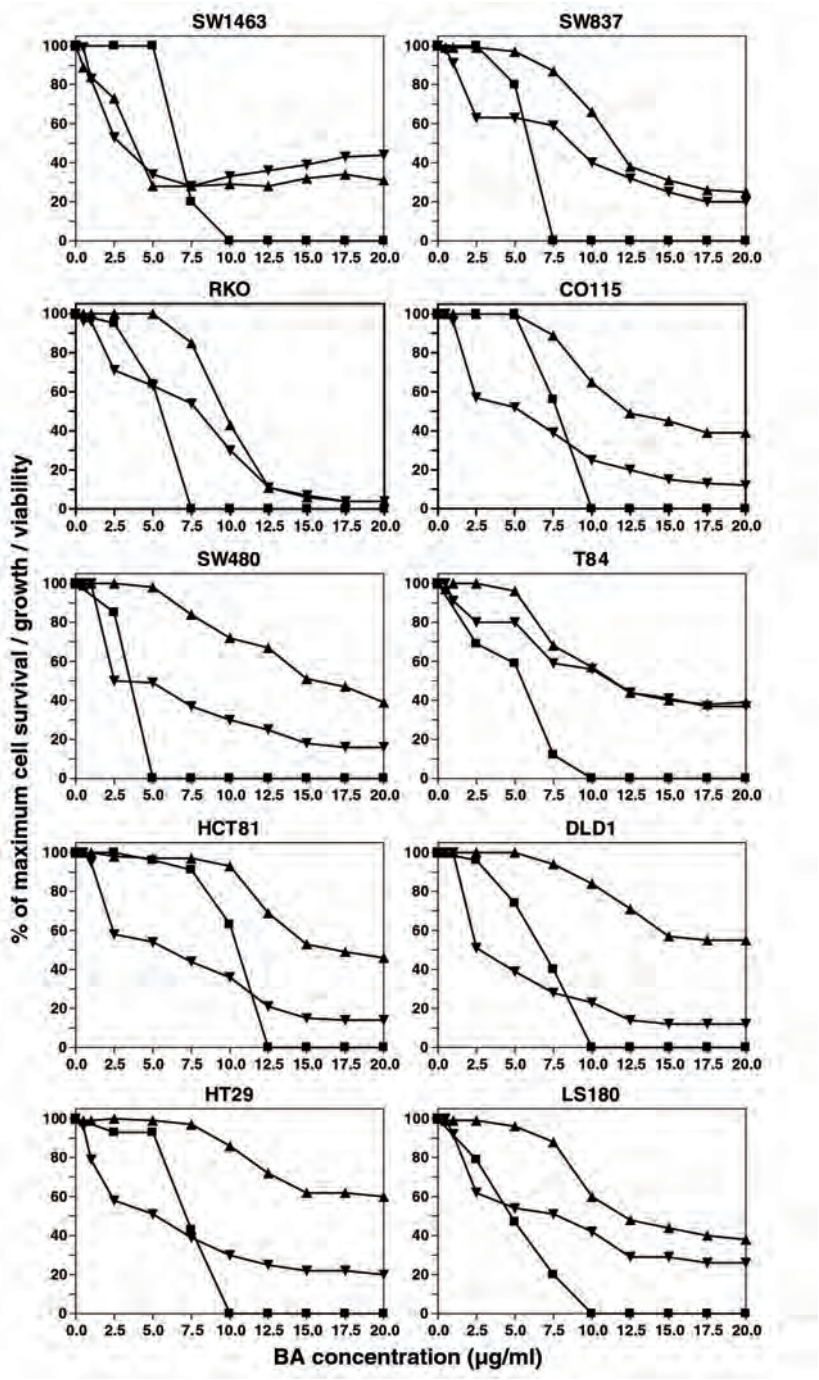

Fig 3. BA sensitivity of 10 colorectal cancer cell lines.
Cytotoxic effects measured with PI exclusion at 48 hr (▲), cell viability measured at 48 hr with MTT conversion (▼) and clonogenic growth (■) were tested for the indicated concentrations of BA.

Sensitivity to BA treatment of breast cancer cell lines

Subsequently, BA treament of 9 breast cancer cell lines (MCF7, SKBR3, MDA231, MDL13E, BT483, BT474, T47D, BT549, ZR-75-1) was analysed.

Two of these cell lines, MCF7 and ZR-75-1, were almost resistant to BA-induced cell death at 48 hr when assayed with PI exclusion (Fig 4). Apart from MCF7 and ZR-75-1, the 7 other breast cancer cell lines reached half maximal lethality with BA concentrations ranging from 5.5 to 16.2 μg/ml (Table 1). In some of these cell lines the highest BA concentration tested (20 μg/ml) induced significant levels of cell death, e.g. BT549 was killed for 95% after 48 hr, others were less sensitive (maximal lethalities induced by 20 μg/ml BA varied between 95% and 44%) (Fig 4). In the clonogenic assay, the 6 breast cancer cell lines that could be tested (BT483, BT474 and ZR-75-1 failed to form colonies at all) were all halted in their clonogenic potential at 7.5 or 10 μg/ml, indicating a blockage of clonogenic growth at relatively low BA concentrations as compared to the other assays (Fig 4, Table 1). It should be noted that MCF7, which completely lacked BA-induced cell death at 48 hr, was among these cell lines. Importantly, this cell line is caspase 3 and 10 deficient [37], which indicates that caspase 3/10 deficiency does not protect MCF7 from an abrogated colony formation induced by BA treatment, which is consistent with the fact that zVAD.fmk did not protect Jurkat T cells (Fig 1E). In the MTT assay we again observed a biphasic character of BA-sensitivity in 7 out of 9 breast cancer lines. This consisted of a sharp decline in MTT conversion at low BA concentrations followed by a more gradual reduction at higher concentrations. Only BT483, lacking the initial high responsive phase, and ZR-75-1, lacking the further reduction in MTT conversion with BA treatment above 2.5 μg/ml, did not show this biphasic response pattern. Thus, ZR-75-1 was the only cell line showing high resistance against BA treatment in both the cell death assay and MTT conversion assay. The other breast cancer cell lines were highly sensitive for BA in the MTT assay and the EC_{50} values in this assay, ranging from 2.4 to 11.6 μg/ml, were considerably lower than the BA concentrations needed for half maximal cell death measured by PI exclusion.

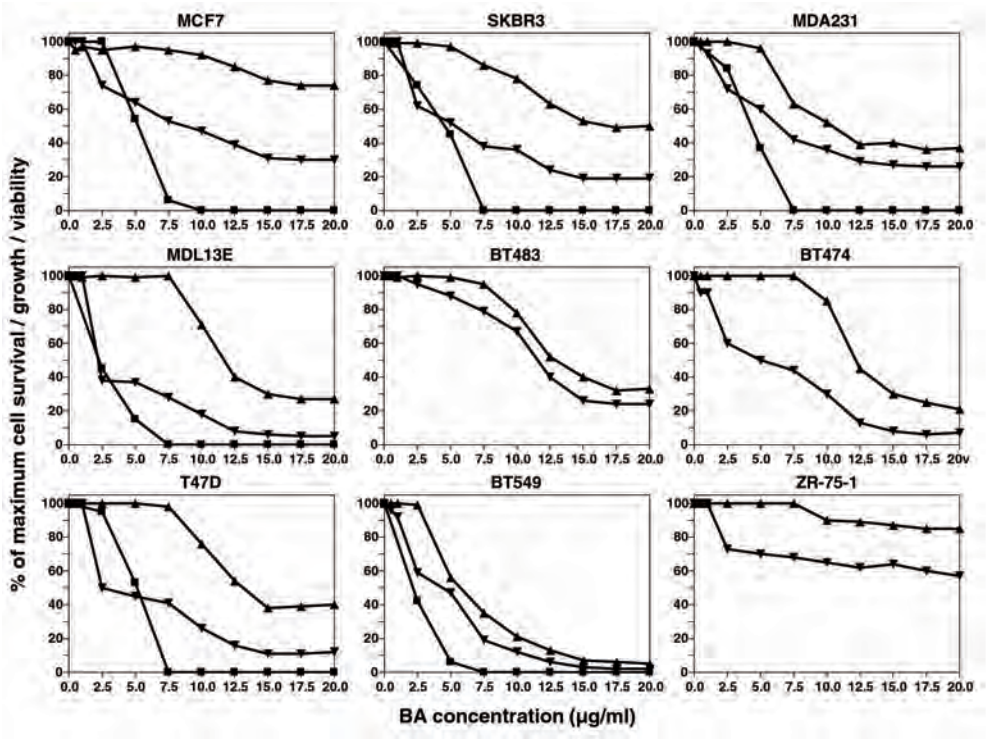

Fig 4. BA sensitivity of 9 breast cancer cell lines.
Cytotoxic effects measured with PI exclusion at 48 hr (▲), cell viability measured at 48 hr with MTT conversion (▼) and clonogenic growth (■) were tested for the indicated concentrations of BA.

Sensitivity to BA treatment of prostate and cervix cancer cell lines

Finally, we tested cell line panels derived from prostate cancer (DU145, PC3, 22Rv1, LNCaP) and from cervix carcinoma (CaSki, HeLa, SiHa). Where prostate cancer is the second cause of death from cancer in man [24], cervical cancer is leading to the highest cancer mortality in women in developing countries [30].

In general, treatment with BA of these cell lines resulted in a similar response pattern as observed in lung, colorectal and breast cancer cell lines. Maximal levels of cell death in these 7 cell lines after treatment with 20 µg/ml BA for 48 hr ranged from 73% to 58% as measured with PI exclusion (Fig 5, Table 1) and the EC_{50} values in this assay varied between 9.6 and 14.3 µg/ml for prostate and cervix cancer cell lines together (Table 1). Clonogenic growth was halted at 7.5 µg/ml for all cell lines, except 22Rv1 which was slightly more sensitive (Fig 5). In all cell lines, a sharp decrease in the level of MTT conversion was again observed at low BA concentrations and the half maximal effect in this assay was reached at markedly lower concentrations of BA than the EC_{50} values in the PI exclusion assay (Table 1).

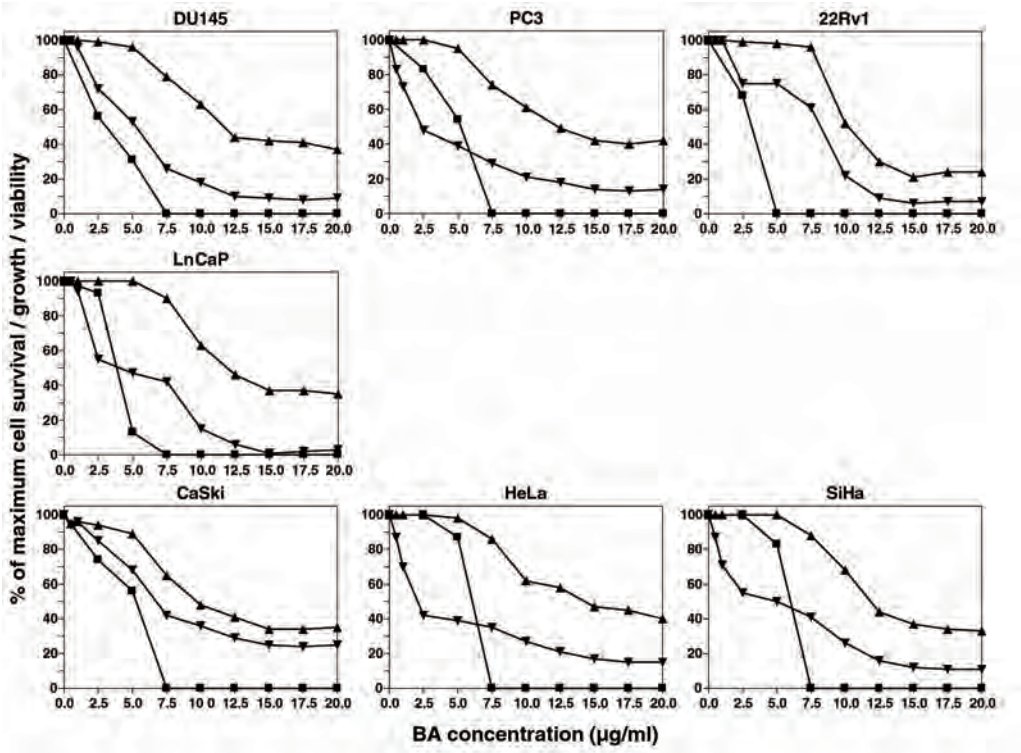

Fig 5. BA sensitivity of 4 prostate cancer cell lines and 3 cervical cancer cell lines.
BA sensitivity of 4 prostate cancer cell lines (DU145, PC3, 22Rv1, LnCaP) and 3 cervical cancer cell lines (CaSki, HeLa, SiHa) as measured with 3 different assays.
Cytotoxic effects measured with PI exclusion at 48 hr (▲), cell viability measured at 48 hr with MTT conversion (▼) and clonogenic growth (■) were tested for the indicated concentrations of BA.

Discussion

Although the efficacy of chemotherapy and other standard therapies for the majority of cancer types has been improved during the last decades, the treatment of most human malignancies is still facing high mortality rates. Moreover, toxic side-effects of the current chemotherapeutical drugs are often causing a severe reduction in the quality of life. Therefore, the development of novel potent, but non-toxic anti-cancer reagents is worth a continuous effort. Since its re-discovery in the 1990s BA has attracted considerable attention as a potential anti-neoplastic drug that may lack toxic effects towards healthy tissues.

The results of the current study show that BA treatment halted in vitro clonogenic growth of all cell lines tested - either derived from lung, colorectal, breast, prostate or cervix cancer - at remarkable uniform concentrations. Twenty seven out of 31 cell lines that were tested stopped to develop colonies at either 7.5 µg/ml or 10 µg/ml and the other 4 cell lines showed only slightly different sensitivities in this assay (Table 1). A much greater variety between the cell lines was observed in

overall cell death after 48 hr. Treatment with 20 μg/ml BA resulted in maximum lethalities at 48 hr ranging from over 90% in 5 cell lines (derived from lung, colon and breast cancer) to less than 25% in breast cancer cell lines MCF7 and ZR-75-1 (Table 1). For MCF7 this may be explained by its deficiency in caspase 3 and 10 [37], with that depriving this cell line from a main component (caspase 3) in the downstream apoptosis pathway. It should be noted, however, that co-treatment of BA together with ZVAD.fmk did not result in a significantly reduced cytotoxicity in Jurkat cells (Fig 1E), suggesting that caspase 3 deficiency is not the primary reason for the resistance of MCF7 after 48 hr. Importantly, the results of long term BA treatment in the clonogenic assay suggest that BA-induced cell death is rather delayed than absent in this cell line.

Despite the big differences in lethality within the different panels (as measured by PI uptake after 48 hr treatment with 20 μg/ml BA) there were no major differences in BA-induced cell death between the panels (Table 1). Our observations indicate an approximate equal sensitivity for BA-induced cell death of lung cancer, colorectal cancer, breast cancer, prostate and cervix cancer.

The anti-tumor activities of BA in the cell death assay after 48 hr either expressed as EC_{50} value or as maximal lethality with 20 μg/ml BA, did not correlate with BA sensitivities in the clonogenic assay. It is of note, for instance, that 4 out of the 5 cell lines that were not half maximally killed with 20 μg/ml BA treatment (SW1573, DLD1, HT29 and MCF7), were not more resistant for BA in the clonogenic assay when compared to the cell lines that did reach half maximal cell death. The most remarkable cell line in this respect was MCF7, which displayed only low level of cell death after 48 hr, while colony formation was completely halted at 10 μg/ml. Therefore, BA treatment induced either strong cytostatic effects or late cytotoxic effects. The latter is more likely as we did not observe living cells after a week culture, indicating that MCF7 cells do die later on in culture.

The MTT conversion assay was included in the current study because it has frequently been used to monitor BA activity [15, 32, 34, 38-43] and because our results in the Jurkat cell line showed an early BA effect at low concentrations in this assay that was missed by monitoring cell death only (Fig 1). In the panels of cancer cell lines, we also observed a sharp drop in MTT conversion at low BA concentrations (Fig 2-5). With the exception of ZR-75-1, all cell lines reached a half maximal reduction in enzymatic MTT conversion at BA concentrations that were consistently lower than those needed for half maximal cell death as measured by PI exclusion (Table 1). For ZR-75-1, which was also refractory in the PI uptake assay, the clonogenic potential could not be assessed. Therefore, this is the only cell line for which we can not make a final judgment of its overall BA sensitivity.

We can state that it is vital to analyze multiple assays in order to determine the efficacy of BA. For instance, the moderate cell lethality observed in some cell lines at 48 hr suggested the existence of cell lines that are (partially) refractory to BA, but monitoring of colony formation revealed that BA was toxic at relatively low concentrations in all cell lines tested (Table 1). On the contrary, our results show

that the MTT assay may lead to an overestimation of the anti-cancer capacity of BA when compared to the inhibition of colony formation. Thus, the current study demonstrates the importance to monitor BA-induced effects by a combination of different assays to allow a comprehensive evaluation of the anti-cancer efficacy of BA. In this light it is important to note that some of the cell lines tested in the current study have previously been reported to be insensitive for BA. For instance, MCF7 (breast cancer) and HT29 (colon cancer) were found resistant when DNA fragmentation was monitored[10], however our approach identified these cell lines sensitive for BA in the MTT and clonogenic assays (Table 1). For MCF7, BA sensitivity in the MTT assay has also been observed by Amico et al. [38]. Similarly, BA-induced effects in LNCaP [1] and DU145 [32] (prostate cancer) were reported to be only minimal, whereas we identify these cell lines as sensitive using the different assays (Table 1). Our study does confirm the BA sensitivity of non-small cell lung cancer cell lines A549 [33] and H460 [15] and prostate cancer line PC3 [34].

In the literature only a few cancer cell lines have been tested for BA-induced effects by the clonogenic assay [17, 44, 45], and none of the cancer cell lines tested in the current study have been monitored before in this assay. Differences between our results and the literature in the assessment of BA sensitivity of some of the current cancer cell lines (HT29, LNCaP, DU145) can be attributed mainly to the inclusion of the clonogenic assay in our study. The multiple mechanisms that possibly contribute to the cytotoxic and / or cytostatic effects of BA render the clonogenic assay, which is monitoring effects after relatively long incubation times and independent of the mechanism, an especially suitable assay to evaluate the anti-cancer potency of BA.

Overall, the results presented here identify cell lines derived from lung, colorectal, breast, prostate, and cervix cancer to be sensitive for BA treatment in vitro. Therefore, our study further substantiate the notion that BA is an anti-cancer reagent with a broad specificity that is not restricted to tumors from neuroectodermal origin only [15, 16]. Although in the cell death and MTT assays clear differences between the cell lines were observed (Table 1) and in the cell death assay partially refractory cell lines were found, the BA concentration at which colony formation was halted appeared remarkably equal for all cell lines tested, irrespective of the originating cancer source. A further evaluation of the potential applicability of BA as drug for the treatment of these clinically most important cancer types now urgently awaits animal studies. The documented low toxicity of BA against human primary cells of healthy tissues in vitro [13, 15, 17], which we confirmed (Fig 1G), as well as the reported absence of toxic side-effect in mice and rats [1, 15, 18], raise hope that the therapeutic window of BA is broad enough to reach therapeutic and/or prophylactic anti-cancer effects in vivo.

Acknowledgements

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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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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*.

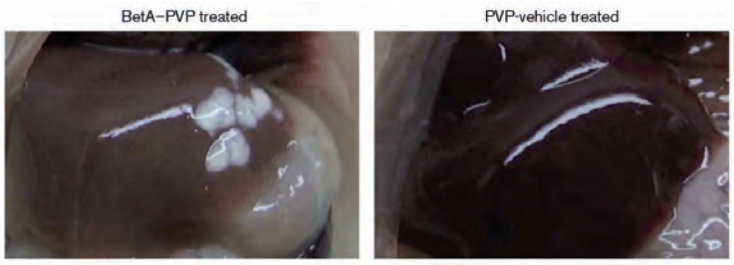

Figure 1: Formation of intra-abdominal deposits of BetA-PVP in treated mice.
White deposits of BetA-PVP complexes were observed on the liver of a representative mouse that received multiple injections of BetA-PVP (left), whereas mice that were injected with PVP as control vehicle only did not show deposits (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.

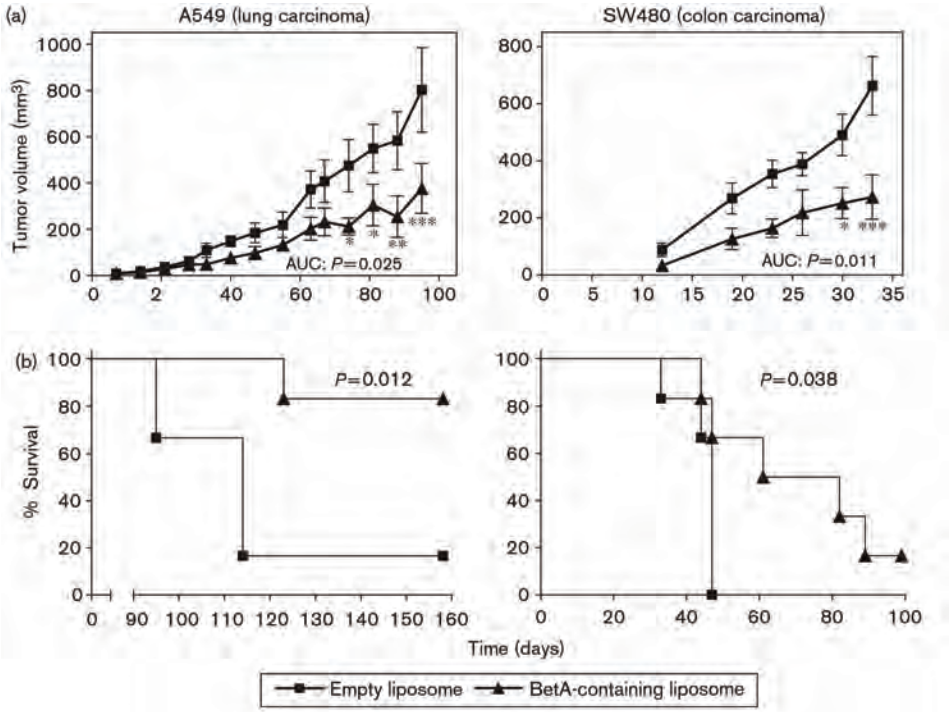

Figure 2: Intravenous administration of BetA-liposomes reduces tumor growth and prolongs survival of tumor-bearing mice.

*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.

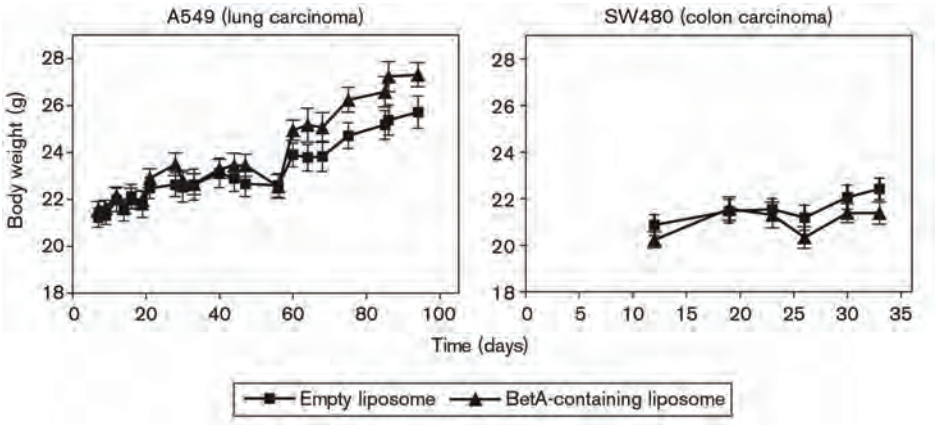

Figure 3: Body weight of tumor-bearing mice treated i.v with BetA-liposomes or control liposomes. *The average body weight per group of six mice bearing either A549 lung cancer tumors (left) or SW480 colon cancer tumors, monitored during the course of the experiment is shown.*

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.

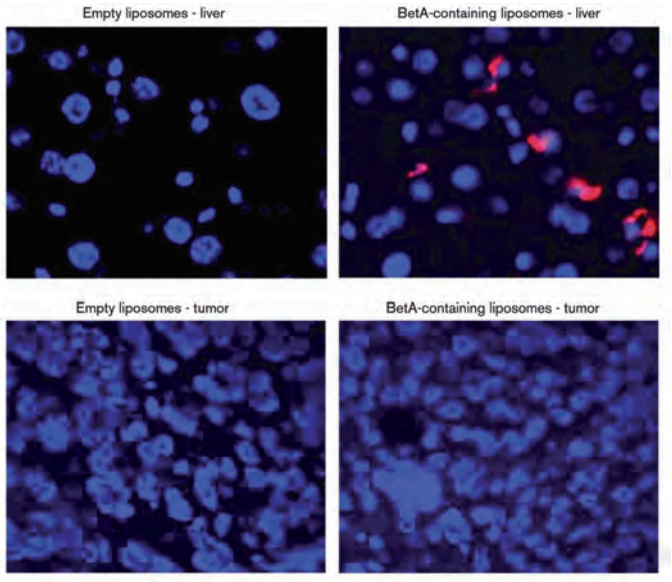

Figure 4: Fate of Rhodamine-PE labeled BetA-liposomes and empty liposomes in vivo.
Mice were injected with 200 µl Rho-PE-labeled BetA-liposomes or empty liposomes and killed after one hour. Organs (liver and kidney) and tumor were isolated and slides were prepared. Slides were stained with 4',6-diamidino-2-phenylindole (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).

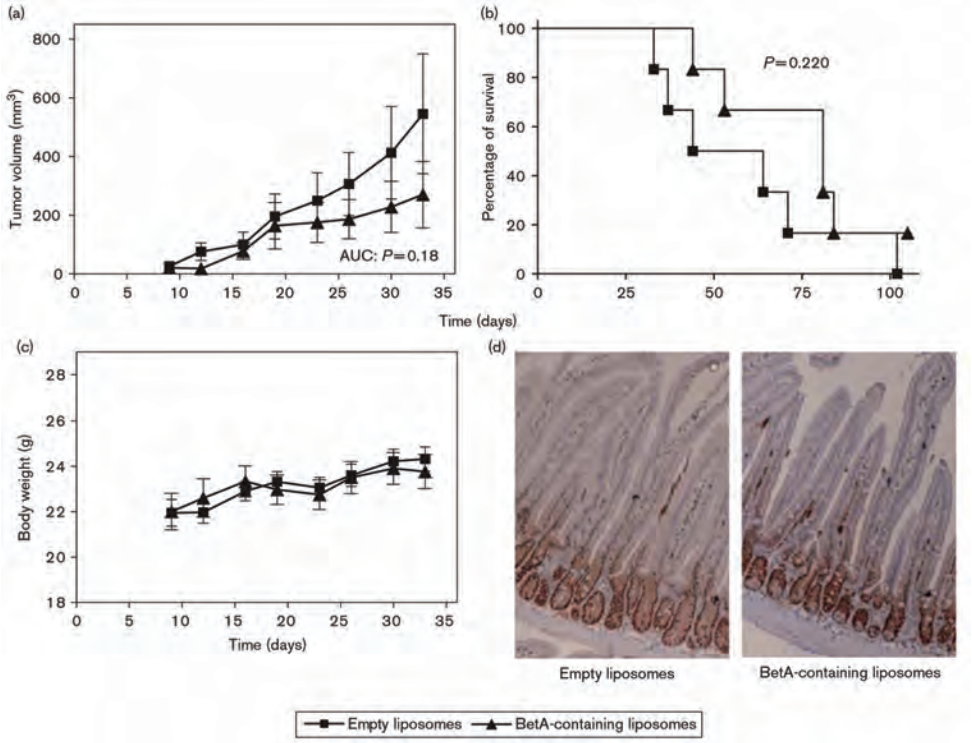

Figure 5: Oral administration of BetA-containing liposomes reduces tumor growth and prolongs survival of SW480 tumor-bearing mice.

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.

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Chapter 4

Betulinic Acid Induces Cytochrome c Release and Apoptosis in a Bax/Bak-Independent, Permeability Transition Pore Dependent Fashion

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Abstract

Betulinic Acid (BetA) is a plant-derived pentacyclic triterpenoid that exerts potent anti-cancer effects in vitro and in vivo, but is non toxic to untransformed cells. In our previous study we observed that BetA consistently induced cell death in a broad panel of tumor cell lines. Apoptosis induced by BetA involves activation of caspases, PARP cleavage and DNA fragmentation and was suggested to depend on the mitochondrial pathway. However, conflicting results have been reported with respect to the role of the pro- and anti-apoptotic members of the Bcl-2 family, which are often aberrantly regulated in tumors and thereby confer growth and survival advantages.

Here we show that BetA-induced apoptosis critically depends on the release of cytochrome c from the mitochondria and formation of the apoptosome. Nevertheless, over-expression of Bcl-2 or Bcl-XL only provides limited protection against BetA-induced apoptosis. More importantly, Bax/Bak deficient cells are as sensitive to BetA as their wild-type counterparts, suggesting that cytochrome c is released in a non-classical fashion. In agreement, pre-incubation with cyclosporin A indicated a crucial role for the mitochondrial permeability transition pore (PTP) in the induction of apoptosis.

Our observations therefore indicate that BetA affects mitochondria and induces cytochrome c release directly via PTP. This is only temporarily prevented by anti-apoptotic members of the Bcl-2 family, but independent of Bax and Bak. These findings help to explain the remarkable broad efficacy of BetA against tumor cells of different origin and its effect in tumor cells that are resistant to other chemotherapeutic agents.

Introduction

Betulinic Acid (BetA) is a naturally occurring triterpenoid that has been initially described to specifically kill melanoma cells via induction of apoptosis [1] and was later shown to have specificity for neuroectoderm-derived tumors. However, it is becoming clear that its efficacy extends to many other cancer cell lines derived from a variety of different malignancies such as leukemia, prostate, ovarian, breast, lung and colon cancer [2-6]. Importantly, BetA-induced apoptosis appears to be independent of p53 [6-8], but does show remarkable selectivity for tumor cells over non-transformed cells.

The extrinsic or death receptor pathway is not involved in BetA-induced apoptosis [9]. However, formation of reactive oxygen species (ROS) and a decrease in the mitochondrial membrane potential have been repeatedly associated with BetA treatment [4, 10, 11], which prompted the hypothesis that BetA induces apoptosis via the mitochondrial pathway [12-14]. This pathway is normally regulated by a carefully balanced interplay between pro- and anti- apoptotic members of the Bcl-2 family. Over-expression of pro-survival molecules, such as Bcl-2, Bcl-XL or Mcl-1 or deletion of pro-apoptotic members, such as Bax and Bak, or alternatively deregulation of BH3-only molecules like Bim or Puma, is often observed in tumors and causes resistance of these cells to intrinsic death stimuli [15].

In agreement with a role for the mitochondria in BetA-induced apoptosis is the observation that over-expression of Bcl-2 or Bcl-XL prevents BetA-induced cytochrome c release, caspase activation and PARP cleavage in SHEP neuroblastoma cells [12, 13]. However, separate studies have shown that BetA rather increases the expression of Bcl-2, but can also modulate the expression of other pro-and anti- apoptotic Bcl-2 family members with distinct outcomes. For instance, Mcl-1 was strongly induced in melanoma cells after treatment with BetA [7], while expression of Bcl-XS and Bax were induced in neuroblastoma cells after treatment with BetA [7, 9]. These conflicting observations make it difficult to assess the role of the Bcl-2 family in BetA-induced apoptosis. Nevertheless, the broad anti-tumor effect of BetA renders it unlikely that induction of apoptosis would strictly depend on the classical Bcl-2 regulated mitochondrial pathway, as this is often disrupted in tumor cells [7, 15]. Previously we have shown that in Jurkat T leukemia cells apoptosis is induced upon incubation with BetA. Both classical and non-classical pathways to apoptosis exist [7, 16, 17] but the mechanism used by BetA remains incompletely defined. We therefore set out to delineate the signaling pathway and specifically the role of the Bcl-2 family regulated pathway in BetA-induced apoptosis in further detail. We show that Bcl-2 does not provide effective protection against BetA-induced apoptosis, especially not at later time points. Moreover, for the first time, we demonstrate that Bax/Bak double deficient mouse embryonic fibroblasts (Bax/Bak DKO MEFs) display cytochrome c release, caspase activation and PARP cleavage upon BetA treatment. This indicates that BetA does not induce a classical mitochondrial pathway to apoptosis. The mitochondria are critically involved though, as inhibition of the

mitochondrial permeability transition (PT) pore by cyclosporin A (CsA) in combination with Bcl-2 over-expression provided effective protection from BetA induced apoptosis.

Materials and methods

Chemicals

Betulinic Acid (≥99% pure, Bioservice Halle) was dissolved at 4 mg/ml in DMSO and aliquots were stored at -80°C. zVAD.fmk (benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone), etoposide, TMRE (Tetramethylrhodamine ethyl ester perchlorate), cyclosporin A and propidium iodide (PI) were purchased from Sigma-Aldrich. Anti-APO-1 was a kind gift from Dr Peter Krammer.

Antibodies

For western blot analysis anti-caspase-3 (AF-605-NA, R&D and #9662, Cell Signaling) was used as well as an antibody specific for cleaved capsase-3 (Asp175, Cell Signaling) for mouse embryonic fibroblasts, furthermore anti-PARP (#9542, Cell Signaling), anti caspase-7 (#9492, Cell Signaling), anti-Bak (#06-536, Millipore) anti-Bax (N-20, Santa Cruz), anti Bcl-XL (B22630, BD; SC-634, Santa Cruz) and anti-Bcl-2 (N-19, Santa Cruz) were used. Anti-cytochrome c for FACS staining was obtained from BD (clone 6H2.B4).

Cells

Jurkat cells over-expressing Bcl-2, Bcl-XL and wild-type (wt) control cells were obtained from Dr Jannie Borst (NKI, Amsterdam), MCF-7/FAS and MCF-7/FAS Bcl-2 or Bcl-XL were obtained from Dr Marja Jäättelä (Danish Cancer Society, Copenhagen) [18]. FADD-deficient, Casp-8- deficient and control Jurkat cells (JA3) were obtained from Dr John Blenis (Harvard Medical School, Boston), Bax/Bak DKO MEFs and wild-type control MEFs were from Dr Stanley Korsmeyer. APAF-1 and Caspase-9 knockout and wild-type MEFs were from Dr Tak Mak (Univ of Toronto, Canada) and Dr Richard Flavell (Yale, New Haven, USA) respectively. SW480 were provided by by J. van Eendenburg and Dr. A. Gorter (dept. Pathology, Leiden University Medical Center, The Netherlands), A549 were from ATCC, and HCT116 lines were from Dr G. C. Chinnadurai (St Louis University School of Medicine, USA) and Dr B.Vogelstein (Johns Hopkins, USA). All cells were cultured in IMDM supplemented with 8% FCS, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin.

DNA fragmentation

Apoptotic DNA fragmentation was measured as previously described [6,7]. Briefly, cells were resuspended in Nicoletti buffer containing 50 µg/ml PI for a minimum of 24 hours. Afterwards DNA content was determined in the resulting nuclei using flow cytometric measurement of PI.

Western blot analysis

Cells were lysed in Triton X-100 buffer on ice and protein was quantified using a BCA kit from PIERCE according to manufactures protocol. 10-15 µg total protein was loaded per lane for SDS-PAGE and blotted onto PVDF transfer membrane (Amersham Biosciences). Blocking of unspecific binding sites was performed overnight at 4^{o}C in 5% low fat milk powder in PBS/0.2% Tween-20 (blocking buffer), blots were then incubated with the primary antibody for two hours at room temperature in blocking buffer, washed and incubated in blocking buffer with a secondary, HRP labelled antibody. For chemiluminescent visualization, ECL from Amersham Biosciences was used.

Cytochrome c release by FACS staining

Cytochrome c release assay was performed according to the protocol of Waterhouse [7,19]. Briefly, cells were trypsinized, washed with PBS and incubated with 50 µg/ml digitonin in PBS with 100 mM KCl for 5-10 minutes. Permeabilization of the cell membrane was assessed using trypan blue exclusion. When the majority of the cells in the aliquot used for trypan blue staining were penetrated by the dye, cells were fixed in 4% paraformaldehyde in PBS for 30 min, washed and incubated in blocking buffer (3% BSA, 0.05% saponin, 0.02% azide in PBS supplemented with normal goat serum, dilution 1:200) for one hour at room temparature. Anti-cytochrome c incubation was done overnight at 4 ^{o}C, cells were washed three times and FITC conjugated secondary antibody was applied for one hour at 4 ^{o}C. After another washing step cells were analyzed by flow cytometry.

Assessment of mitochondrial membrane potential

Cells were incubated in growth medium containing 25 nM TMRE for 15-20 minutes at 37 ^{o}C, resuspended in HEPES buffer, pH 7.4, containing 25 nM TMRE and analysed by flow cytometry.

Results

BetA-induced apoptosis depends on the apoptosome.

To determine whether apoptosis is induced in a classical caspase-dependent fashion, Jurkat cells were treated with BetA in the presence or absence of the pan-caspase inhibitor zVAD.fmk. In line with previous observations, BetA induced caspase-3 and PARP cleavage as well as DNA fragmentation and all these effects were inhibited in the presence of 20 µM zVAD.fmk (Fig. 1A) [6, 12, 13].

The death receptor or extrinsic pathway is not involved in BetA-induced apoptosis [2, 9, 11, 20]. In agreement, Jurkat cells deficient in either FADD (FAS Associated Death Domain) or caspase-8, two crucial mediators in the extrinsic pathway, revealed similar sensitivity to BetA, whereas these cells were fully protected against CD95-induced apoptosis (Fig. 1B). These results suggest that BetA is able to induce caspase activation in a death receptor-independent and therefore likely a mitochondria dependent fashion. To validate whether the apoptosome, the signaling complex for capase-9 activation, is crucial for downstream caspase activation we used cells devoid of a functional apoptosome due to deletion of either Apaf-1 or caspase-9. MEFs lacking caspase-9 or Apaf-1 and wild-type MEFs were treated with various concentrations of BetA and apoptosis induction was analyzed after 24 h. Importantly, processing of both caspase-3 and PARP was blocked in caspase-9 deficient cells and in cells lacking Apaf-1 (Fig. 1C), indicating that the apoptosome is a key platform for downstream caspase activity in BetA-treated cells.

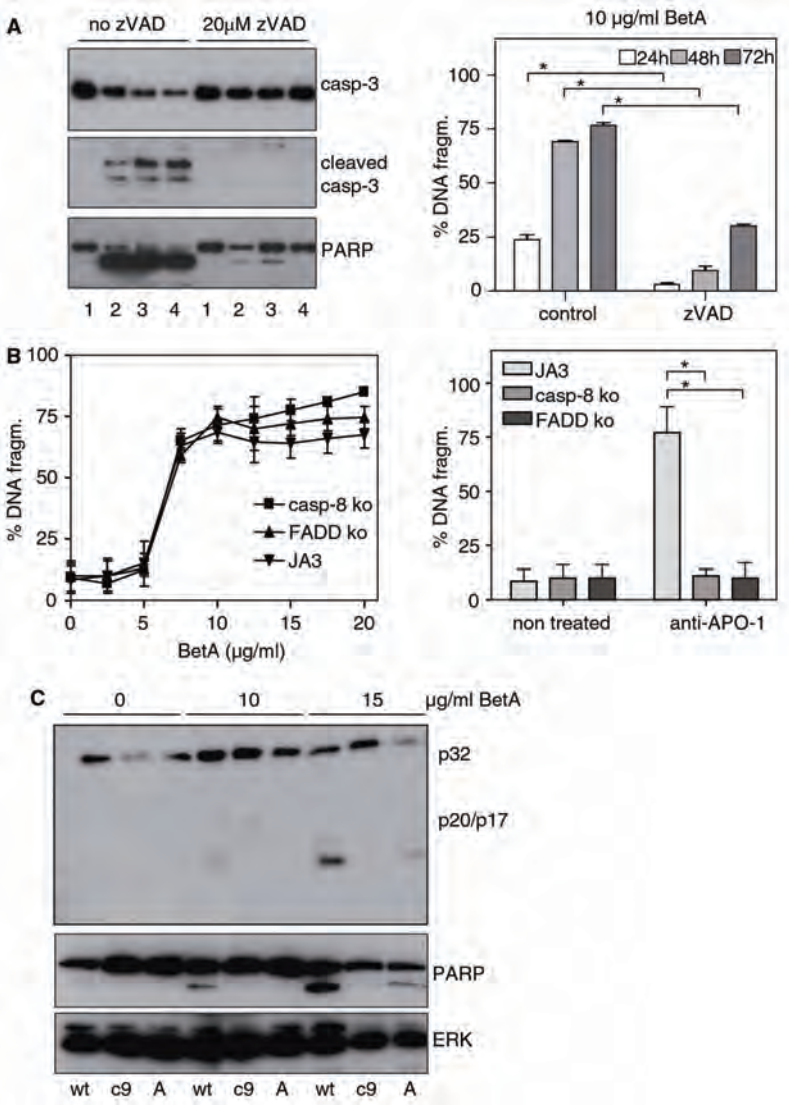

***Fig. 1.* BetA induced apoptosis depends on the apoptosome.**

A. Jurkat cells were pretreated with 20 μM zVAD.fmk for 2 h before addition of 10 μg/ml BetA. After 24 h of treatment cells were subjected to western blot analysis (lanes: 1: non treated; 2: Betulinic Acid; 3: 5 μg/ml Etoposide; 4: 1 μg/ml anti-APO-1). DNA fragmentation of BetA treated cells was monitored after 24, 48 and 72 h. Etoposide and anti-APO-1 were included as a control for caspase-3 and PARP cleavage.

B. Jurkat control (JA3), FADD deficient (FADD ko) and caspase-8 deficient (casp-8 ko) cells were treated with indicated concentrations of BetA or anti-APO1 (1 μg/ml) for 48 h and subjected to nicoletti analysis.

C. Wild-type (wt), caspase-9 knockout (C9) and Apaf-1 knockout (A) mouse embryonic fibroblasts (MEF) were treated with 10 or 15 μg/ml BetA for 24 h and caspase-3 and PARP cleavage was analysed by immunoblotting.

*Symbols: *: statistical significant difference (t-test) $p < 0.05$*

Anti-apoptotic Bcl-2 family members partially prevent BetA-induced apoptosis

Previously, the neuroblastoma cell line SHEP was shown to become resistant to BetA when over-expressing Bcl-2 [9, 12, 13]. This would fit the role of cytochrome c in the induction of apoptosis. In agreement with these observations we detected that BetA-induced DNA fragmentation after 24 h was reduced, albeit partially, by Bcl-2 over-expression in Jurkat cells (Fig. 2B, 2C). Surprisingly, this protective effect was lost at later time points and after 72 h of treatment DNA fragmentation was induced as effectively in Bcl-2 over-expressing cells as compared to control cells (Fig. 2C). In contrast, etoposide-induced DNA fragmentation was prevented by Bcl-2 at all time points analyzed (Fig. 2A). This indicates that the level of Bcl-2 was sufficient to provide protection against a typical mitochondrial-dependent drug, but that BetA can circumvent this protection and BetA induced apoptosis is only delayed by Bcl-2.

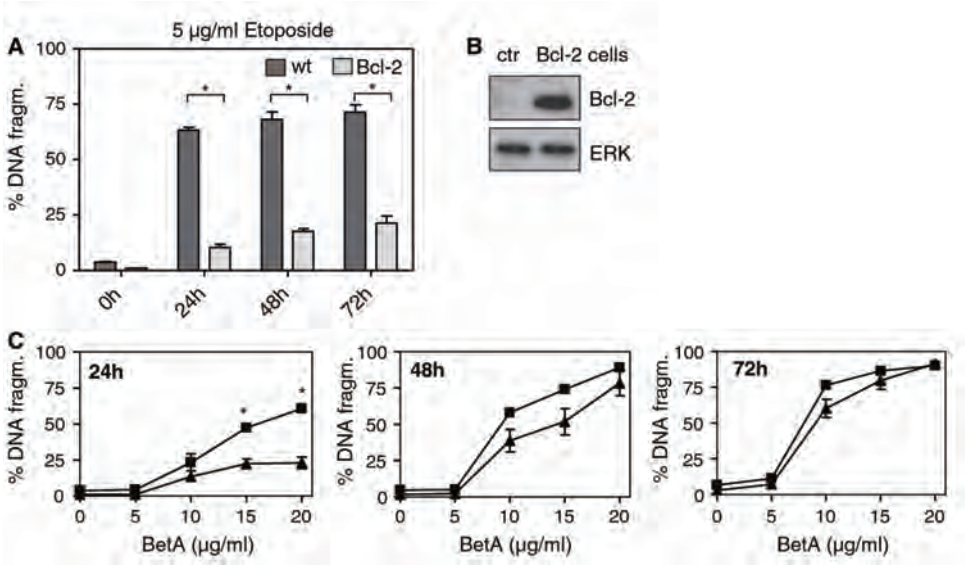

***Fig. 2.* Bcl-2 over-expression delays BetA induced apoptosis in Jurkat cells.**
A. Vector control and Bcl-2 over-expressing cells were treated with 5 µg/ml Etoposide for 24, 48 and 72 h and DNA fragmentation was measured.
B. Vector control (ctr) and Bcl-2 over-expressing (Bcl-2) Jurkat cells were analyzed for Bcl-2 expression by immunoblot analysis.
C. Vector control(■) and Bcl -2 over-expressing (▲) Jurkat cells were treated with indicated concentrations of BetA for 24, 48 and 72 h and DNA fragmentation was assessed.
*Symbols: *: statistical significant difference (t-test) p< 0.05*

To further dissect the role of the Bcl-2 family in BetA-induced apoptosis and to generalize these observations we used different lines expressing high levels of anti-apoptotic Bcl-2 family members. The breast cancer cell line MCF-7 has been shown to be relatively resistant to BetA-induced apoptosis as measured by PI

exclusion [6] and DNA fragmentation [9]. Nevertheless, MCF-7 cells are as sensitive as other tumor cells when clonogenic survival is measured [6]. MCF-7 lacks functional caspase-3 due to a frameshift mutation [21], which could explain its resistance to BetA-induced apoptosis. Indeed, when we measured DNA fragmentation by means of FACS analysis of propidium iodide stained nuclei, we did not observe a sub-G1 peak, which is indicative for DNA fragmentation (data not shown). To determine whether BetA was incapable of activating caspases in MCF-7 we also analyzed PARP cleavage, which is a general substrate for executioner caspases and is not necessarily impaired in cells lacking caspase-3 [22]. Interestingly, PARP was clearly processed 48 h after BetA addition (Fig. 3A). This is likely due to the activation of caspase-7, which was cleaved after BetA treatment (Fig. 3A). Similar to Jurkat cells, MCF-7 cells over-expressing Bcl-2 or Bcl-XL [18] were also clearly sensitive to BetA-induced caspase-7 cleavage and PARP processing, although partial protection was evident (Fig. 3B). Similarly, DNA fragmentation in Ramos cells over-expressing Bcl-2 or Mcl-1 was not prevented upon BetA treatment (data not shown), which further substantiates our conclusion that anti-apoptotic Bcl-2 family members do not provide effective protection against BetA-induced apoptosis, especially not at later time points.

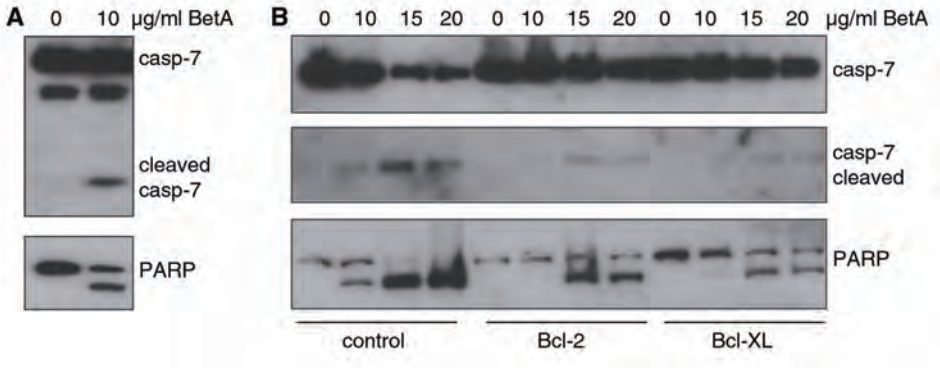

Fig. 3. ***Bcl-2 or Bcl-XL over-expression partially prevents BetA-induced apoptosis in MCF-7 cells.***
A. MCF-7 cells were treated with 10 µg/ml BetA for 48 h and PARP processing and caspase-7 cleavage was assessed.
B. MCF-7 FAS, MCF-7 FAS Bcl-2 and MCF-7 FAS Bcl-XL were treated with 10, 15 or 20 µg/ml BetA for 48 h and immunoblot staining was performed to assess caspase-7 and PARP cleavage. Immunoblot staining for capsase-7 and PARP was performed on the same blot.

Bax/Bak double deficient cells are sensitive to BetA-induced apoptosis

Because expression, interaction and regulation of Bcl-2 family member proteins is complex and can differ in distinct cell types [23, 24] we decided to make use of cells lacking both Bax and Bak. Different models exist describing the interaction between the pro-and anti-apoptotic molecules [25], however, it is agreed upon that Bax or Bak are required for classical cytochrome c release from the mitochondria and subsequent caspase activation [25, 26]. Indeed, MEFs lacking Bax and Bak (Fig. 4A) were resistant to starvation-induced apoptosis and death while wild-type MEFs readily showed caspase-3 activation (Fig. 4B) and morphological cell death (Fig. 4C). To determine if BetA-induced apoptosis was still observed in Bax/Bak DKO, we performed western blot analysis using antibodies specific for PARP and cleaved caspase-3. Strikingly both PARP and caspase-3 were processed as efficiently in the wild-type and Bax/Bak DKO MEFs (Fig. 4D). This suggests that BetA-induced apoptosis is activated in a Bax/Bak-independent, but mitochondria-dependent fashion. To rule out the possibility that these findings are selective for MEFs we also obtained HCT116 colon cancer cells that were made deficient for both Bax and Bak [27, 28]. Similar to the MEFs, we observed that BetA-induced apoptosis was readily induced in cells lacking Bax and Bak (Fig 4E), indicating that BetA induces apoptosis independent of these pro-apoptotic Bcl-2 family members.

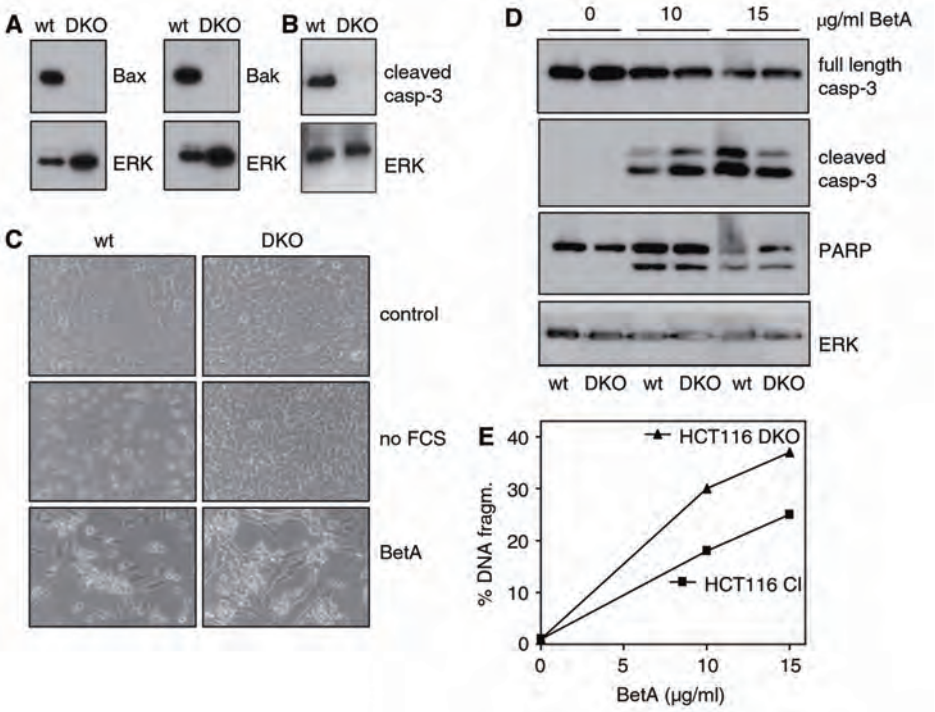

Fig. 4. ***Bax/Bak DKO MEFs are sensitive to BetA-induced apoptosis.***
A. Bax and Bak expression was determined in MEFs by immunoblot analysis.
B. Cells were functionally tested by washing three times with PBS and incubation in medium without serum (no FCS) for 24 h and subsequently tested for the presence of cleaved caspase-3 (casp-3 cleaved).
C. Wild-type (wt) and Bax/Bak double deficient (DKO) mouse embryonic fibroblasts were treated with indicated concentrations of BetA for 24 h or subjected to FCS withdrawal and photographed under a phase-contrast microscope at 100x magnification.
D. Wild-type (wt) and Bax/Bak double deficient (DKO) cells were treated with 10 or 15 µg/ml BetA for 24 h and immunoblot staining for cleaved caspase-3 and PARP processing was performed. ERK was used as a control for equivalent protein loading.
E. HCT116 CL (Bax KO) and HCT116 DKO (Bax KO and Bak KD) were incubated with 10 or 15 µg/ml BetA and DNA fragmentation was tested with nicoletti. Expression of Bax and Bak were validated by western (not shown).

Cytochrome c is released upon BetA treatment in Bax/Bak DKO MEFs

Although Bax and Bak appear to be dispensable, the apoptosome is crucial for BetA induced apoptosis. Functional apoptosome formation requires the release of cytochrome c from the mitochondria. We therefore analyzed whether cytochrome c was released upon BetA treatment. In untreated wild-type MEFs cytochrome c was detected at high levels in the mitochondria using intracellular FACS analysis (Fig.

5A). Treatment with BetA or FCS starvation for 24 h resulted in the appearance of a second peak that signifies cells that have released cytochrome c from their mitochondria, indicating that BetA and starvation both induce cytochrome c release. Importantly, cytochrome c release was also detected in casp-9 and APAF-1 KO MEFs (data not shown), which shows that their lack of apoptosis induction is not due to a lack of mitochondrial dysfunction. As Bax/Bak DKO MEFs undergo apoptosis as efficiently as wild-type, our results could point to a Bax/Bak-independent release of cytochrome c. In agreement, a similar percentage of Bax/Bak DKO cells displayed cytochrome c release upon BetA treatment (Fig. 5B). It is important to note that the level of cytochrome c retained in the mitochondria of treated cells appeared slightly higher in Bax/Bak DKO cells (Fig. 5A). This indicates that the number of cells with cytochrome c release is the same, but that the amount of release per cell seems to be less, potentially due to a lack of amplification. BetA thus releases cytochrome c in a Bax/Bak-independent fashion.

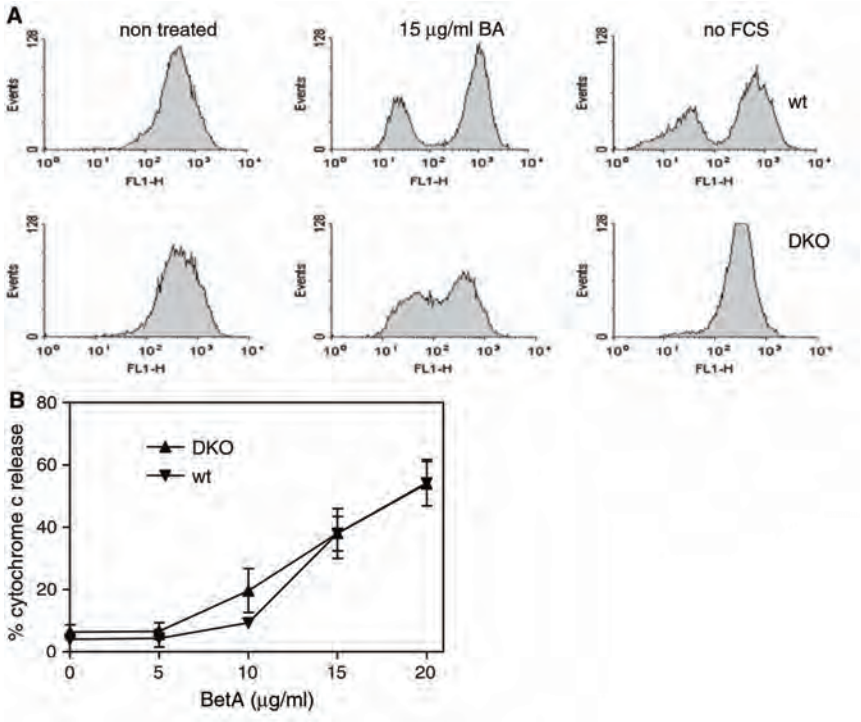

Fig. 5. BetA induces Bax/Bak independent cytochrome c release.
Wild-type (wt) and Bax/Bak double deficient (DKO) mouse embryonic fibroblasts (MEFs) were treated for 24 h with indicated concentrations of BetA or subjected to FCS withdrawal and intracellular staining for cytochrome c release was performed. Representative FACS histograms for cytochrome c release are shown (A), release was quantified using CellQuest software (B).

BetA induced mitochondrial depolarization and apoptosis is dependent on the PT pore

Combined these observations indicate that apoptosis induction by BetA is independent of Bax and Bak, but is, at least partially, affected by Bcl-2 or Bcl-XL over-expression. This suggests that the pathway induced by BetA is not a classical mitochondrial Bcl-2 family-dependent one. Previous findings have indicated that under specific conditions, anti-apoptotic Bcl-2 family members can be cleaved and thereby converted into pro-apoptotic molecules directly facilitating cytochrome c release [29, 30, 31]. Although such a pathway could explain our current observations, no evidence of cleavage of either Bcl-2 or Bcl-XL was detected upon BetA treatment (Fig. 6A). Moreover, the levels of Bcl-2 and Bcl-XL compared to a control protein ERK-2 were unaffected upon BetA treatment.

The role of the mitochondrial permeability transition (PT) pore is controversial in apoptosis signaling. Some results strongly indicate that it is a secondary effect that is observed only upon downstream caspase activation, while other observations rather propose a causal role for the PT [32]. Opening of the PT pore results in membrane depolarization and is suggested to also lead to cytochrome c release plus subsequent apoptosis. The composition of the pore that plays a role in apoptosis is still a matter of debate. Initial observations indicated that the pore consists of VDAC, ANT and cyclophilin D [33]. However, recent findings on knockouts lacking all ANT or VDAC forms suggest a more complicated picture [34, 35], at least when looking at the role of this pore in apoptosis. Nonetheless, induction of PT can be directly affected by Bcl-2 family members [36]. We therefore tested the role of PT using TMRE to measure the mitochondrial membrane potential. We found that BetA induced a significant mitochondrial depolarization in Jurkat cells after 24 h, which was independent of caspase activation (Fig. 6B). Importantly, depolarization was reduced in cells over-expressing Bcl-2 (Fig. 6C). Etoposide, a more classical mitochondria-dependent compound, induced depolarization even more dramatic in control cells, but this effect was completely blocked by Bcl-2 (Fig. 6D) and to some extent caspase-dependent (not shown). This again points to the fact that BetA utilizes a different mechanism to target the mitochondria. To more directly analyze the involvement of the PT pore in BetA-induced apoptosis we made use of cyclosporin A (CsA), an inhibitor of cyclophilin D that prevents opening of the pore and thereby depolarization [36]. In agreement, CsA reduced BetA-induced loss of TMRE staining by almost 50% in Jurkat control cells and completely prevented mitochondrial depolarization (Fig. 6C) and cytochrome c release (Fig. 6E) in Jurkat-Bcl-2 cells. In contrast, CsA did not have any protective effects on etoposide treated cells (Fig. 6D). More importantly, apoptosis as measured by DNA fragmentation after 72 h of BetA treatment was substantially inhibited by CsA alone and almost completely when it was combined with Bcl-2 over-expression (Fig. 6F).

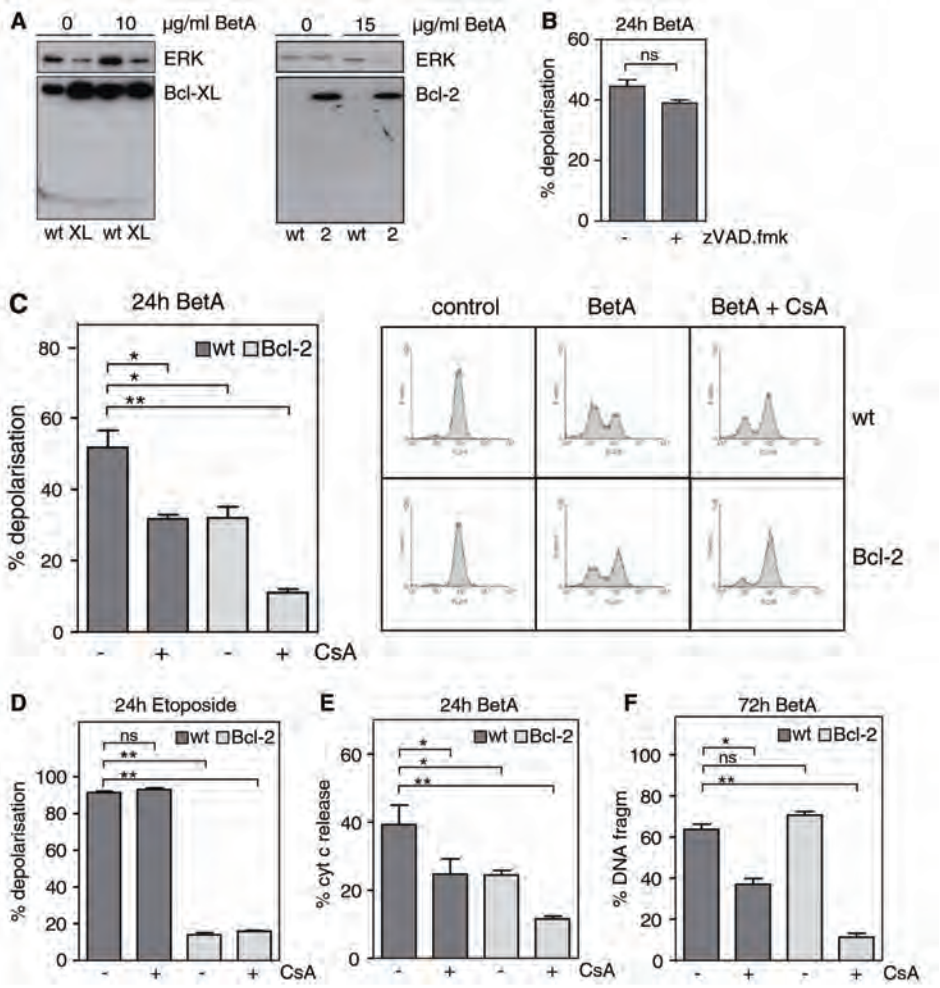

Fig. 6. ***BetA induced mitochondrial depolarization and apoptosis is dependent on the PT pore.***
A. BetA treated Jurkat wild-type (wt), Bcl-2 or Bcl-XL (XL) over-expressing cells were lysed after 24 h of treatment and stained with anti Bcl-2 or anti Bcl-XL antibody. Erk is shown as loading control.
B. Jurkat wild-type cells were pre-treated with 20 µM zVAD-fmk for one hour before BetA addition (10 µg/ml). After 24 h mitochondrial depolarization was measured using TMRE.
C. Jurkat wild-type and Bcl-2 over-expressing cells were pre-incubated with or without 5 µg/ml cyclosporin A (CsA) for 45 minutes and exposed to 10 µg/ml BetA for 24 h. Mitochondrial depolarization was measured using TMRE. Representative FACS histograms are shown and depolarization was quantified using CellQuest software.
D. Experiment was performed as described above (C) but instead of treatment with BetA, cells were treated with 5 µg/ml etoposide for 24 h.
E. Jurkat wild-type and Bcl-2 over-expressing cells were pre-treated with or without 5 µg/ml cyclosporin A (CsA) for 45 minutes and exposed to 10 µg/ml BetA for 24 h and cytochrome c release was assessed using intracellular FACS staining.
F. Jurkat cells were treated as described above (E) and after 72 h DNA fragmentation was measured.
*(Statistics: t-test: Fig B ; one-way ANOVA: Fig C,D,E,F; symbols: n.s.: not significant; *: P < 0.05; **: P < 0.01)*

BetA induced PT-pore dependent apoptosis is not cell type dependent

Because Jurkat cells are very sensitive to CsA at higher concentrations (Fig. 7) it is difficult to assess if CsA could also provide complete protection to BetA-induced apoptosis in cells without Bcl-2 over-expression. In order to test this and to generalize our findings we used A549 (lung cancer cell line) and SW480 (colon cancer cell line), which tolerate higher concentrations of CsA (up to 50 µg/ml). Strikingly, in these cell lines we clearly observed that CsA completely blocked BetA induced apoptosis, even at high concentrations (15 µg/ml) of BetA (Fig 7). Similar results were obtained using HCT116 cells lacking Bax and Bak (Fig 7); again CsA provided very effective protection. This indicates that the mechanism of BetA induced apoptosis depends on the PT pore and is broadly applicable in a variety of tumor cells.

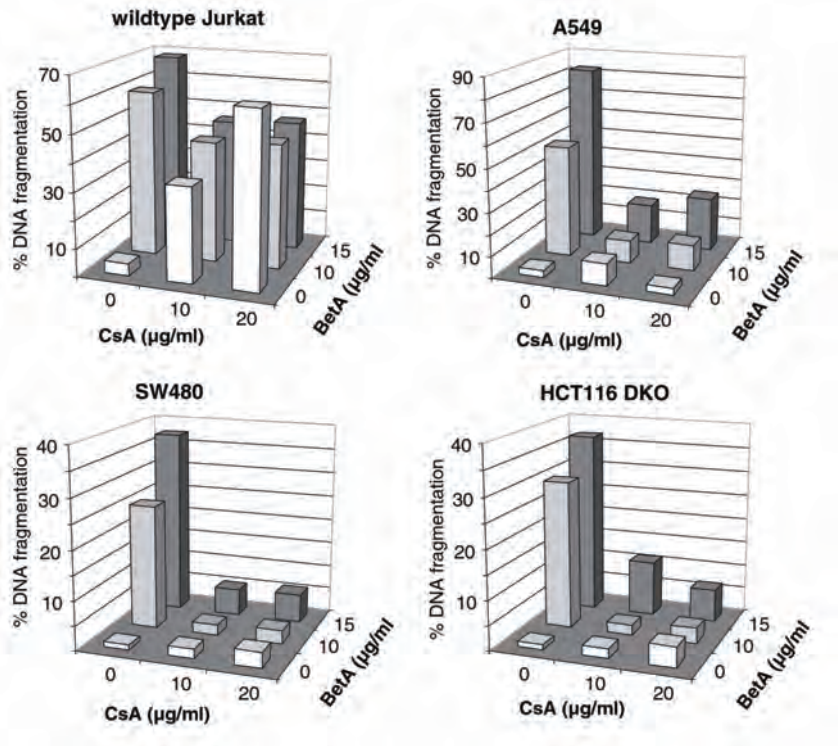

***Fig. 7* BetA induced PT-pore dependent apoptosis is not cell type dependent**
Jurkat wildtype, A549 (lung cancer cell line), SW480 and HCT116 Bax/Bak DKO (colon cancer cell lines) were pre-treated for one hour with increasing concentrations of CsA and subjected to 10 or 15 µg/ml BetA for 48 h after which DNA fragmentation was assessed.

Discussion

BetA is a very potent compound that is capable of killing a plethora of tumor cells. Here we demonstrate that BetA induces apoptosis in a manner that is dependent on the apoptosome, but is not affected by a lack of pro-apoptotic Bcl-2 family members Bax and Bak. BetA appears to target the mitochondrial PT pore directly and mitochondrial depolarization indeed is shown to be prevented by CsA. The anti-apoptotic Bcl-2 family members provide some protection, but this is at best limited and overcome at later time points. Multiple tumor cell lines have been shown to resist classical mitochondrial death pathways as they have a disturbed ratio of pro- and anti- apoptotic Bcl-2 family members [15]. Our report now indicates that such disturbances are to a large extent irrelevant for the apoptosis induced by BetA.

Previous studies using the neuroblastoma cell line SHEP have demonstrated BetA-induced apoptosis to be completely abrogated by Bcl-2 over-expression [12, 13], which is in apparent contrast with our data. However, the same group showed that over-expression of Bcl-2 in Jurkat cells only provided about 65% protection after 24 h treatment [2]. Similarly, it has been shown in two human glioma cell lines and in melanoma cells that over-expression of Bcl-2 only partially reduced caspase-3 activity and cell death after BetA-treatment [11, 20]. This observation is now corroborated by us, but extended to later time points and other cell lines. We found that the partial protective effect is not detected at all at later time points where the sensitivity to BetA in Bcl-2 over-expressing cells and wild-type cells was similar (Fig. 2C). It is important to note that this failure to protect is not due to the level of Bcl-2 (Fig. 2B) as etoposide-induced death is blocked at all time points tested (Fig. 2A). Previously, Chintharlapalli et al. reported that BetA directly affects transcription factors sp1, 3 and 4 and that this would lower transcription of survivin as well as Bcl-2 [37, 38]. As such, BetA could lower Bcl-2 expression and thereby induce apoptosis. Although this is likely to affect sensitivity of tumor cells indirectly, we do not believe that this is the explanation for the differential sensitivity that we observe between BetA and etoposide. First and most important, Bcl-2 and Bcl-XL in our system are not dependent on their endogenous promoter, but on the strong CMV or EBV promoter and are thus not affected by sp1, 3, 4 down regulation. Secondly, even in the non-over-expressing Jurkat lines, we do not observe a decrease in Bcl-2 or Bcl-XL expression 24 hr after BetA treatment.

The Bcl-2 independency of our system is even further supported by our observation that BetA-induced apoptosis is independent of the pro-apoptotic Bcl-2 family members Bax and Bak. In Bax/Bak DKO MEFs caspase-3 and PARP processing was induced with equal efficiency as in wild-type cells (Fig. 4D). Previously, Liby et al. showed that Bax/Bak DKO MEFs were sensitive to BetA derivatives, but resisted BetA induced apoptosis [39]. It is difficult to provide a rational for this discrepancy, especially as we also observe BetA-induced apoptosis in Bax/Bak deficient HCT-116 cells. We do observe a slight difference in cytochrome c release though, which is less pronounced in Bax/Bak DKO MEFs (Fig. 5A), but

importantly, the amount of cells releasing cytochrome c is identical (Fig. 5B). The small difference in cytochrome c release is therefore either the result of less mitochondria per cell that release cytochrome c or that less cytochrome c is released per mitochondria. We believe this signifies the absence of an amplification loop initiated by active caspase-3, which cleaves Bid and thereby targets the mitochondria to induce maximal release. In agreement, caspase-8 processing occurs as an event downstream of the mitochondria in BetA treated neuroblastoma cells [12], and both caspase-8 and Bid-cleavage were shown for BetA treated Jurkat cells [2]. Bcl-2 over-expression and Bax/Bak deficiency could therefore disallow this amplification and cells would thereby show less cytochrome c release.

Mitochondrial membrane depolarization is a heavily debated issue in apoptosis signaling. There is agreement on its occurrence, but the causal role for cytochrome c release is clearly disputed [32]. We show that etoposide-induced loss of TMRE is blocked by Bcl-2 (Fig. 6D), and that BetA-induced depolarization is only partially prevented by Bcl-2 (Fig. 6C) but appears to serve a causal role as suggested by the inhibition of apoptosis by CsA (Fig. 6F). These observations point to a model in which BetA directly targets the pore and thereby allows cytochrome c release and downstream caspase activation. Bcl-2 family members have been shown to directly bind VDAC and regulate its pore forming activity. The pro-apoptotic member Bax promotes opening, while the anti-apoptotic members induce closure [40]. A direct Bcl-2 family member-independent opening of the PT pore by BetA is consistent with our current observations. These would be independent of Bax and Bak, but could be hampered by Bcl-2 or Bcl-XL, which independently promote closure of the pore [41]. The fact that CsA prevents pore opening and reduces apoptosis significantly, combined with the observation that this effect is complete in Bcl-2 over-expressing Jurkat cells adds to this hypothesis. It is important to note though that higher concentrations of CsA are capable of complete protection against BetA-induced apoptosis in three separate tumor lines. In Jurkat cells we failed to obtain full protection due to the high toxicity of CsA itself on these cells. However, A549 lung carcinoma cells, SW480 colon carcinoma cells as well as Bax/Bak deficient HCT-116 colon carcinoma cells all withstood CsA up to concentrations of 50μg/ml and showed complete inhibition of BetA-induced apoptosis by CsA. Previous observations have shown that bongkrekic acid, a separate PT pore inhibitor, can also prevent BetA-induced cytotoxic effects [13] and thus support this model even further. We therefore believe that BetA targets the PT pore directly and thereby induces mitochondrial-dependent apoptosis. Other compounds, such as gossypol [42], A23187/ArA [43] and chelerythrine [44] have recently also been shown to induce Bax/Bak-independent cytochrome c release. Although for A23187/ArA-induced release this is suggested to be a serine protease dependent mechanism, chelerythrine, which shares some structural similarity with BetA, can induce release from isolated mitochondria. Although chelerythrine was developed as a Bcl-XL inhibitor, it also appears to induce mitochondrial permeabilization and apoptosis in Bax/Bak DKO cells, which is prevented by CsA [44]. Combined, this may point to a mechanism in which the lipophilic characteristics of these

compounds provide them with the means to target the outer mitochondrial membrane and thereby potentially affect the PT pore. In agreement with this idea is the observation that the PT pore opening is affected by the cholesterol content of the outer mitochondrial membrane [32]. As cholesterol and BetA are structurally related, BetA could either modify this effect or have similar activity itself. Importantly, some tumor cells have been shown to contain higher cholesterol levels in their mitochondrial membrane and lowering this sensitizes them to apoptosis [32]. More importantly, reconstituted PT pores in artificial membranes localize to cholesterol areas and can be opened using long chain fatty acids [45], indicating that lipids have a strong modulating effect on the pore. Although these data provide an appealing explanation for the broad effects of BetA on tumor cells, it still remains difficult to understand why it is without effect on untransformed cells. As mentioned, the lipid content of the mitochondria in tumor cells may be different to some extent, but this is unlikely to explain the selectivity for tumor cells. Especially as the changes observed in tumor cells rather prevent then facilitate apoptosis induction. It is possible though that another component associated with the PT-pore is a crucial determinant in the toxicity exerted by BetA. This component, hexokinase is associated with the pore on the cytoplasmic side and regulates pore opening as well as the level of glycolysis in a cell [46]. It is well established that tumor cells mainly utilize glycolysis for their ATP generation. This so called Warburg effect is potentially regulated by a differential expression level of hexokinase II in tumor versus normal cells [47]. Inactivation of hexokinase II using 3-bromopyruvate displays similar tumor selectivity as BetA [46]. In summary, we conclude that our study provides an explanation as to why BetA is a very effective and broadly applicable anti-cancer agent, even against tumor cells which have acquired resistance to other, Bcl-2 family dependent, apoptosis inducing treatments. Future experiments will provide more mechanistic insight into the exact mechanism by which BetA modulates the PT-pore and whether the tumor selectivity involves PT pore sensitivity and/or hexokinase activity.

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Chapter 5

Mitochondrial Damage Triggered by Betulinic Acid Induces Autophagy in Tumor Cells

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Manuscript in preparation

Abstract

Betulinic acid (BetA) is a naturally occurring triterpenoid with strong anti-cancer activity. It is effective against common cancer types and was shown to induce apoptosis via a direct effect on the mitochondria. This is largely independent of Bax and Bak, but can be inhibited by cyclosporin A, an inhibitor of the permeability transition pore. Importantly, blocking apoptosis does not rescue cells, indicating that alternative, caspase-independent cell death pathways such as necrosis or autophagy might be activated. Autophagy was initially discovered to act as a survival pathway, induced upon stress signals or starvation, but also plays a role in cell death. We hypothesized that upon BetA treatment autophagy is activated as a response to the mitochondrial damage. In agreement, mitochondrial concentric cristae are induced by BetA and preceded the formation of autophagosomal structures. Importantly, cyclosporin A prevented the induction of autophagy, suggesting that autophagy is activated as a response to the mitochondrial damage induced by BetA. However, autophagy-deficient cells showed enhanced BetA-induced cell death, which let us to conclude that autophagy is induced as a survival pathway rather than a cell death pathway.

Introduction

BetA is a potent new anti-cancer agent with cytotoxic effects on cancer cells including cells derived from therapy resistant or relapsed tumors [1-5] whilst being non-toxic for healthy cells [6-8]. It was initially proposed to have a direct effect on the mitochondria and to induce Bcl-2 family of proteins-dependent apoptosis [9-11]. However, we previously showed that even though Bcl-2 over-expression can provide short term protection, Bax/Bak double-deficiency does not protect cells from BetA induced apoptosis [12]. Moreover, caspase inhibition was not able to rescue BetA-treated Jurkat T leukemia cells from cell death. There was no difference observed in cell death as measured by PI exclusion in cells subjected to BetA in the presence or absence of the pan-caspase inhibitor zVAD.fmk. At the same time, caspase-3 and PARP cleavage were effectively blocked [4]. A separate study also concluded that treatment of SHEP neuroblastoma cells with BetA and TRAIL involves, next to caspase-dependent, caspase-independent mechanisms [13]. Despite the existence of multiple downstream cell death mechanisms, cyclosporin A (CsA), an inhibitor of the permeability transition pore, was able to protect BetA treated cells from cytochrome c release, apoptosis and cell death [12]. This suggests that apoptosis is not the sole cell death pathway in BetA-treated cells, but that cell death depends on mitochondrial insults that are counterbalanced by CsA. We therefore hypothesized that the mitochondrial damage induced by BetA is not compatible with life and that other cell death pathways apart from apoptosis are induced in BetA-treated cells. Alternative cell death pathways include necrosis, mitotic catastrophe and autophagic cell death [14]. Necrosis or necroptosis, a highly regulated form of necrosis, is induced upon strong cytotoxic insults, mechanical injuries of cells or simply when apoptosis is blocked for example in the presence of zVAD.fmk [15]. Typical features include swelling, rupture of organelle membranes as well as the outer cell membrane and as a result the cell contents are released, often causing inflammation *in vivo* [14,16,17]. Mitotic catastrophe – as the name suggests – is induced upon treatments resulting in impairment of mitosis. It typically occurs when cell-cycle checkpoints are defective [18] and it is morphologically characterized by the presence of enlarged, multinucleated cells [14].

Autophagy is a highly conserved and complex process, which is activated typically during nutrient starvation. It involves several steps including sequestration, degradation and amino acid/peptide generation [19]. This is achieved via induction of an isolation membrane that surrounds cytoplasmic components including organelles (cargo) and formation of the so called autophagosome. The autophagosome is surrounded by a double membrane, which allows the subsequent fusion of the outer membrane with the lysosome to form the autophagolysosome. Lysosomal enzymes will then degrade the cargo to release monomeric units to the cytosol to be reused [19,20].

Autophagy serves primarily as a survival pathway but in recent years autophagy formation has also been associated with programmed cell death [21].

Here we describe the formation of autophagosomal structures upon treatment with BetA and their role in BetA-induced cytotoxicity. The formation of these autophagosomal structures was prevented in the presence of CsA. Furthermore we show that the mitochondrial damage induced by BetA involves morphological changes of the mitochondria, namely the formation of concentric cristae.

Material and Methods

Chemicals/antibodies:
Betulinic acid (BioSolutions Halle, Germany, ≥99% purity) was dissolved in DMSO at 4 mg/ml and stored at -80°C. Propidium iodide (PI), ionomycin, 3-Methyladenine (3-MA) and cyclosporin A were purchased from Sigma-Aldrich. C14-valine (CFB75-50uCi) was purchased from Amersham and rapamycin from VWR. Anti-LC3 antibody (51-11) and anti-cytochrome c (clone 6H2.B4) were obtained from MBL and BD respectively.

Cells:
Caspase-9 knockout (Casp-9 ko) and control mouse embryonic fibroblasts (MEFs) were from Dr. Richard Flavell (Yale, New Haven, USA) HeLa cells and MCF-7 cells were derived from the ATCC, while MCF-7 cells over-expressing LC3-GFP were generated as described [22]. ATG5 knockout and its control MEF were kindly provided by Noboru Mizushima [23] and the ATG7 knockout and their respective control MEF were obtained from Masaaki Komatsu [24]. All cells were cultured in IMDM supplemented with 8% FCS, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin.

Cell death analysis and cytochrome c release by FACS staining:
Cells in suspension were incubated with 1 µg/ml PI prior to analyzing them via flow cytometry. Cytochrome c release was performed as previously described [12,25]. Briefly, after trypsinization, cells were incubated with 50 µg/ml digitonin in PBS with 100 mM KCl for 5-10 minutes. An aliquot of cells was used for trypan blue staining to monitor membrane permeabilization. Cells were fixed using 4% paraformaldehyde in PBS. After 30 minutes, cells were washed and incubated in blocking buffer (3% BSA, 0.05% saponin, 0.02% azide in PBS supplemented with normal goat serum, dilution 1:200) for one hour at room temperature. Anti-cytochrome c staining was performed according to standard protocols and cells were subsequently analyzed by flow cytometry.

LC3 fluorescence microscopy:
MCF-7 cells over-expressing LC3-GFP were cultured in 24 well plates and treated with BetA and/or CsA for 20 hr. HeLa cells were transiently transfected with tandem fluorescent LC3 (mRFP-EGFP-LC3, kindly provided by Yoshimori [26]) using Fugene 6 transfection reagent according to manufacturer's instruction (Promega, Leiden, The Netherlands). 24 hours after transfection cells were treated with 10 µg/ml BetA. After treatment cells were fixed using 4% paraformaldehyde/PBS and subsequently analysed by microscopic analysis using a Zeiss Axiovert 200 fluorescent microscope.

Western blot analysis:
MCF-7 cells over-expressing LC3-GFP were lysed in Triton X-100 buffer and subjected to protein quantification using a BCA kit (PIERCE). 15 µg protein per lane were applied for SDS-PAGE. For subsequent blotting a PVDF transfer membrane (Amersham Biosciences) was used. Blocking of unspecific binding sites was performed overnight at 4°C in 5% low fat milk powder in PBS/0.2% Tween-20 (blocking buffer). Blots were then incubated with the primary antibody for 2 h at room temperature in blocking buffer. Membranes were washed and incubated again in blocking buffer with a secondary, HRP (horse radish peroxidase) labeled antibody. For chemiluminescent visualization, ECL (Amersham Biosciences) was used.

Degradation of long lived proteins:

25000 MCF7 cells were seeded in 12 well plates. The next day cells were labelled with C-14 valine (50 µCi/ml 1:250 diluted in cell culture medium). After one day a cold chase using cell culture medium only was performed before adding BetA or rapamycin for various time points. Supernatants were collected separately from cells and both were precipitated for 30 minutes on ice in 10% TCA (trichloroacetic acid). Precipitates were collected via centrifugation for 10 minutes at 10000 rpm and subsequently dissolved in 0.5m NaOH. Radioactivity in supernatant and cell samples was measured and the ratio determined. The relative degradation ratio in the control cells was set to "one" and compared to the degradation ratio in BetA and rapamycin treated samples.

Electron microscopy:

Cells were fixed overnight in 4% paraformaldehyde in 0.1M Na-cacodylate buffer (Ph 7.4). After fixation the cells were washed in 0.1M Na-cacodylate buffer (Ph 7.4) and osmicated for 1 hour in 1% OsO_4 in distilled water and washed in distilled water. For contrast enhancement in the electron microscope, cells were block stained overnight in 1.5% aqueous uranyl acetate. Dehydration was done through a series of ethanol's and embedded in epon LX-112 (ladd) and polymerized for 48 hours at a temperature of 60 °C. Ultrathin sections of 80 nm were cut on a Reichert EM UC6 with a diamond knife, collected on formvar coated grids and stained with uranyl acetate and lead citrate. Sections were examined with a Fei Technai-12 electron microscope.

Results

Caspase-9 deficiency does not prevent BetA-induced cell death and cytochrome c release

Our previous results showed that caspase inhibition with the chemical pan-caspase inhibitor zVAD.fmk did not provide protection from BetA-induced cell death despite clearly blocking apoptotic features such as caspase cleavage, PARP processing and DNA fragmentation [4]. BetA-induced apoptosis has been linked to the mitochondrial pathway of apoptosis [9-11]. The initiator caspase for this pathway is caspase-9, which is activated in a complex called the apoptosome. MEFs deficient for caspase-9 are able to resist BetA-induced caspase-3 and PARP processing [12]. Because zVAD.fmk only blocked apoptotic features, but not cell death in BetA-treated cells, we decided to investigate if cells deficient for APAF-1 or caspase-9 die upon BetA treatment. We subjected wild-type, APAF-1 and caspase-9 deficient MEFs to BetA or serum withdrawal for 24 hours and subsequently analyzed cell death. Whereas the APAF-1 and caspase-9 deficient cells subjected to serum withdrawal were clearly protected from cell death, we observed no defect in BetA-induced cell death as measured by PI exclusion in these cells (Fig 1A). As expected, cytochrome c release was also still observed upon BetA treatment of caspase-9 and APAF-1 deficient MEFs (Fig 1B). These results suggest that the damage induced by BetA at the mitochondrial level is not capable of inducing apoptosis in apoptosome-deficient cells, but still is incompatible with life.

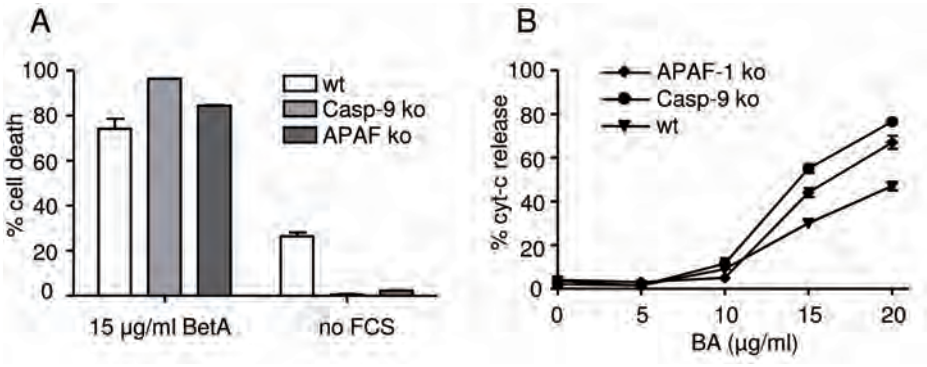

Figure 1. Caspase-9 deficiency does not prevent from BetA induced cell death

(A) Wildtype (wt), APAF-1 deficient (APAF ko) or caspase-9 deficient (casp-9 ko) mouse embryonic fibroblasts were subjected to 15 µg/ml BetA or FCS withdrawal for 24 hours. Cell death was assessed via PI exclusion.

(B) Wildtype (wt), caspase-9 deficient (casp-9 ko) and APAF-1 deficient (APAF-1 ko) mouse embryonic fibroblasts were treated with various concentrations BetA for 21 hours and cytochrome c release was assessed.

BetA induces mitochondrial morphology changes

To further evaluate the nature of the mitochondrial damage induced by BetA we analyzed BetA-treated cells via transmission electron microscopy (TEM). The mitochondria showed a typical structure in untreated HeLa cells with classic longitudinal cristae being observed. In contrast, in BetA-treated HeLa cells the mitochondria were more electron dense with a marked change in the inner structure of the mitochondria. Concentric instead of longitudinal cristae were observed concentric (Fig 2A). To rule out that the induction of concentric cristae is a cell type-specific effect, we also analyzed BetA-treated MCF7 cells and obtained similar results. TEM of control MCF7 cells displayed a less clear-cut mitochondrial morphology, but also in MCF7 cells BetA treatment disrupted the mitochondrial structure and induced the formation of concentric cristae (Fig 2B). Pre-treatment with CsA, which we reported to prevent BetA-induced death, only slightly weakened these effects on the mitochondria (Fig 2B), suggesting that it either is a to a large extent independent effect or upstream of the CsA inhibited step.

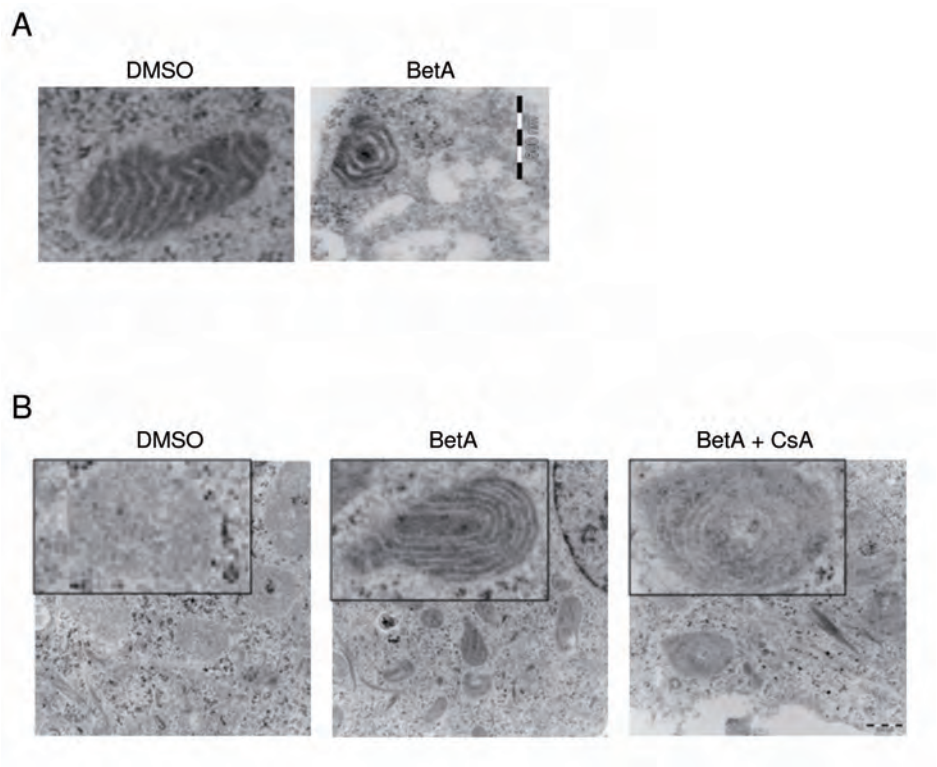

Figure 2. BetA induces mitochondrial morphology changes

(A) HeLa cells were subjected to 7.5 µg/ml BetA for 18 hours and analyzed via electron microscopy.

(B) MCF7-LC3-GFP cells were treated for 18 hours with 10 µg/ml BetA alone or in combination with 5 µg/ml CsA and analyzed via electron microscopy.

BetA induces autophagy

BetA induces severe mitochondrial changes (Fig 2), which implies that energy maintenance may be hampered as well and mitochondrial replacement and/or repair is required. Mitochondrial damage has been associated with autophagy, with the term mitophagy specifically describing the elimination or turnover of mitochondria via autophagy [27]. Autophagosome-formation requires two conjugation systems, one involving LC3-I (light chain-I) conjugation to phosphatidylethanolamine (PE). A common way to monitor autophagy is to analyze processing of LC3-I to PE-conjugated LC3-II via immunoblotting. The amount of LC3-II closely correlates with the number of autophagosomes, but it is important to note that LC3-II itself is also degraded during the process of autophagy, therefore results have to be evaluated with caution [28,29]. Ionomycin, a classic inducer of autophagy [22], resulted in enhanced LC3-II levels in MCF7 cells as compared to the DMSO-treated control cells (Fig 3A). BetA had a similar effect on LC3 processing in these cells, but was clearly more potent than ionomycin (Fig 3A). For classic, starvation-induced autophagy, class III phosphoinositide 3-kinase (PI3K) is required [22]. In agreement, ionomycin-induced autophagy, which is associated with this mTOR (mammalian target of rapamycin) regulated pathway, is prevented by 3-methyladenine (3-MA), an inhibitor of class III PI3K. BetA-treated cells on the other hand did not show a decrease in LC3-II in the presence of 3-MA (Fig. 3A), suggesting that BetA-induced autophagosome-formation might be induced via a different pathway or is too potent to be inhibited by 3-MA.

Conjugated LC3 (LC3-II) is associated with autophagic membranes and accordingly fusion of LC3 to green fluorescent protein (GFP) can be used to detect autophagosomes [29]. In untreated cells, LC3-GFP is evenly dispersed in the cells, with a slight preference for the nucleus, whereas in cells where autophagy is induced, LC3 translocation is evident [29] (Fig. 3B). In MCF7-LC3-GFP cells treated with BetA, autophagosomal structures were clearly distinguishable (Fig 3B). However, LC3-translocation was only limited after 6 hours of BetA treatment, while treatment with the classic autophagy-inducer rapamycin, already caused a high level of LC3-translocation at this timepoint. After 20 hours on the other hand LC3-GFP punctuate staining was massive in the BetA-treated cells and even more pronounced than after treatment with rapamycin (Fig 3B).

Both, detection of LC3-II via immunoblotting and LC-3-GFP translocation only resemble the amount of autophagosomes present at a certain time-point, but do not give information about the cause of this phenotype. It is possible that an increased number of autophagosomes is caused by an inhibition of the basal autophagic flux (e.g. via inhibition of fusion of autophagosomes with lysosomes or defective degradative function of lysosomes) rather than by induction of more autophagy [30]. Especially because 3-MA did not block BetA induced LC3 processing and because lysosomal cell death is an important alternative cell death pathway we analysed whether BetA acted as an inhibitor rather than as an inducer of autophagy.

To evaluate this possibility we measured degradation of long lived proteins, which are in part degraded by autophagy. Rapamycin clearly induced degradation of long lived proteins after both 6 and 20 hours (Fig 3C). For BetA-treated cells degradation of proteins appeared delayed after 6 hours, but at 20 hours it was more pronounced as compared to rapamycin-induced degradation (Fig 3C). This is consistent with the timing observed for the appearance of autophagosomes and indicates that activation of autophagy is likely the reason for an increased number of autophagosomes in BetA-treated cells. Because degradation of long lived proteins is not solely specific for autophagy and cannot discriminate between proteasomal- and autophagosomal degradation we verified our results by a more specific assay using tandem fluorescent LC3 tagged by RFP and eGFP [26]. As RFP is pH stable, it will remain fluorescent after fusion of the autophagosome with the lysosomal compartment, while GFP loses its fluorescent activity. This therefore allows detection of autophagic flux by simply observing the formation of red fluorescent lysosomes from green/red fluorescent autophagosomes. Using this approach we observed that BetA treatment induced a functional autophagic flux (Fig 3D).

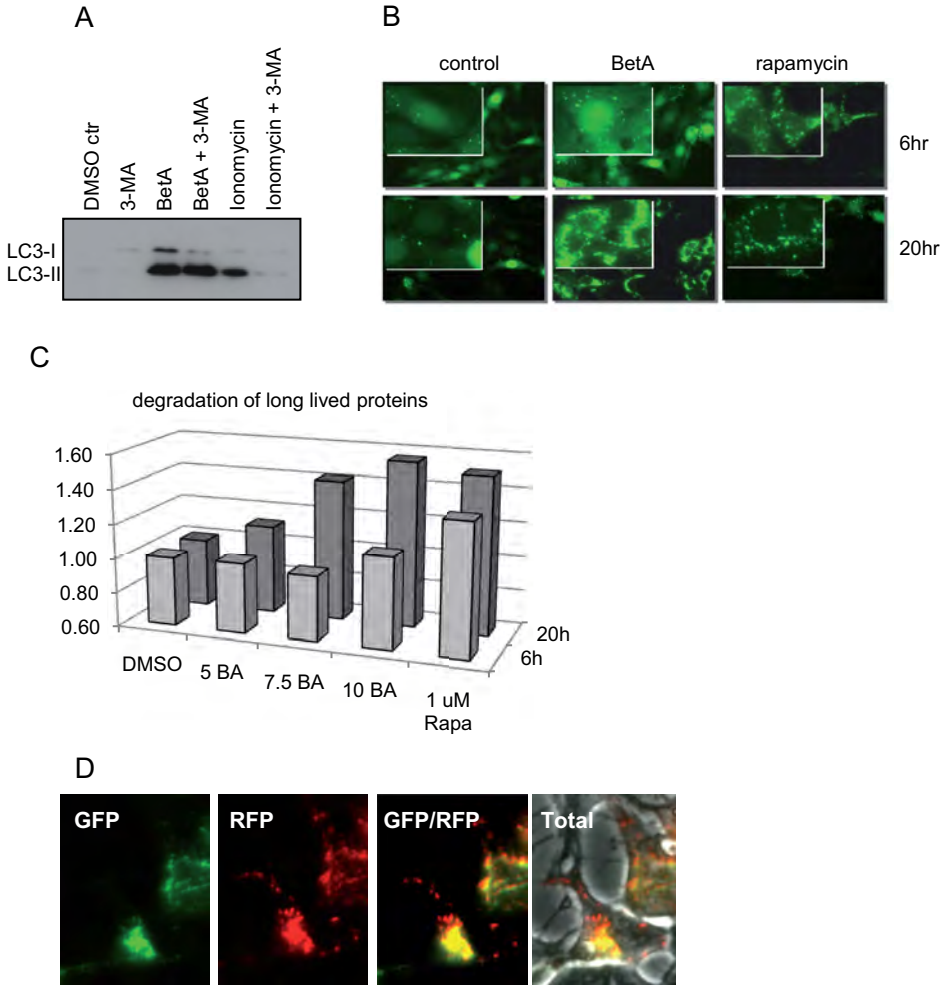

Figure 3. BetA induces autophagy

(A) MCF7-LC3-GFP cells were treated with either 5 µg/ml BetA or 10 µM Ionomycin for 48 hours either alone or in combination with 10 mM Methyladenine (3-MA). For combination treatments cells were pre-incubated with 3-MA for 1 hour. LC3 processing was assessed via immunoblotting. Results for endogenous LC3 are shown.

(B) MCF7-LC3-GFP cells were treated with 10 µg/ml BetA or 1 µM rapamycin and after 6 and 20 hours cells were analyzed under a fluorescence microscope.

(C) MCF7 cells were treated with indicated concentrations of BetA or rapamycin and after 6 and 20 hours degradation of long lived proteins was measured.

(D) Transiently transfected HeLa cells were treated with 10 µg/ml BetA for 20 hours and analyzed by fluorescence microscopy.

Autophagy serves as a rescue pathway, not as an alternative cell death pathway in BetA-treated cells

Autophagy, even though initially regarded as a cell survival pathway, has a role in dying cells as well and has been suggested to serve as a balancing mechanism between cell survival and cell death [20]. We hypothesized that an over-response in autophagy could explain the cell death observed after BetA treatment in cells deficient in the execution of apoptosis.

Autophagy is detectable already at low, non-toxic BetA concentrations (5 µg/ml) and we reasoned that it is primarily induced as a rescue pathway, but that at higher BetA concentrations autophagy is induced beyond a certain threshold and shifts the balance from cell survival to cell death [31]. To test this theory we used mouse embryonic fibroblasts (MEFs) deficient for either ATG5 or ATG7, two crucial regulators of autophagy and treated them with various concentrations of BetA. Interestingly, in the absence of autophagy BetA-induced cell death was even more enhanced (Fig 4A, B), indicating that autophagy serves primarily as a survival pathway in BetA-treated cells and does not play a role as an alternative cell death pathway. Also zVAD.fmk, a pan-caspase inhibitor could not prevent enhanced cell death in autophagy-impaired MEFs (Fig 4A, B). These results suggest that BetA-treated cells ultimately die via an autophagy as well as caspase -independent mechanism.

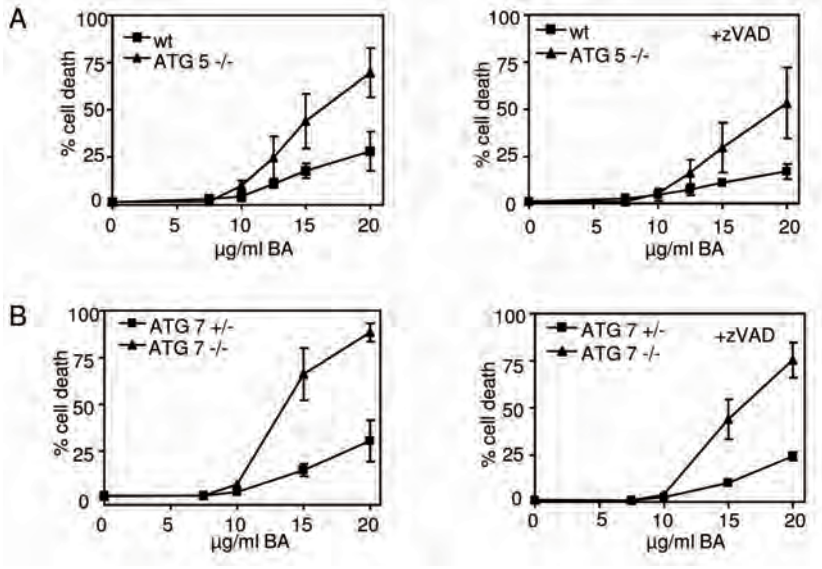

Figure 4. Autophagy serves as a rescue pathway, not as an alternative cell death pathway in BetA treated cells

Mouse embryonic fibroblasts lacking either ATG5 (A) or ATG7 (B) and their respective control cells were subjected to different concentrations of BetA for 24 hours in the presence or absence of 20 µM zVAD.fmk and cell death was measured via PI exclusion.

Cyclosporin A blocks BetA induced formation of autophagosomes

Typical inducers of autophagy include rapamycin, a direct inhibitor of mTOR (mammalian target of rapamycin), nutrient starvation and free cytosolic calcium [22,32] whereas 3-methylalanine (3-MA), wortmannin and PI3 kinase inhibitors, can block autophagy induced by afore mentioned triggers. BetA-induced autophagy, however, could not be blocked by 3-MA (Fig 3A) and wortmannin (data not shown). Previously, we have shown that cyclosporin A (CsA) is able to prevent BetA-induced cytochrome c release and apoptosis [12]. To test whether BetA-induced autophagy is also prevented by CsA we treated MCF7-LC3-GFP cells with BetA alone or in combination with CsA. Autophagosome formation was clearly inhibited in the presence of CsA (Fig 5), suggesting that BetA-induced autophagy is a consequence of the BetA triggered mitochondrial damage.

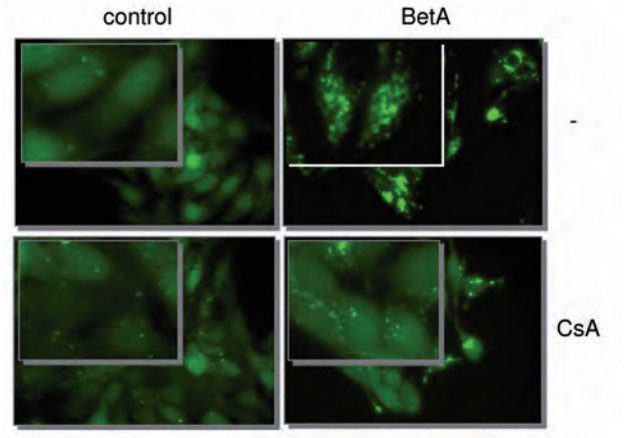

Figure 5. CsA blocks BetA-induced autophagosome formation

MCF7-LC3-GFP cells were treated with 10 µg/ml BetA for 24 hours in the presence or absence of 5 µg/ml cyclosporin A (CsA) and analyzed under a fluorescence microscope.

Discussion

BetA has been found to be a promising anti-cancer agent with apoptosis-inducing effects, which are independent of Bax and Bak but dependent on the permeability transition pore [12]. Here we show that BetA-induced cellular responses are complex and we reveal autophagy as a phenomenon induced by BetA (Fig 3). Although BetA has clear cell death inducing capacities, the autophagic response is induced as a survival pathway and not as an alternative cell death pathway (Fig 4A, 4B). This might explain why BetA-treated cells can withstand cell death for a longer period of time than typical apoptosis-inducers like CD95 activation or etoposide treatment. The latter two cause clear apoptotic features in Jurkat T leukemic cells already after 12 hours or earlier, whereas the first evidence of apoptosis/cell death upon BetA treatment is occurring only after 24 hours in these cells [4,33].

Even though our results suggest that autophagy does not primarily serve as a cell death pathway it is conceivable that it does play an indirect role in the toxicity exerted by BetA. Autophagy is already induced at low, non-toxic BetA concentrations (5 μg/ml, Fig 3A and data not shown), again hinting to a survival rather than a cell death-inducing function for autophagy. At such low BetA concentrations autophagy may succeed in re-establishing cell homeostasis with the outcome that cells survive [31]. At higher BetA concentrations, however, autophagy may keep the balance up for a limited period of time, after which the damage induced by BetA is too massive to be successfully counteracted by autophagy and results in cell death. The definite role of autophagy in this context still has to be unravelled, but autophagy is likely not crucial for BetA-induced cell death because cells deficient for ATG5 or ATG7 are not protected and are in fact even more sensitive to BetA-induced cell death (Fig 4).

An interesting connection between BetA-induced apoptosis and autophagy is the fact that both are efficiently inhibited by CsA, an inhibitor of the permeability transition pore (Fig 5) [12]. This hints to a scenario in which both pathways are triggered by a common upstream event that is related to the function of CsA. At this stage the precise molecular targets of BetA are not known but the mitochondria have been clearly shown to be involved. Interestingly we found that in BetA-treated MCF7 cells the mitochondrial structure is strongly affected (Fig 2B). Combined with the lipophilic nature of BetA, this might indicate an effect of BetA on mitochondrial membranes. Of note, it has been reported previously that BetA-induced damage is possibly associated with mitochondrial damage because alpha-DL-tocopherol (vitamin E), a lipophilic antioxidant was able to prevent tumor cells from BetA-induced apoptosis [34]. In contrast, hydrophilic antioxidants such as L-ascorbic acid (vitamin C) and N-acetyl-L-cysteine (l-NAC) did not provide protection against BetA induced cytotoxicity [34]. Here we show that the inner structure of the mitochondria was completely remodeled upon BetA treatment with the formation of concentric cristae (Fig 2A, 2B). How these concentric cristae relate to BetA-induced apoptosis and autophagy is not yet clear but such structures

have been associated with mal-functioning mitochondria, for example in patients with Barth syndrome [35]. Barth syndrome is characterized by a mutation in the tafazzin gene. Tafazzin is an important cardiolipin remodeling enzyme [36]. Cardiolipin is a crucial structural mitochondrial lipid that orchestrates oxidative phosphorylation. Moreover, it is involved in opening of the permeability transition pore and release of cytochrome c from the mitochondria [37]. Our preliminary observations indicate that BetA indeed modifies cardiolipin (data not shown), which may thus be an important trigger for the observed changes in the mitochondrial structure, contributing to the opening of the permeability transition pore and the subsequent release of cytochrome c. Mitochondrial damage would in that scenario first be expected to activate autophagy (mitophagy) in order to eliminate damaged mitochondria, but if the damage is too massive and can't be controlled by autophagy anymore, it would result in release of cytochrome c to the cytosol, activation of the caspase cascade and apoptosis. In cells with impaired apoptosis the mitochondrial damage would then also not be compatible with life and cells would die via other, caspase-independent mechanisms. Importantly, CsA, a specific inhibitor of PT-pore opening did not appear to sufficiently prevent the structural changes in the mitochondria, although it quite effectively blocked cell death and autophagy. This suggests that mitochondrial changes precede PT-pore opening and that the detrimental effects can be prevented when the pore is kept in a closed conformation. Further experiments are needed to prove this hypothesis, but our current data do provide compelling evidence for a non-classical, but central role of the mitochondria in BetA-induced cell death.

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Chapter 6

Betulin is a Potent Anti-Tumor Agent That is Enhanced by Cholesterol

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Abstract

Betulinic Acid (BetA) and its derivatives have been extensively studied in the past for their anti-tumor effects, but relatively little is known about its precursor Betulin (BE). We found that BE induces apoptosis utilizing a similar mechanism as BetA and is prevented by cyclosporin A (CsA). BE induces cell death more rapidly as compared to BetA, but to achieve similar amounts of cell death a considerably higher concentration of BE is needed. Interestingly, we observed that cholesterol sensitized cells to BE-induced apoptosis, while there was no effect of cholesterol when combined with BetA. Despite the significantly enhanced cytotoxicity, the mode of cell death was not changed as CsA completely abrogated cell death. These results indicate that BE has potent anti-tumor activity especially in combination with cholesterol.

Introduction

Triterpenoids are extensively studied for the potential use as anticancer agents. One of the most promising compounds in this class is Betulinic Acid (BetA), but its effect is limited by the poor solubility of the compound. A lot of effort is therefore put into the development of derivatives of BetA with the goal to develop even more powerful compounds and to achieve better solubility for enhanced in vivo administration [1–3]. BetA has been modified at many different positions including C1-4, C-20, C-28 and A-, D- and E ring with different outcomes [2,4]. For example, Kvasanica et al found 3beta-O-phthalic esters from BetA more cytotoxic and polar in comparison to BetA itself [5]. In contrast, generation of different C-28 ester derivatives did not result in enhanced cytotoxicity [4]. On the other hand, C-28 amino acid conjugates made by Jeong et al showed improved selective toxicity and solubility [6] and a C-3 modified BetA derivative has shown promising results in a human colon cancer xenograft model [2].

BetA can be found in numerous different plants, but it can also be obtained by a simple 2 step reaction from its more abundantly available precursor molecule Betulin (BE) [3]. BE is easily isolated and therefore plays an important role as raw material for the production of BetA and other biologically active compounds [7]. BE itself has been shown in the past to only possess limited or no cytotoxic effects on cancer cells [5,8]. For example it was shown to be inactive against MEL-2 (melanoma) cells when compared to other BetA derivatives [9]. Several other melanoma lines (G361, SK-MEL-28) leukemia lines (HL60, U937, K562), and neuroblastoma (GOTO, NB-1) cell lines were also found to be more resistant to BE than to other tested lupane triterpenes [10]. In contrast, a recent report found BE to be active against colorectal (DLD-1), breast (MCF7), prostate (PC-3) and lung (A549) cancer cell lines [11], and for A549 it was shown that apoptosis was induced [12]. Apoptosis is one of the major cell death pathways induced by anti tumor agents. In principle, two main pathways can be distinguished, the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway with the latter being regulated by the Bcl-2 family of proteins [13]. Numerous studies have shown that BetA induces apoptosis via the mitochondrial pathway [14–17], however, to our knowledge, it is currently not clear how BE induces cell death. Here we show that apoptosis induction by BE does not involve the death receptor pathway, but is dependent on the mitochondria. Nevertheless, similar as we have previously shown for BetA [17], cytochrome c release and caspase activation occur independently of the Bcl-2 family proteins but are blocked in the presence of cyclosporin A (CsA), an inhibitor of the mitochondrial permeability transition (PT) pore. Furthermore we found that cholesterol strongly enhances the cytotoxic effects induced by BE but not BetA. Our results suggest that BE should not be regarded as an inactive precursor, but as a potent anti-tumor agent.

Materials and methods

Chemicals:
Betulin ≥98% pure; Sigma -Aldrich, St Louis, MO, USA) and Betulinic acid ≥99% (pure; BioSolutions Halle, Germany) were dissolved in DMSO at 4 mg/ml, cholesterol (Sigma-Aldrich) was dissolved at 5 mM in DMSO. Aliquots were kept frozen. Propidium iodide (PI), zVAD.fmk (benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone), etoposide and cyclosporin A were purchased from Sigma-Aldrich, Mitosox was obtained from Invitrogen (Carlsbad, CA, USA).

Antibodies:
Anti-PARP (#9542; Cell Signaling Technology, Danvers, MA, USA) and anti-cytochrome c (clone 6H2.B4; BD Biosciences, San Diego, CA, USA) were used.

Cell lines:
A549 and Hela were obtained from the ATCC, FADD-deficient, Caspase 8- deficient and control Jurkat cells (JA3) were kindly provided by Dr John Blenis (Harvard Medical School, Boston), Jurkat cells over-expressing Bcl-2 by Dr Jannie Borst (NKI, Amsterdam) and Bax/Bak double knockout (DKO) mouse embryonic fibroblasts (MEFs) and wild-type control MEFs were from Dr Stanley Korsmeyer.

Cell death analysis:
Overall cell death was assessed as previously described [18] by PI exclusion assay. Briefly, cells were incubated with 1 µg/ml PI and measured by flow cytometry.

DNA fragmentation:
Cells were incubated in Nicoletti buffer containing 50 µg/ml PI for at least 24 hours before analysis via flow cytometry.

Western blot analysis (immunoblotting):
Cells were lysed using Triton X-100 buffer and for protein quantification a BCA kit from PIERCE was used. SDS-PAGE was performed and proteins were transferred onto a PVDF transfer membrane (Amersham Biosciences). Blocking of unspecific binding sites was achieved by incubation of the membrane in 5% low fat milk powder in PBS/0.2% Tween-20 (blocking buffer) for 1 hour at room temperature. Primary antibody incubation was performed overnight at 4°C and secondary antibody (HRP labeled) incubation for 2 hours at room temperature. For chemiluminescent detection ECL from Amersham Biosciences was used in combination with a LAS-3000 imaging system.

ROS detection:
For ROS measurements the highly selective dye for mitochondrial superoxide Mitosox was used. Cells were incubated with 5 µM Mitosox in pre-warmed tissue culture medium at 37°C for 10 min before flow cytometry analysis.

Cytochrome c release by FACS staining:
Cytochrome c release was measured as previously described by Waterhouse et al [19]. First, outer cell membrane permeabilization was achieved by incubation for 5-10 minutes with 50 µg/ml digitonin in PBS containing 100 mM KCl. Cells were then fixed in 4% paraformaldehyde for 30 minutes at room temperature, washed and incubated in blocking buffer (3% BSA, 0.05% saponin, 0.02% azide in PBS supplemented with normal goat serum, dilution 1:200). Anti cytochrome c incubation was done overnight at 4°C and for flow cytometric detection a FITC conjugated secondary antibody was applied.

MTT assay:
Cells were incubated in the presence of 40 µg/ml MTT reagent for 2 hours at 37°C. During the incubation period appearance of purple formazan structures was followed by phase-contrast light microscopy.

Results

Cholesterol strongly enhances cytotoxic effects of BE but not BetA

Previously we have shown that BetA induces cell death in Jurkat T leukemia cells in a concentration and time-dependent fashion [18]. Here we show that low concentrations (5 µg/ml) of BetA are non toxic up to 48 hours incubation and show limited cell death after 72 hours (Fig 1A). In contrast, when 7.5 µg/ml BetA or more is used almost all cells are PI positive after 48 to 72 hours (Fig 1A). To analyze whether Betulin (BE), the precursor of BetA, is capable of inducing cell death we titrated BE on Jurkat T Leukemia cells. In contrast to previous reports we show here that BE is capable of killing cells, but required higher concentrations than BetA. However, it appeared that cell death induced by BE is more efficient after 12 hours when compared to BetA and maximum cell death is achieved after 24 hours (Fig 1C).

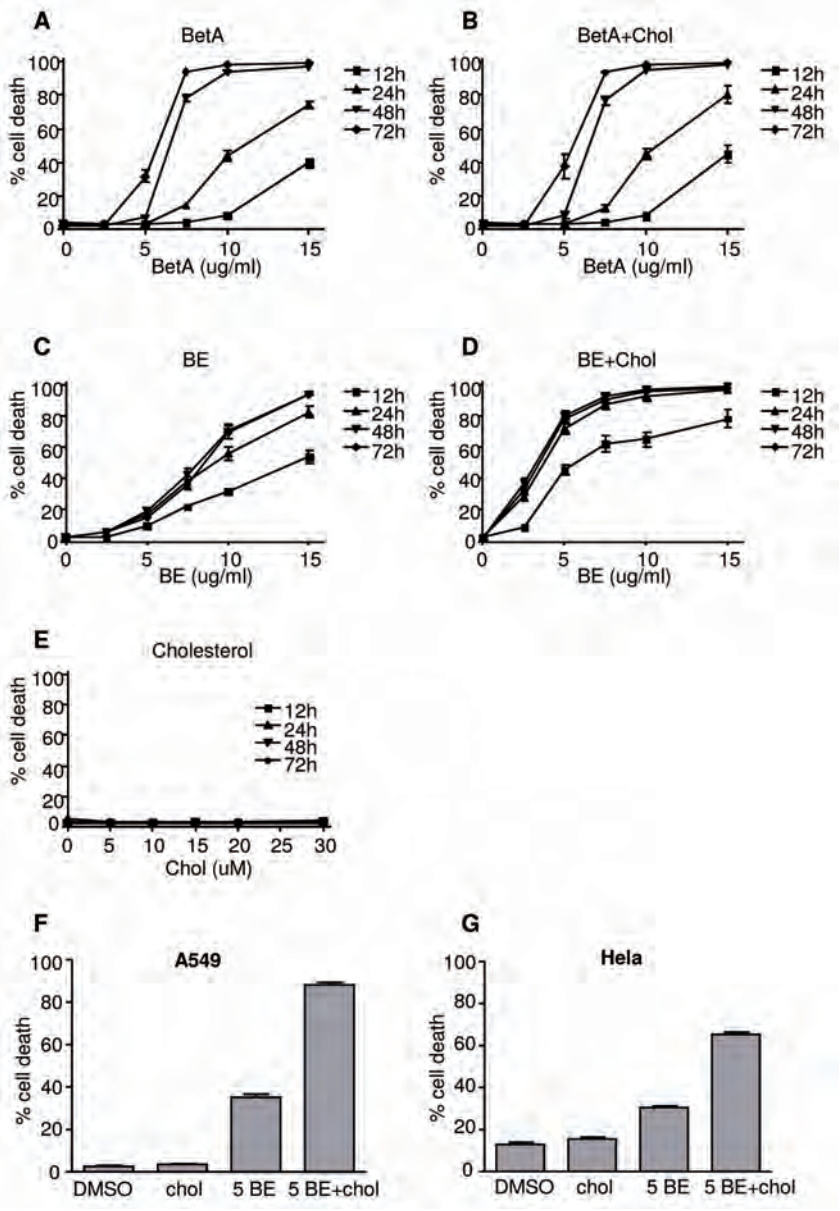


Figure 1. Cholesterol strongly enhances cytotoxic effects of BE but not BetA
Jurkat cells were treated with the indicated concentrations of BetA (A), BetA in combination with 5 µM cholesterol (B), BE (C), BE in combination with 5 µM cholesterol (D) or various concentrations of cholesterol only (E). Cell death was monitored after 12, 24, 48 and 72 hours using PI exclusion. A549 lung cancer (F) and HeLa cervix carcinoma (G) cell lines were treated with 5 µM cholesterol (chol), 5 µg/ml BE (5 BE) or the combination of 5 µg/ml BE with 5 µM cholesterol (5 BE + chol) and after 24 hours cell death was analyzed via PI exclusion.

We have found previously that when using the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay to measure BetA [18] or BE (unpublished data) induced cytotoxicity, results were much more pronounced when compared to other assays such as PI exclusion and clonogenic survival [18]. This decrease in MTT conversion is likely the result of a direct effect of BetA on the mitochondria and was accompanied by a different morphological appearance of the formazan precipitates. While normal formazan formation shows a punctuate appearance, BetA and BE-induced formazan formation shows the rapid appearance of needle-like structures on the cell surface (supplem. Fig 1). Interestingly, cholesterol, which shares some structural similarities with BE and BetA, has been reported to have a comparable effect in the MTT assay [20–22] (supplem. Fig 1). This suggests that cholesterol, BetA and BE may share common targets in the cell. To clarify if this feature is related to the cytotoxicity of these compounds we decided to analyze the effect of cholesterol on cell death and combine cholesterol with either BetA or BE and measure PI exclusion after various time points. Cholesterol itself did not induce cell death in Jurkat cells (Fig 1E) and it did not enhance cytotoxicity of BetA at all time points measured (Fig 1B). However, the combination of BE with cholesterol resulted in massive cell death in Jurkat cells even when very small concentrations of BE were used (2.5 and 5 µg/ml BE, Fig 1D). To rule out that this is a cell type specific effect we analyzed cell death in A549 (lung cancer) and HeLa (cervix cancer) cells exposed to either BE or BE in combination with cholesterol. In both cell lines the combination treatment resulted in massive cell death whereas BE by itself was only minor toxic at the concentration used (Fig 1F, 1G).

BE/Cholesterol induces apoptosis in Jurkat cells

To identify the nature of cell death induced by BE/Cholesterol we investigated the apoptotic pathway. Apoptosis has been previously reported to be the cell death pathway induced by BE in A549 lung cancer cells [12]. We assessed DNA fragmentation as an apoptosis read-out in Jurkat cells treated for 24 hours with either cholesterol, BE or the combination of both. In cells treated with cholesterol only, DNA fragmentation was completely absent (Fig 2A), consistent with the lack of cell death. BE at 5 µg/ml showed only moderate DNA fragmentation. However, when combined with cholesterol DNA was clearly fragmented (Fig 2A). To verify these results we performed immunoblotting for the classical caspase target PARP and observed similar effects: Upon BE treatment PARP was processed to some extent and this was strongly enhanced by addition of cholesterol (Fig 2B). Importantly, both, DNA fragmentation and PARP cleavage were blocked when cells were pre-treated with zVAD.fmk (a pan-caspase inhibitor) confirming that both are caspase-mediated events (Fig 2A, 2B).

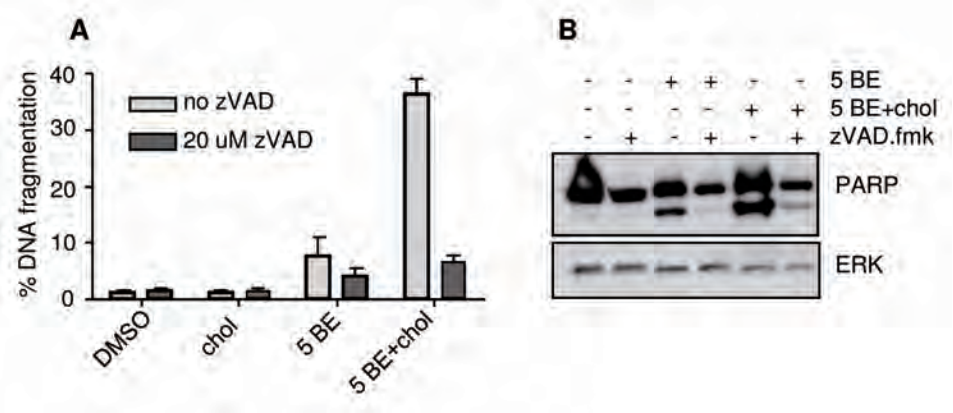


Figure 2. BE/cholesterol induces apoptosis in Jurkat cells
(A) Jurkat cells were pretreated with 20 µM zVAD.fmk for at least one hour prior to addition of either DMSO, 5 µM cholesterol (5 Chol), 5 µg/ml BE (5 BE) or 5 µg/ml BE in combination with 5 µM cholesterol (5 BE+chol). After 24 hours DNA fragmentation was assessed by FACS analysis of propidium iodide (PI) stained nuclei.
(B) Jurkat cells were treated as described in (A) but after 24 hours PARP cleavage was assessed by immunoblotting. ERK was used as a control for equal protein amounts.

The death receptor pathway is not involved in BE/cholesterol induced apoptosis

Cholesterol is an important constituent of cell membranes where it plays a crucial role in maintaining integrity and fluidity [23]. In addition, cholesterol-enriched micro-domains, so called lipid rafts, are important signal transduction platforms [24], which have been related to apoptosis [25] and changes in plasma cholesterol levels have been associated with Fas-FADD complex formation and caspase-8 activation [26,27]. BetA has been shown to induce apoptosis independently of the extrinsic pathway [28]. However, because of the strong apoptosis-enhancing effects of cholesterol when combined with BE, we decided to investigate the involvement of this pathway by applying BE/cholesterol on Jurkat cells either deficient of FADD or caspase-8. Recently we showed that the FADD and caspase-8 deficient cells were completely resistant to Fas-induced apoptosis [17]. Here this resistance was further confirmed using TRAIL (Figure 3A). Despite the resistance towards the extrinsic pathway, neither cell line showed decreased DNA fragmentation when treated with BE/cholesterol (Figure 3B), indicating that the death receptor pathway is not involved in BE/ cholesterol-induced apoptosis.

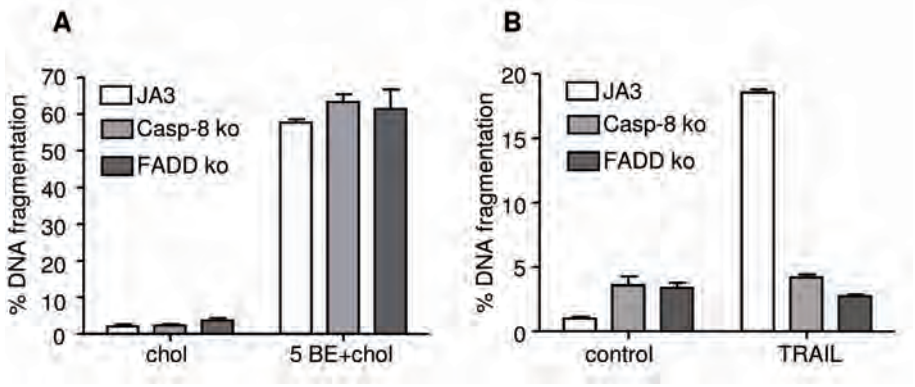


Figure 3. The death receptor pathway is not involved in BE/cholesterol induced apoptosis
Jurkat control (JA3), FADD deficient (FADD ko) or caspase-8 deficient (Casp-8 ko) cells were treated with TRAIL (0.5 mg/ml plus 1 mg/ml anti-FLAG) (A) or with either 5 mM cholesterol (chol) or 5 mg/ml BE in combination with 5 mM cholesterol (5BE+chol) and after 24 hours DNA fragmentation was analyzed.

BE/cholesterol induced apoptosis is mechanistically related to BetA induced apoptosis

BetA induced apoptosis has been clearly linked to the mitochondria [14–17] with the consistently described features of cytochrome c release and induction of reactive oxygen species (ROS) [28–31]. These events were initially described to be Bcl-2 family dependent [15,16], however, our recent evidence suggests only a minor role for the Bcl-2 family proteins. Instead we proposed a direct effect on the PT-pore [17]. To test if BE/cholesterol induces apoptosis via similar mechanisms as BetA we investigated the mitochondrial pathway of apoptosis.

BE/cholesterol showed clear cytochrome c release in Jurkat cells. Importantly, there was only a slight difference in cytochrome c release in the Bcl-2 over-expressing cells (Fig 4A), but this difference was statistically not significant (paired t-test). Jurkat cells over-expressing Bcl-2 were completely resistant to etoposide (Fig. 4A). In contrast to the lack of effect of Bcl-2 over-expression, CsA provided almost complete protection (Fig 4A). To determine if ROS are produced upon BE/cholesterol treatment we used a dye specifically detecting mitochondrial superoxide. Both wildtype as well as Bcl-2 over-expressing cells showed clear increase in ROS, strikingly this was again abolished in the presence of CsA (Fig 4B). To verify that these events resemble the amount of apoptosis and overall cell death we measured DNA fragmentation and PI exclusion respectively. Bcl-2 over-expression did not provide any protection whereas CsA effectively prevented both, apoptosis and cell death (Fig 4C and 4D). In order to find out if Bcl-2 over-expression causes a delay in apoptosis as is the case with BetA [17] we performed a kinetic analysis. Cell death and DNA fragmentation were measured after various time points from 0–24 hours. At all time points we did not observe any difference in sensitivity to BE/cholesterol, further underscoring the lack of inhibition by Bcl-2

(Figure 4E and 4F). These results suggest that BE/cholesterol kills Jurkat cells by inducing mitochondrial damage that leads to cytochrome c release and apoptosis which is independent of Bcl-2.

To further determine the efficacy of BE/cholesterol and to find out if Bax and Bak are involved in BE/cholesterol induced cytotoxicity we used Bax/Bak double-knockout (DKO) mouse embryonic fibroblasts (MEFs). DKO MEFs are resistant to drugs such as etoposide, staurosporine, UVC or actinomycin D, all targeting the Bcl-2 family regulated mitochondrial pathway [32]. We measured PI exclusion and found DKO MEFs to be sensitive to BE/cholesterol, as a control for the functionality of the cells etoposide was included (Fig 5A). We assessed if apoptosis was induced like in BetA treated cells by analyzing PARP cleavage. PARP was clearly processed in wildtype as well as in DKO MEFs, suggesting that Bax and Bak are not essential in BE/cholesterol induced apoptosis (Fig 5B). Also cytochrome c release was not prevented in DKO MEFs (Fig 5C), further substantiating that Bax and Bak are not required for BE/cholesterol mediated cytotoxicity. Similar to Jurkat cells, CsA provided complete protection against cell death (Fig 5A), apoptosis (Fig 5B) and cytochrome c release (Fig 5C), confirming the crucial role for the mitochondrial permeability transition in BE/cholesterol induced cytotoxicity.

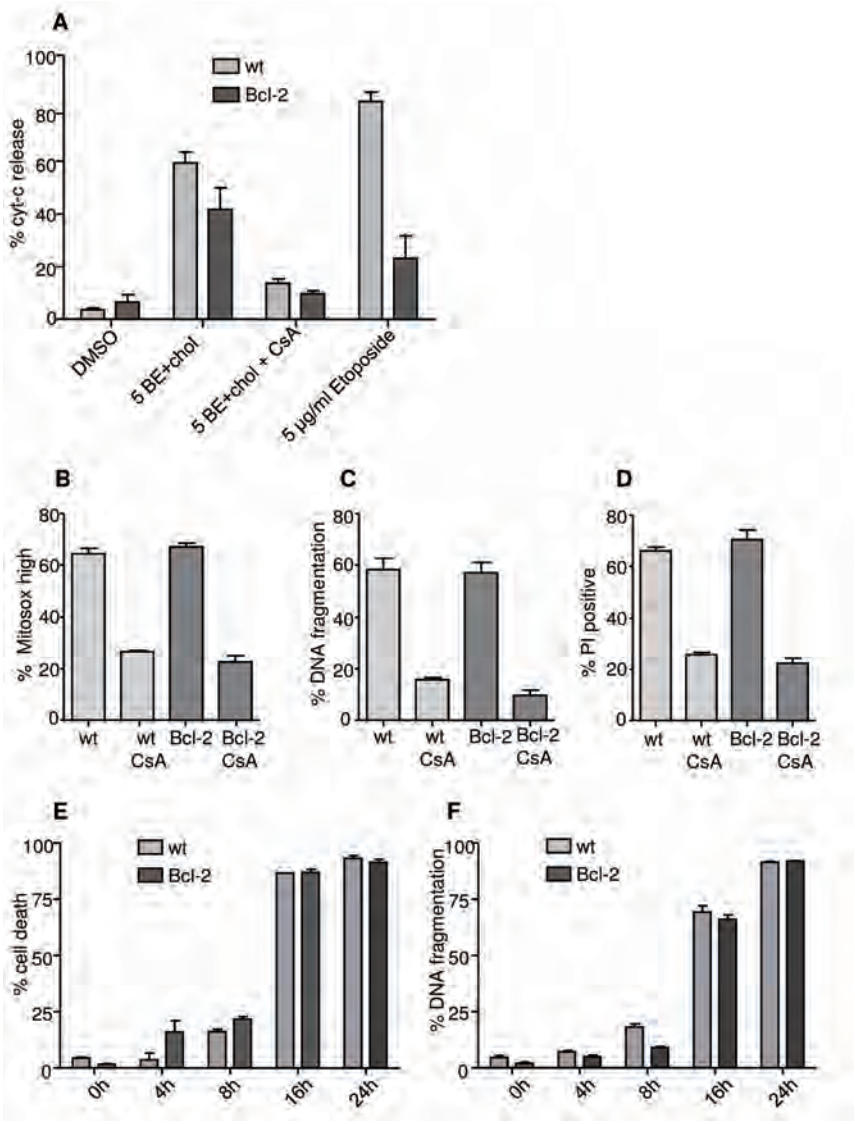


Figure 4. BE/cholesterol induced apoptosis is not affected by Bcl-2 over-expression but is inhibited in the presence of cyclosporin A

(A) Jurkat control (wt) or Bcl-2 over-expressing cells (Bcl-2) were treated as indicated (5BE = 5 µg/ml BE; chol = 5 µM cholesterol; CsA = 5 µg/ml cyclosporin A), after 24 hours intracellular staining for cytochrome c release was performed.

(B, C, D) Jurkat control (wt) or Bcl-2 over-expressing cells were treated with 5 µg/ml BE/ 5 µM cholesterol either in the absence or presence of 5 µg/ml cyclosporin A. After 24 hours ROS (B), DNA fragmentation (C) and overall cell death (D) were assessed by FACS analysis.

(E, F) Jurkat control (wt) or Bcl-2 over-expressing cells were treated with 5 µg/ml BE/ 5 µM cholesterol and PI exclusion (E) or DNA fragmentation (F) were measured after 0, 4, 8, 16 and 24 hours.

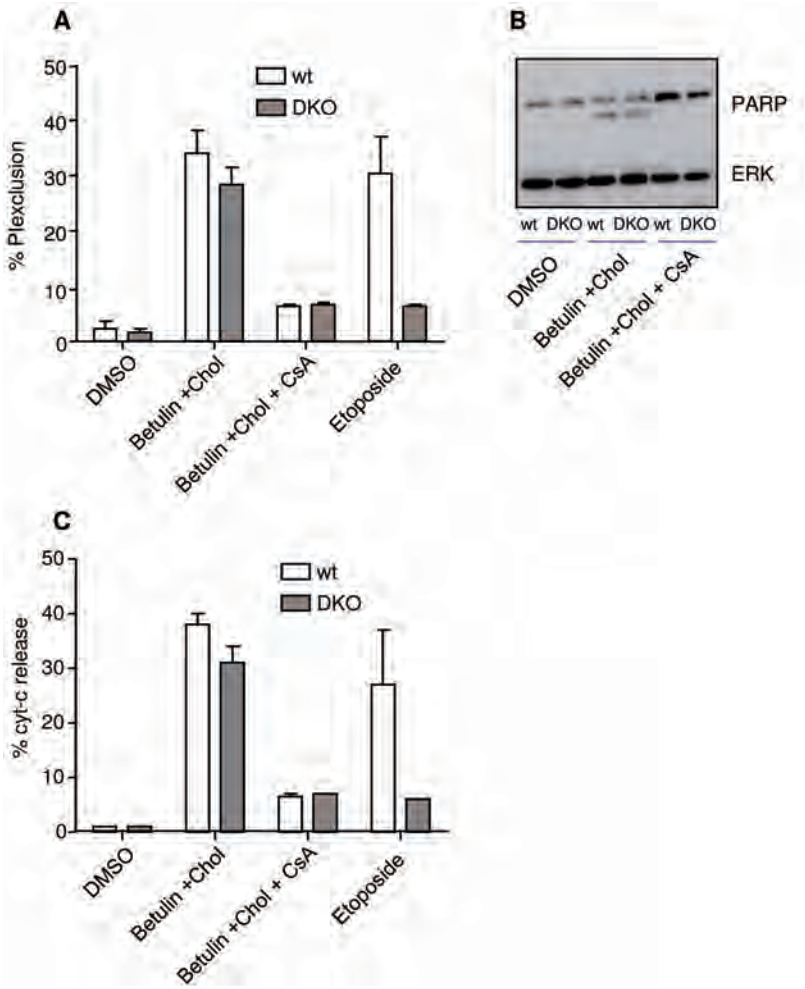


Figure 5. BE/cholesterol induced apoptosis is independent of Bax/Bak
(A) Wildtype (wt) or Bax/Bak double knockout (DKO) mouse embryonic fibroblasts (MEFs) were treated as indicated and after 24 hours cell death was assessed by PI exclusion. Etoposide was included as a control for functionality of the cells.
(B) Wt or DKO MEFs were treated as indicated and after 24 hours cells were subjected to immunoblotting to determine PARP processing. ERK was used as control for equal protein amounts.
(C) Wt and DKO MEFs were treated as indicated for 24 hours before measuring cytochrome c release by intracellular FACS staining.

Discussion

BE is a natural compound, which contains derivatives that have been shown to possess strong anti-tumor properties [7,33]. Here we provide evidence that BE itself, especially in combination with cholesterol (BE/cholesterol), is very potent in killing cancer cells in vitro (Fig 1). BE/cholesterol induces apoptosis in a similar manner as BetA and does not involve the extrinsic pathway of apoptosis (Fig 3), but instead apoptosis depends on the mitochondrial pathway (Fig 4). However, as we reported for BetA, this pathway is activated in an unconventional manner as cytochrome c release and apoptosis are induced in cells over-expressing Bcl-2 (Fig 4) or in cells deficient for Bax/Bak (Fig 5), while both events are blocked by CsA (Fig 4 and 5). This indicates that permeability transition is pivotal in the process of BE/cholesterol induced cytotoxicity.

Despite the strong similarities, and the almost identical structure of BE and BetA, there are also important differences in comparison to BetA induced apoptosis. We previously showed that Bcl-2 over-expression delayed BetA-induced apoptosis [17], but curiously in the case of BE/cholesterol it has very limited effect on the amount of cytotoxicity induced (Fig 4) . Furthermore, CsA by itself provides much stronger protection in the case of BE/cholesterol in Jurkat cells, while BetA treated Jurkat cells are only completely protected when a combination of CsA with Bcl-2 over-expression is used.

This difference between BetA and BE/cholesterol is even more remarkable when considering the time dependency of cytotoxicity of both molecules: For BetA the maximum effect requires around 48-72 hours and a dose of 7.5-10 µg/ml (Fig 1A, 1B), while BE/cholesterol induced death is already maximum at 24 hours. Nevertheless, CsA is capable of providing efficient protection.

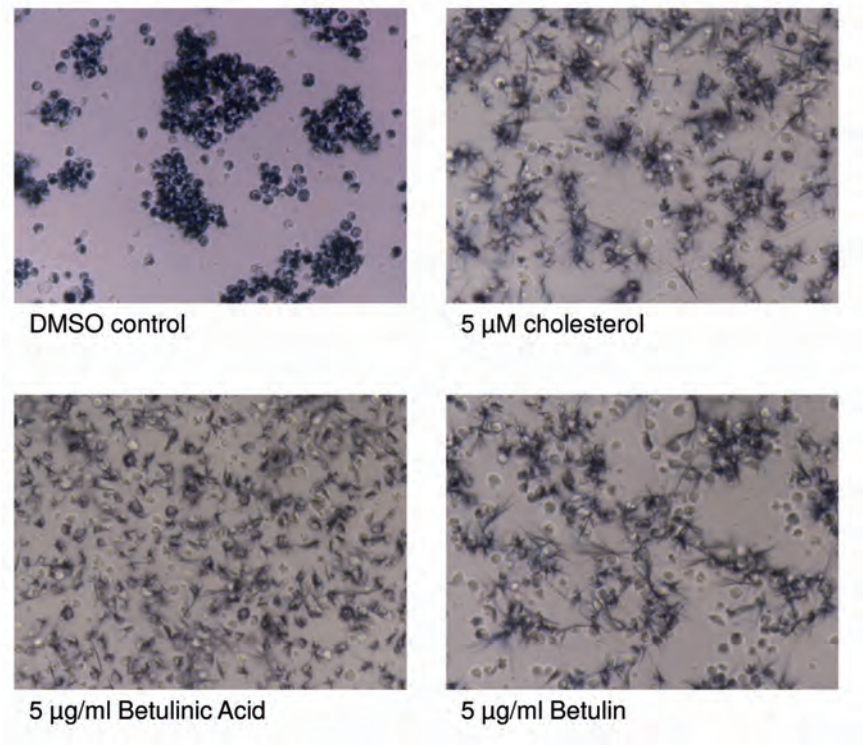
Striking is the fact, that cholesterol strongly enhances the cytotoxic effects of BE but not BetA (Fig 1B, 1D) whilst being completely non-toxic on its own, even at very high concentrations (Fig 1E). Currently we do not know the mechanism by which cholesterol acts as a “cytotoxicity-amplifier” for BE but it likely involves membrane integrity. Cholesterol is abundantly present in the plasmamembrane and it is possible that changes in cholesterol content can affect the amount of BE that is taken up by a cell.

The effect on MTT conversion to formazan (MTT measures mitochondrial enzymatic activity [20,34]) by all three compounds, BetA, BE and cholesterol, suggests a common target in the mitochondria. Even though this is clearly not directly related to cytotoxicity, as cholesterol on its own is completely non-toxic, it may point to a mechanism that sensitizes cells to BE. It is not clear how this is orchestrated but it could involve the mitochondrial membrane, for instance mitochondrial PT pore opening. The exact composition of the pore has yet to be established but adenine-nucleotide-translocator (ANT), voltage-dependent-anion-channel (VDAC) and cyclophilin D are discussed as core components in the currently accepted model [35]. PT pore opening is influenced by the amount of

cholesterol present in the mitochondrial membrane, cholesterol affects VDAC function [35] and impairs ANT mediated PT through altered membrane fluidity [36]. So cholesterol-induced effects on the PT pore may facilitate BE-induced opening. Why this then does not influence BetA-induced opening is unclear at this point and will require further investigation. In this light it is also important to realize that Bcl-2 over-expression delays BetA-induced apoptosis [17], while CsA can only partially prevent the induction of apoptosis. This suggests that BetA may has a direct effect on the PT pore, which is blocked by CsA and maybe also induces a more classical Bcl-2-dependent pathway to cytochrome c release. This latter seems absent when using BE and may be the reason these compounds react slightly different to CsA and potentially also cholesterol.

To further evaluate the anti-tumor properties of BE/cholesterol *in vivo* studies will be required. Preliminary results from a pharmacokinetic study using triterpene extract (TE) mainly consisting of Betulin suggest that it is safe; no signs of toxicity were observed in rats or dogs in a subchronic toxicity study [37]. Another study investigated the effects of BE on the central nervous system (CNS) with the conclusion that there was no effect of BE on muscle tone and coordination in mice; doses up to 100 mg/kg bodyweight were used [38]. Interestingly another study explored the antinociceptive properties of Betulin in mice and results suggest that it is even more active than aspirin and paracetamol [39].

It will be interesting to explore the combined effects of BE and cholesterol *in vivo*. Because cholesterol is ubiquitously present in the body it is unlikely that additional applied cholesterol is useful for *in vivo* effects of BE as an anti-tumor agent. Our results indicate that the amount of cholesterol necessary (5 μM) for enhanced *in vitro* effects of BE are about 1000 times lower than normal plasma cholesterol levels in humans (5 mM). However the fast majority of this cholesterol is contained in LDL or HDL and it is therefore difficult to assess whether there is sufficient free cholesterol available to potentiate BE-induced apoptosis in vivo. Adding more cholesterol may not bear any significance though, but application of cholesterol containing Betulin-liposomes may be an interesting mode of applying this cytotoxic agent. In summary we conclude that Betulin by itself and in combination with cholesterol is a potent anti-cancer agent *in vitro* and warrants further investigation *in vivo*.



Supplem Figure 1. Effects of BetA, cholesterol and BE on MTT assay
Jurkat cells were treated as indicated, incubated with MTT reagent and photographed under a phase-contrast microscope.

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Chapter 7

General Discussion and Summary

Betulinic acid (BetA) is a member of naturally occurring, plant derived triterpenoids with strong anti-cancer effects. Since the mid-90th of the last century it has been extensively studied as a potential new anti-cancer and anti-HIV drug. However, whereas derivatives of BetA have already reached clinical trial phase for HIV indication, its development as anti-cancer drug is still in preclinical stage. The step towards patients is hindered by two complicating features of BetA, which clearly constitute the biggest challenge for its further development. Firstly, its unique and distinct mechanism of inducing tumor cell death that involves several pathways. Although this would by itself not preclude clinical development, the complicated interactions between those pathways in different tumor types combined with a currently undefined, but potentially antagonizing interaction with other commonly used treatments makes development difficult and will require better insight and identification of the initial intracellular target of BetA. Secondly, the highly lipophilic character of BetA is hampering *in vivo* studies and thus clinical development as well as applicability. Sophisticated formulation strategies are needed to reach therapeutic concentrations at the target location, namely the tumor tissue. These features of BetA and of its non-oxidized precursor Betulin are summarized and discussed in this chapter.

The role of apoptosis in BetA-induced cell death

BetA was initially described as an apoptosis-inducing compound specific for melanoma [1]. It was quickly thereafter established that tumor cells from neuroectodermal origin are sensitive and that the BetA-induced apoptosis pathway did not involve the death receptor CD95 and, importantly, was also not p53 dependent [2]. Instead, loss of mitochondrial membrane potential (MMP) and cytochrome c release was observed in BetA-treated cells, indicating that the mitochondria are involved. Further analysis using isolated mitochondria indeed revealed that BetA directly affects mitochondria [3,4], in contrast to other known triggers of the mitochondrial pathway of apoptosis, such as doxorubicin, cisplatinum or etoposide, acting upstream of the mitochondria [4]. Results obtained with Bcl-2 over-expressing SHEP neuroblastoma cells led to a model in which BetA induces apoptosis in a fashion that is dependent on the Bcl-2 family of proteins [4]. However, subsequent studies employing other cell types did not lead to consistent results in regards of the role of the Bcl-2 proteins [5]. Furthermore, it became also clear that the effect of BetA is not restricted to cancer cells derived from melanoma and neuroectodermal tumors but that it has a much broader efficacy [5]. But again, partly conflicting results were obtained in different studies. For example, glioma cells were initially found to be resistant [1] whereas in subsequent studies glioma cells as well as other brain tumor cells were sensitive towards BetA [6,7]. Also lung cancer cell lines were resistant in initial reports [1,2] whereas another study found different lung cancer cell lines (as well as cells derived from ovarian carcinomas) to be sensitive towards BetA [8]. Furthermore, BetA-induced cytotoxicity data were not yet known for other prevalent cancer

types, like colon and breast cancer at the time (2003) the work for this thesis started. Thus, one of the important issues that needed to be addressed was the reason underlying the contradictory results in published studies and the tumor selectivity of BetA-induced cytotoxicity.

We first revealed that findings regarding the sensitivity of tumor cells for BetA were highly dependent on the type of measurement used. Big differences were observed between results obtained with apoptosis readouts, overall cell death assays, MTT assay (measuring cell viability) and clonogenic survival (**chapter 2**). This explained why for example lung cancer cell lines were resistant when specific apoptosis was measured [2] but sensitive in an anti-proliferative assay (MTT) [8]. Also HT-29 colorectal carcinoma and MCF-7 breast carcinoma cells were initially found to be resistant when an apoptosis readout was used [2] whereas in other reports, including ours, these cell lines were found BetA-sensitive when clonogenicity was analyzed (**chapter 2**). MCF-7 cells are known to lack functional caspase-3 with the consequence that downstream apoptotic events in these cells are impaired [9]. As such, it is not surprising that in apoptosis-specific readouts MCF-7 was not found to be sensitive to BetA. Thus, with the knowledge that different readouts lead to differential results the reports are not necessarily contradicting, but instead provide clues to the working mechanisms of BetA. Our finding that the pan-caspase inhibitor zVAD.fmk prevented BetA-treated cells from undergoing apoptosis, but on the other hand did not provide protection against cell death, prompted the idea that BetA-induced cytotoxicity does not depend on the downstream apoptosis machinery, including caspase activity **(chapter 2, 4, Fig 1)**. Despite the often contradictory results, the central involvement of the mitochondria in BetA-induced cell death (and apoptosis) is uniformly agreed upon as is described below.

The role of the mitochondria in BetA induced cytotoxicity

In SHEP neuroblastoma cells BetA-induced cytotoxicity is clearly dependent on Bcl-2 because over-expression of this protein provided protection against BetA-induced apoptosis as measured by caspase and PARP cleavage [2], mitochondrial permeability transition, cytochrome c release and DNA fragmentation [3,4]. Subsequent studies with tumor cells over-expressing Bcl-2 derived from other tumors types (T-cell leukemia, melanoma) provided less clear results, because the protective effect of Bcl-2 over-expression was only limited [10,11]. Furthermore, when expression levels of different members of the Bcl-2 family were analyzed in tumor cells derived from various cancer types, ambivalent results were obtained [7,12-15]. Apoptosis induction that is dependent on the Bcl-2 family of proteins requires Bax and/or Bak for cytochrome c release and as such Bax/Bak deficient cells are rendered resistant to a range of apoptotic stimuli [16]. However, in **chapter 4** we showed that murine embryonic fibroblasts and human colon cancer cells (HCT 116) lacking functional Bax and Bak are sensitive to BetA with apoptotic features being induced, including cytochrome c release. Bcl-2 was able to

provide limited and short term protection in Jurkat and MCF7 cells. This indicates that changing the Bcl-2 rheostat in favour of anti-apoptosis at best results in a delay of BetA-induced apoptosis. Interestingly, in all cases (including SHEP neuroblastoma cells) inhibition of the permeability transition pore using bongkrekic acid [4] or Cyclosporine A **(chapter 4)** prevented BetA-induced cytotoxicity. Together, these results indicate that classical Bcl-2-family-dependent cytochrome c release is dispensable in BetA-induced cytotoxicity, while the permeability transition pore appears to be crucial **(chapter 4, Fig 1)**.

The precise molecular target(s) of BetA in the mitochondria are still unknown and it is also not clear to date if the effects on the permeability transition pore are direct or indirect. Results of the MTT assay point to the fact that the enzymatic activities in the mitochondria are affected by BetA treatment. Even in normal cells, which are resistant to BetA-induced cell death, a considerable decrease in MTT dye conversion was observed (our unpublished results). If and how this is related to BetA-induced cytotoxicity remains to be identified. Also, electron microscopy pictures showed clearly altered mitochondrial structures resembling concentric cristae, which have previously been observed in patients with Barth syndrome [17] **(chapter 5)**. This rare disease is characterized by a mutation in the tafazzin gene causing alterations in cardiolipin [18]. Cardiolipin, a phospholipid that is located mainly in the inner mitochondrial membrane, is involved in numerous mitochondrial processes, such as electron transport chain activity and apoptotic processes including mobilization of cytochrome c [19,20]. It is conceivable that BetA as a highly lipophilic molecule might either directly or indirectly affect cardiolipin or other lipophilic components in the mitochondria. In agreement, we observed a rapid change in cardiolipin upon BetA treatment that resembles the changes observed in Barth syndrome patients. That is, cardiolipin becomes more saturated in its fatty acid side chains and thereby loses flexibility and functionality [18]. As cardiolipin interacts with proteins that make up the PT pore, it is reasonable to assume that such a modification can result in pore opening and subsequently cell death. Although this is difficult to prove, the observation that addition of saturated fatty acids synergizes with BetA to modify cardiolipin *and* induces cell death at least indicates that this modification is of importance **(chapter 5, Fig 1)**.

The direct modification of the mitochondria by BetA suggests that BetA is an ideal candidate for combination treatment or adjuvant therapy of cancer. Due to its distinct working mechanism it might prove useful as a chemosensitizing agent, especially in regards with its apparent lack of toxicity on normal cells. In vitro experiments testing anti-cancer effects of a combination of BetA with TRAIL [21], doxorubicin, etoposide [22] or irradiation [13] indeed gave promising results. Also entirely new treatment regimens can be envisaged by combining BetA with other new potential drugs targeting the mitochondria such as gossypol [23], chelerythrine [24] or ABT-737 (a BH3 mimetic) [25]. Even though all three compounds have been identified as inhibitors of Bcl-2 proteins, only ABT-737 was found to induce cell death via apoptosis. Chelerythrine and gossypol on the other hand are

associated with mitochondrial damage which includes cytochrome c release [26], similar to BetA. Considering that the mitochondria are crucial organelles, a combination of mitochondria damaging drugs with different specific targets might proof useful to induce mitochondrial damage that is not compatible with survival of the tumor cells. Especially when higher concentrations of the single compounds are not feasible or unreachable in vivo this approach could be envisaged.

The role of autophagy in BetA-induced cytotoxicity

Interestingly, we discovered that BetA-treatment led to a rapid and rather massive accumulation of autophagosomes or more specifically aggregation of LC3-GFP **(chapter 5)**. This accumulation is the result of an enhanced level of autophagy and was inhibited in the presence of Cyclosporine A **(chapter 5)**. These data indicate that the trigger for induction of autophagy is the mitochondrial damage induced by BetA and not a direct autophagy-inducing effect of BetA. Initially, we hypothesized that autophagy might serve as an alternative cell death pathway in cells with impaired apoptosis. Such scenarios have been reported for example for etoposide and staurosporine [27]. Autophagy depends on lysosomal degradation and drug-affected lysosomes in turn have also been reported to be causative in cell death [28]. Therefore, it will be important to analyze whether such a lysosomal cell death mechanism is induced during BetA-treatment. By its lipophilic character, BetA may directly or indirectly influence the lysosomal membrane properties causing lysosomal membrane permeabilization or, alternatively, BetA may act as a lysosomotropic agent like siramesine. Siramesine is a lipophilic compound that shares some interesting features with BetA such as induction of p53-, Bcl-2- and caspase independent cell death and induction of cytoprotective autophagosomes [29-31].

As is the case for siramesine, also for BetA we found autophagy to act as a survival pathway **(Fig 1)**, as cells lacking the essential autophagy gene products (ATG5 or ATG7) were found to be even more susceptible to BetA-induced killing **(chapter 5)**. Especially when considering the potent cell death-inducing effects of BetA, it first seemed contradictory that in parallel a massive survival response is induced. However, the latter phenomenon might simply reflect the amount of damage induced by BetA and may also explain why BetA-treated cells in general survive longer as compared to cells treated with other compounds, like etoposide or anti-APO-1. It will be interesting to explore the amount of autophagy induced in healthy cells upon BetA-treatment, because these are much more resistant towards BetA as compared to tumor cells. In case autophagy would be also induced in resistant, healthy cells this would further substantiate that autophagy serves as an intracellular survival mechanism after treatment with BetA.

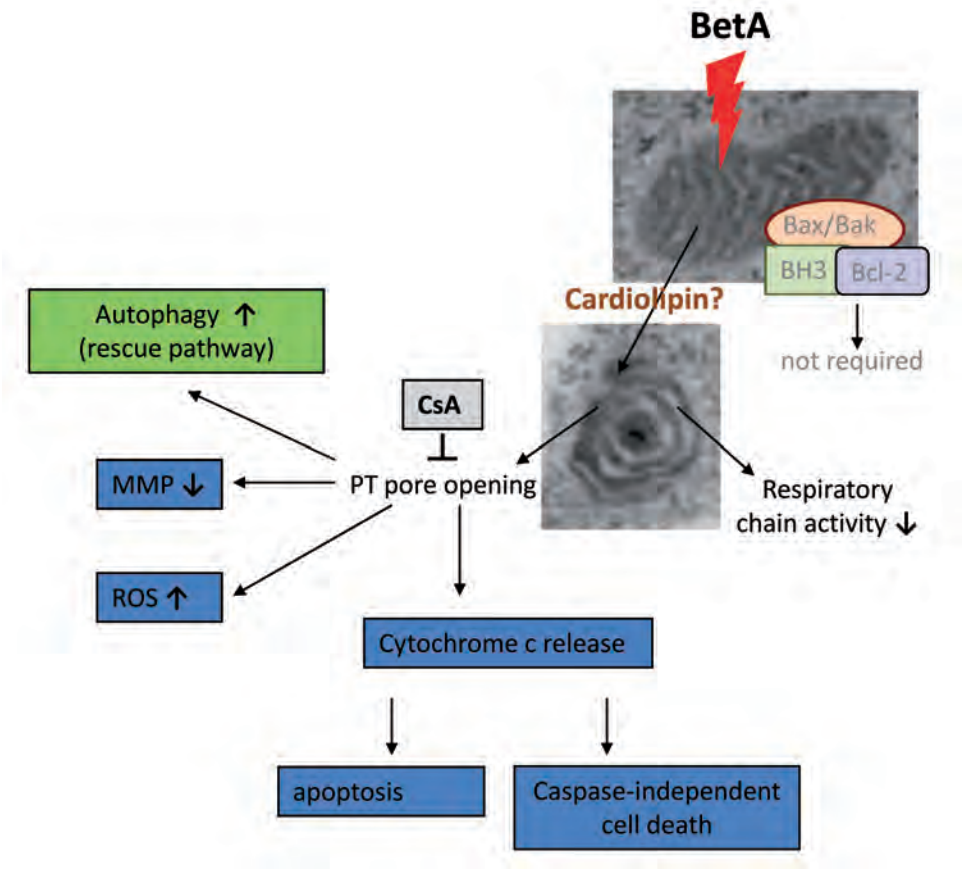

Figure 1: Model of BetA induced cytotoxic effects on tumor cells:

BetA treatment of tumor cells leads to massive remodelling of the mitochondria which is possibly induced through changes in cardiolipin side chain saturation. BetA induced permeability transition (PT) pore opening and reduction of respiratory chain activity might also be indirect as consequence of cardiolipin modifications. PT pore opening in turn results in cytochrome c release and subsequent apoptotic and non apoptotic cell death as well as an increase in reactive oxygen species (ROS), reduction of the mitochondrial membrane potential (MMP) and enhanced autophagy. All PT pore opening related events can be inhibited in the presence of the PT-pore inhibitor Cyclosporine A (CsA).

Differential effects of BetA on tumor and healthy cells

A quite unique characteristic of BetA is its differential effect on tumor cells and healthy cells. Tumor cells are sensitive to BetA, largely independent of their origin, whereas healthy cells are to a large extent resistant (**chapter 1)** [8,12,13]. This hints to a scenario in which BetA – rather than targeting a specific pathway as is the case for other anti-tumor agents – hits a key-element that is vital for all tumor cells. Tumor cells are characterized by a number of alterations as compared to normal cells including virtually unlimited growth and replication, growth signal

autonomy and apoptosis- as well as anti-growth signal resistance [32]. Another major characteristic of tumor cells includes their changed metabolism. As was first shown by Otto Warburg, the glycolytic rate in tumor cells is much higher than in normal cells, even in the presence of oxygen [33]. Since the mitochondria are heavily affected by BetA and therefore likely also the metabolism is changed in BetA-treated cells, it is conceivable that this so-called Warburg effect may help to explain why healthy cells in contrast to tumor cells survive BetA-treatment. To prove this hypothesis, it needs to be established whether healthy cells are affected differently by BetA or if the initial response is the same but that healthy cells then manage to recover in a way that tumor cells cannot. The results of the MTT assay may point to the second scenario because also resistant cells show a sharp decrease in MTT conversion upon BetA-treatment (our unpublished results). Similar results were obtained when measuring ATP synthesis from glutamate/malate (our unpublished results) that appeared to be affected in BetA-resistant cells to an even greater extent than ATP synthesis in tumor cells (unpublished observation). Therefore, it can be hypothesized that in normal cells, pathways such as autophagy are activated which discard of damaged organelles (in particular mitochondria; coined as mitophagy [34]) and that these healthy cells, whilst being deprived of ATP remain in a "resting state" until energy becomes available again (e.g. through new mitochondria replacing damaged ones). Tumor cells, on the other hand, would initially keep proliferating with the extra energy-gain from enhanced glycolysis. Eventually, however, the energy requirements are not met any longer via glycolysis without sufficient support from the mitochondria and, thus, subsequently cells might die via a phenomenon that is called metabolic catastrophe. Metabolic catastrophe specifically describes the killing of tumor cells by means of inhibition of energy production or other modulations of tumor cell metabolism [35].

Another difference between tumor cells and healthy cells is that the mitochondria of tumor cells contain highly elevated cholesterol levels as compared to normal cells [36]. Since BetA as a lipophilic molecule will likely co-localize with lipophilic parts of the cells such as mitochondrial membranes it is conceivable that the difference in cholesterol levels on mitochondrial membranes on cancer and healthy cells may also contribute to the differential effects of BetA. In case BetA would be specifically localized to cholesterol rich areas, membranes containing more cholesterol could be affected stronger e.g. via disturbed membrane fluidity [36] (see also in vivo section below, BetA liposome formulations with and without cholesterol) If that would be the case, one can speculate that the differential effect is already induced at the level of the plasma cell membrane. Of note, BetA and analogues have been reported to be effective as antimalarial agents via modifying erythrocyte membranes [37].

Until now it is not clear if and how BetA enters a cell but it can be envisaged that lipid rafts may play a role. Lipid rafts are protein containing sphingolipid and

cholesterol enriched areas which function as signalling and trafficking platforms [38].

BetA *in vivo*

The application of BetA has currently not yet progressed into the clinic. However, a derivative of BetA, Bevirimat, which is active against HIV-1 by inhibiting virus maturation [39], has been tested in phase I and II trials (NCT00511368, NCT01097070, NCT00967187). Furthermore, of note, currently a phase I/II study in Illinois is recruiting participants to test the safety and efficacy of a 20% BetA ointment for topical treatment of dysplastic nevi (ClinicalTrials.gov Identifier: NCT00346502).

The first *in vivo* application of BetA by Pisha et al. resulted in a complete inhibition of tumor growth as well as regression of established tumors in a melanoma xenograft mouse model. In this study, BetA was co-precipitated with polyvinylpyrrolidone (BetA-PVP) and applied i.p. at concentrations up to 500 mg per kg bodyweight without observing any signs of systemic cytotoxicity [1]. Remarkably, no follow-up studies using BetA-PVP were published (except for one, analyzing the pharmacokinetic properties as well as BetA tissue distribution [40]) which may indicate that the used BetA formulation was suboptimal for *in vivo* application. Other groups investigating the efficacy of BetA *in vivo*, applied BetA either i.p. (in an ethanol/Tween-80/water formulation) or orally (using corn oil as vehicle). Although these studies were less successful than the aforementioned study, they also demonstrated a favourable anti-tumor effect of BetA *in vivo* [8,41]. At the start of the currently discussed thesis project, the biggest challenge for treatment with the very lypohilic BetA was to find a suitable way for its *in vivo* application. As we failed to reproduce positive treatment effects with any of the reported BetA formulations we set out to investigate whether liposomes could function as a delivery vehicle for BetA. Liposomes are vesicles made up of a phospholipid bilayer and an aqueous core and are used as carrier for some lipophilic drugs such as doxorubicin [42]. Liposomes exist with different sizes, either large liposomes or small liposomes (0.1 – 0.2 μm), also referred to as long-circulating liposomes. The latter class has distinct advantages because this type of liposomes is passively targeted to the site of tumor through enhanced permeability in the tumor blood vessels as compared to blood vessels in non-tumor tissue [43]. BetA incorporation in small liposomes turned out to be less efficient as hoped and, furthermore, the incorporation of BetA rendered liposomes more rigid as compared to control liposomes. Therefore, different types of liposomes were investigated for their BetA payload and rigidity upon BetA incorporation. We developed a suitable liposomal BetA formulation for *in vivo* application using large liposomes assembled without cholesterol. Normally, cholesterol is used to stabilize liposomes, but because BetA fulfils a similar role the liposomes could be assembled without cholesterol and were still stable. Thus the absence of cholesterol rendered the liposomes stable without being too rigid. Interestingly, both oral and i.v.

administration resulted in significantly reduced tumor growth, although under the current treatment regiments BetA could not prevent tumors growth completely. This indicates that an improvement of the current formulation is still necessary **(chapter 3)**. In this context it will be interesting to also explore other approaches, such as use of cyclodextrins as a drug vehicle or the development of BetA-micelles or emulsions. Polymeric micelles are nano-assemblies of amphiphilic block co-polymers that hold great promise as drug carriers for hydrophobic drugs [44]. Similarly, isotropic mixtures of oils called self-emulsifying drug delivery systems (SEDDS) might be an alternative approach for oral delivery of BetA [45]. Also cyclodextrins, which consist of oligosaccharides and have a broad application, including foods and cosmetics, should be tested as potential BetA delivery method to increase in vivo solubility [46].

Anti-cancer activity of Betulin:

Betulin, the precursor molecule of BetA, is due to its broad availability in the plant kingdom considered as an important source for the latter one [47]. Conflicting results were obtained previously with regards to its efficacy against cancer cells [48-52]. We found it to be effective against various cancer cell lines using PI exclusion assay **(chapter 6).** A recent report also demonstrated sensitivity of various cancer cell lines to Betulin via MTT assay [53]. However, our unpublished results suggest that similar to BetA, also for Betulin this readout is not consistent with other apoptosis or cell death readouts. Interestingly, as a serendipitously acquired observation, the presence of cholesterol strongly enhanced the cytotoxicity induced by Betulin. Importantly, such a phenomenon was not observed when cells were co-treated with BetA and cholesterol, although the mechanisms of tumor cell death induction by both compounds are very similar. Bcl-2 over-expression or Bax/Bak double-deficiency had no protective effects whereas the presence of Cyclosporine A provided complete protection from Betulin/cholesterol-induced apoptosis and cell death **(chapter 6)**. It is important to analyze in future experiments the effects of Betulin in combination with cholesterol on healthy cells. In this context it will be critical to establish *in vivo* models to test the safety and efficacy of Betulin. Especially harmful with regards to safety might be the strong cytotoxic-enhancing effects of cholesterol, which may also be induced by cholesterol present in blood plasma in animals and humans. An advantageous application of Betulin and cholesterol as compared to BetA, on the other hand, could be envisaged by using cholesterol-containing liposomes as a carrier for Betulin. Because of the strongly enhanced cytotoxicity in the presence of cholesterol, the Betulin concentrations reached might be sufficient for effective tumor growth inhibition.

Relevance and outlook

A major problem for treatment of oncology patients are adverse events induced by anti-cancer treatments. These can often be severe and have a huge impact on the quality of life for the patient. Therefore it is crucial to find new therapies with less severe adverse drug reactions. BetA being non-toxic might be the perfect candidate, although in itself it has only moderate anti-tumor effects *in vivo* in formulations suitable for human treatment. The reason for that is likely that a high concentration is required as compared to other chemotherapies and that such a high concentration is difficult to reach *in vivo*. As such BetA might be an ideal addition in chemotherapy regimens or suitable as an adjuvant or neo-adjuvant therapy due to its unique anti-cancer properties. These importantly include lack of cytotoxicity in healthy cells *in vitro* [8,12,13,54] as well as *in vivo* [1] whereas at the same time BetA or BetA-derivatives exert very strong anti-tumor effects, even in cells derived from therapy resistant and refractory tumors [8,10,41,55-57]. Because of its non-toxicity, adding BetA to the treatment regimen will likely not pose an extra health disadvantage to patients whereas it should significantly increase the effectiveness of the treatment.

Although we made several important steps as discussed in this thesis, one of the most important challenges still is the unraveling of the precise molecular target(s) of BetA in order to fully understand those unique anti-cancer mechanisms. This will help to pre-clinically develop BetA itself. Based on that knowledge it may be possible to design entirely new anti-cancer drugs or BetA derivatives in a precise, targeted way. The advantage of potential new drugs in comparison to BetA and Betulin could be enhanced solubility and therefore easier *in viv*o application. Also for the development of combination therapies, with either BetA itself or drugs derived from it, it will be important to understand the pathways involved in order to predict if effects will be more likely synergistic/additive or antagonistic.

In cancer treatment, the specificity of the therapy is the cornerstone making a drug or treatment attractive. Therefore, deciphering how healthy cells manage to escape the effects of BetA whilst tumor cells cannot remains a prime goal and, once discovered, will be a huge step towards exposing the Achilles heel of cancer.

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Nederlandse Samenvatting

Betuline zuur (BetA) behoort tot de in de natuur veel voorkomende pentacyclische triterpenoiden. Triterpenoiden maken deel uit van de terpenoide familie. Deze familie bestaat uit een grote en diverse groep van plantaardige stoffen waarvan er vele onderzocht worden als potentieel geneesmiddel. In een belangrijk artikel uit 1995 werd de antikanker activiteit van BetA tegen melanoma voor het eerst beschreven. Het celdood mechanisme waarop de antikanker werking van BetA berust in deze studie was apoptose. Snel daarna werd duidelijk dat ook cellen van andere neuroectodermale kanker soorten gevoelig zijn voor BetA en gedetailleerde studies toonden aan dat BetA een direct effect heeft op de mitochondriën. Deze cellulaire organellen hebben een centrale rol bij het apoptotische proces, waarin ook eiwitten van de Bcl-2 familie een belangrijke rol spelen. De groep van Fulda toonden aan dat SHEP neuroblastoom cellen met overexpressie van Bcl-2 resistent zijn voor BetA-geïnduceerde apoptotische kenmerken zoals het vrijkomen van cytochroom C uit de mitochondriën. Aanvullende studies waarbij tumorcellen van verschillende andere weefsels gebruikt werden, leidden echter niet tot een consistent model van het mechanisme van BetA-geïnduceerde celdood en in het bijzonder de rol van de Bcl-2 familie eiwitten daarbij. Tevens waren er tegenstrijdige resultaten over de gevoeligheid van andere tumorcellen dan melanoom en neuroblastoom cellen voor BetA.

In **hoofdstuk 2** beschrijven wij de effecten van BetA op cellijnen afkomstig van darm, long, borst, prostaat en baarmoederhals kanker, waarbij we gebruik maken van verschillende experimentele methoden. De resultaten bleken sterk afhankelijk van de gebruikte methode. Zo werd 48 uur na behandeling met BetA de levensvatbaarheid van de cel en de hoeveelheid celdood gemeten met respectievelijk de MTT test en de PI test. De resultaten waren verrassend: zo bleek dat de half maximale effectieve concentratie van BetA in de MTT test veel lager lag dan de half maximale effectieve concentratie in de PI test. Tevens onderzochten we het anti-proliferatieve effect van BetA middels de kolonie-formatie assay. De resultaten van deze test zijn niet tijdsafhankelijk: in plaats van het meten van de status op een bepaald tijdspunt geeft deze test een eindpunt situatie weer waarin het aantal overlevende en nog delende cellen wordt beoordeeld. Opmerkelijk was dat de concentratie van BetA waarbij er geen kolonie gevormd werden praktisch gelijk lag voor alle geteste cellijnen. Bovendien observeerden wij dat in de aanwezigheid van de pan-caspase remmer zVAD-fmk de apoptotische kenmerken zoals het knippen van het eiwit PARP en de fragmentatie van DNA enerzijds wel bijna geheel geblokkeerd werden, terwijl anderzijds de pan-caspase remmer toch geen bescherming bood tegen celdood. Deze resultaten leerden ons dat het cytotoxische effect van BetA op tumorcellen complexer is dan alleen de inductie van klassieke apoptose.

In de mitochondriale route van apoptose zijn twee pro-apoptotische leden van de Bcl-2 familie, Bax en Bak, essentiële spelers voor het vrijkomen van cytochroom C uit de mitochondriën en de daaropvolgende activatie van caspases. Cellen waarin Bax of Bak ontbreken ondergaan nog steeds apoptose, maar cellen waarin beide eiwitten ontbreken zijn over het algemeen resistent tegen medicijnen die werken op de mitochondriale route van apoptose. Toen wij dit echter voor BetA onderzochten, zoals beschreven in **hoofdstuk 4**, observeerden wij dat cellen waarin Bax en Bak ontbreken nog steeds gevoelig zijn voor BetA en er ook nog apoptotische verschijnselen, zoals het vrijkomen van cytochroom C, optreden. Deze resultaten toonden aan dat de klassieke Bcl-2 familie afhankelijke route niet nodig is in BetA-geïnduceerde cytotoxiciteit. Daarentegen waren cyclosporine A en bongkrekic acid, beide remmers van de mitochondriale permeabiliteits-transitie (PT) poriën, in staat om zowel apoptose als celdood te blokkeren in cellen die met BetA behandeld waren. Dit suggereerde dat de PT poriën cruciaal betrokken zijn bij de door BetA geïnduceerde cytotoxiciteit. Deze bevinding is van groot belang omdat veel tumorcellen resistent zijn tegen medicijnen die zich richten op klassieke, Bcl-2-familie afhankelijke apoptose. Het feit dat niet alleen apoptose maar ook celdood geremd kan worden in de aanwezigheid van remmers van de PT poriën suggereert dat ter plekke van deze poriën het lot van de met BetA behandelde cellen bepaald wordt. Deze bevindingen kunnen ook de sterk overeenkomende anti-proliferatieve concentratie van BetA voor de verschillende geteste cellijnen in de kolonie-formatie test, zoals beschreven in **hoofdstuk 2**, verklaren.

In **hoofdstuk 5** is het onderzoek naar de aard van het effect van BetA op de mitochondriën beschreven. De mitochondriën vertoonden morfologisch gezien een opmerkelijk veranderde modellering na behandeling met BetA. De mitochondriale binnenmembranen (de cristae) verloren hun gebruikelijke langgerekte structuur en vormden concentrische ringen. Deze morfologische verandering is al eerder geconstateerd in de mitochondriën van patiënten met het zogenaamde Barth syndroom. Karakteristiek voor deze zeldzame ziekte zijn de veranderingen in een mitochondriaal fosfolipide, genaamd cardiolipine, met tot gevolg een verzwakte functie van de mitochondriën. De drastische morfologische effecten veroorzaakt door BetA op de mitochondriën impliceerden ook dat de voornaamste functie van deze organellen, het verzorgen van de energiebehoefte van de cel in de vorm van ATP, waarschijnlijk is aangedaan. Dit leidde tot het onderzoeken van mogelijkerwijs door BetA behandeling geïnduceerde autofagie. De inductie van autofagie is vooral bekend als een overlevingsmechanisme in reactie op stress signalen zoals het verlies van ATP of organelschade, maar kan ook een alternatieve route tot celdood zijn. Wij ontdekten dat BetA behandeling een gigantische mate van autofagie induceert. Dit werd - net als vrijkomend cytochroom C en celdood - geblokkeerd door de co-behandeling van de cellen met cyclosporine A. Deze resultaten toonden aan dat de effecten van BetA op de mitochondriën en de inductie van autofagie verwante gebeurtenissen zijn en niet onafhankelijke geïnduceerde processen. In experimenten met cellen waarin de autofagie-route defect is, konden we vervolgens aantonen dat autofagie door BetA geïnduceerd

word als een overlevingsmechanisme en niet als alternatieve celdood route dienst doet. De sterke inductie van autofagie zou kunnen verklaren waarom cellen ondanks de vroege effecten op de mitochondriën in staat zijn om een behandeling met BetA net zo lang te overleven als de celdood die door klassieke apoptose middelen wordt geïnduceerd.

Wij hebben de antikanker effecten van BetA ook *in vivo* onderzocht. Ondanks dat er al enkele publicaties over de toepassing van BetA in proefdieren waren, werd in geen van deze studies een BetA formulatie gebruikt die geschikt is voor toediening in mensen. Daarom was onze focus op het vinden van een formulatie die zowel efficiënt is als ook geschikt voor humane toediening. Door het feit dat BetA een sterk vettige (lipofiele) stof is en daarom slecht oplosbaar in water, is het relatief ingewikkeld om een geschikte formulatie te ontwikkelen die aan voorgenoemde eisen voldoet. We besloten om liposomen te testen als mogelijke drager van BetA in een medicijn. Liposomen zijn kleine blaasjes met een dubbel-membraan welke uit fosfolipiden is opgebouwd. Ze kunnen goed worden gebruikt voor de inbouw en systemische toediening van lipofiele stoffen. In **hoofdstuk 3** tonen we de resultaten van muizen die oraal of intraveneus behandeld zijn met BetA-bevattende liposomen. De tumorgroei was aanzienlijk verminderd vergeleken met de groei in de controle groep. Zeer belangrijk is dat er geen systemische toxiciteit werd veroorzaakt door de behandeling met de BetA-liposomen, zelfs niet na enkele maanden van behandeling. Onze *in vivo* resultaten demonstreren dat de BetA-lipsomen geschikt zijn voor een effectieve behandeling van tumordragende muizen, hoewel een nog verdergaande groeivertraging van de tumoren nastrevenswaardig is. We gebruikten liposomen met een grote diameter, die niet in mensen in de bloedbaan mogen worden ingespoten. Daarom moet er nu verder gezocht worden naar een andere efficiënte manier om BetA toe te dienen, zowel intraveneus (in de aderen) als ook oraal.

Ook onderzochten we het antikanker effect van betuline. Betuline is de chemische voorloper van BetA, wat zeer rijkelijk beschikbaar is in de natuur, bijvoorbeeld in de bast van de witte berk, en zodoende gemakkelijk en goedkoop te verkrijgen is. Eerder was aangetoond dat betuline slechts een beperkt effect heeft op tumorcellen, doch deze beperkte effecten werden over het algemeen niet in detail beschreven. Wij ontdekten dat de aard van de antikanker effecten van betuline *in vitro* vergelijkbaar zijn met die van BetA, zoals wordt gerapporteerd in **hoofdstuk 6**. Ondanks dat de cytotoxische eigenschappen van betuline vergelijkbaar zijn met BetA, zagen we ook belangrijke verschillen tussen de manier van celdood veroorzaakt door deze twee verwante stoffen. Het opmerkelijkst was dat de gezamenlijke toediening van cholesterol het cytotoxische effect van betuline erg versterkte terwijl dit niet het geval was als we de BetA behandeling combineerde met cholesterol. Deze ontdekking is vooral van belang in het kader van *in vivo* studies, omdat cholesterol alomtegenwoordig aanwezig is in alle humane en dierlijke cellen. Toekomstige experimenten zullen moeten uitwijzen of deze unieke eigenschap van de combinatie van betuline met cholesterol mogelijk gebruikt kan worden op een therapeutische manier of dat deze combinatie mogelijk (te)

schadelijk is voor normale niet-maligne cellen. Een ander belangrijk verschil tussen behandeling met BetA, vergeleken met die van betuline en cholesterol samen, is de andere kinetiek van de cytotoxische effecten die worden geïnduceerd. Betuline met cholesterol veroorzaakt een veel snellere celdood vergeleken met BetA, en daarnaast ontdekten we ook dat de beschermende effecten van cyclosporine A meer uitgesproken waren.

We concluderen uit onze studies dat BetA en mogelijk ook betuline, veelbelovende kandidaten zijn voor verdere evaluatie als antikanker medicijn. Dit is te danken aan de unieke manieren waarop ze hun cytotoxiteit tegen kankercellen uitoefenen terwijl gelijkertijd normale cellen niet getroffen worden. Afgezien van de klinische potentie van BetA als chemotherapeutisch geneesmiddel, helpt de studie naar BetA (en betuline) ons ook om de kwetsbare punten van tumorcellen te ontdekken. Het in kaart brengen daarvan is van grote waarde voor het verder ontwikkelen van krachtige antikanker geneesmiddelen.

Curriculum Vitae

Franziska Müllauer was born November 2nd 1978 in Zell am See, Austria and graduated from the Bundesrealgymnasium Zell am See in 1997. After spending 12 months in the USA as an au-pair, she returned to Austria and commenced her tertiary studies, simultaneously undertaking "Genetics and Molecular biology" at the Paris Lodron University Salzburg and "Biomedical Science" at the Landeskliniken Salzburg.

In 2002 she graduated from both programs and took up a position as a research assistant at the Innsbruck Medical University in the Laboratory of Professor Andreas Villunger (Division of Developmental Immunology). This role was undertaken parallel to pursuing a master's program in Molecular Biology at the Leopold-Franzens-University Innsbruck.

Franziska graduated with distinction in 2005 and, in October that same year, moved to the Netherlands where she joined the Laboratory for Experimental Oncology and Radiobiology (LEXOR) at the Academisch Medisch Centrum (AMC). It was here in her role as an assistant in opleiding, under the close supervision of Prof. Dr. Jan Paul Medema and Dr. Jan Kessler, that the work for this thesis was performed.

In April 2009 she took a position as a Clinical Research Associate at the Wilhelminenspital, Center for Hematology and Oncology (Vienna, Austria) on multiple myeloma trials.

In September 2009, Franziska's interest in clinical affairs led her to a role with Quintiles (a Clinical Research Organisation), where she was contracted to Amgen as an Associate for Clinical Trials Operations.

Since December 2010 she has held a position as a Regulatory Affairs Officer at Kwizda Pharma in Vienna.

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Franziska