Betulinic acid, a natural compound with potent anticancer effects

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New therapies using novel mechanisms to induce tumor cell death are needed with plants playing a crucial role as a source for potential anticancer 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 to be relatively nontoxic for healthy cells. In-vivo preclinically applied BetA showed some remarkable anticancer 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 antitumor activity has been related to its direct effects on mitochondria where it induces Bax/Bak-independent cytochrome-c release. ***Anti-Cancer Drugs*** **21:215–227 © 2010 Wolters Kluwer Health | Lippincott Williams & Wilkins.**

Anti-Cancer Drugs 2010, **21**:215–227

Keywords: apoptosis, betulinic acid, cancer, chemotherapy, triterpenoids

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Received 22 October 2009 **Revised form accepted** 18 November 2009

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 semisynthetic 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 antitumor activity [2]. Detailed investigations revealed the disappearance of microtubules and the appearance of crystal structures upon vinca alkaloid treatment [3,4]. By now, the molecular anticancer mechanism of these compounds has been identified to be the destabilization of microtubules, which leads to G_2/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 [7]to promote microtubule assembly. It was approved by the Food and Drug Administration 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 anticancer 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 anticancer treatments, among which the triterpenoids gained much attention lately because of their highly promising results in preclinical studies.

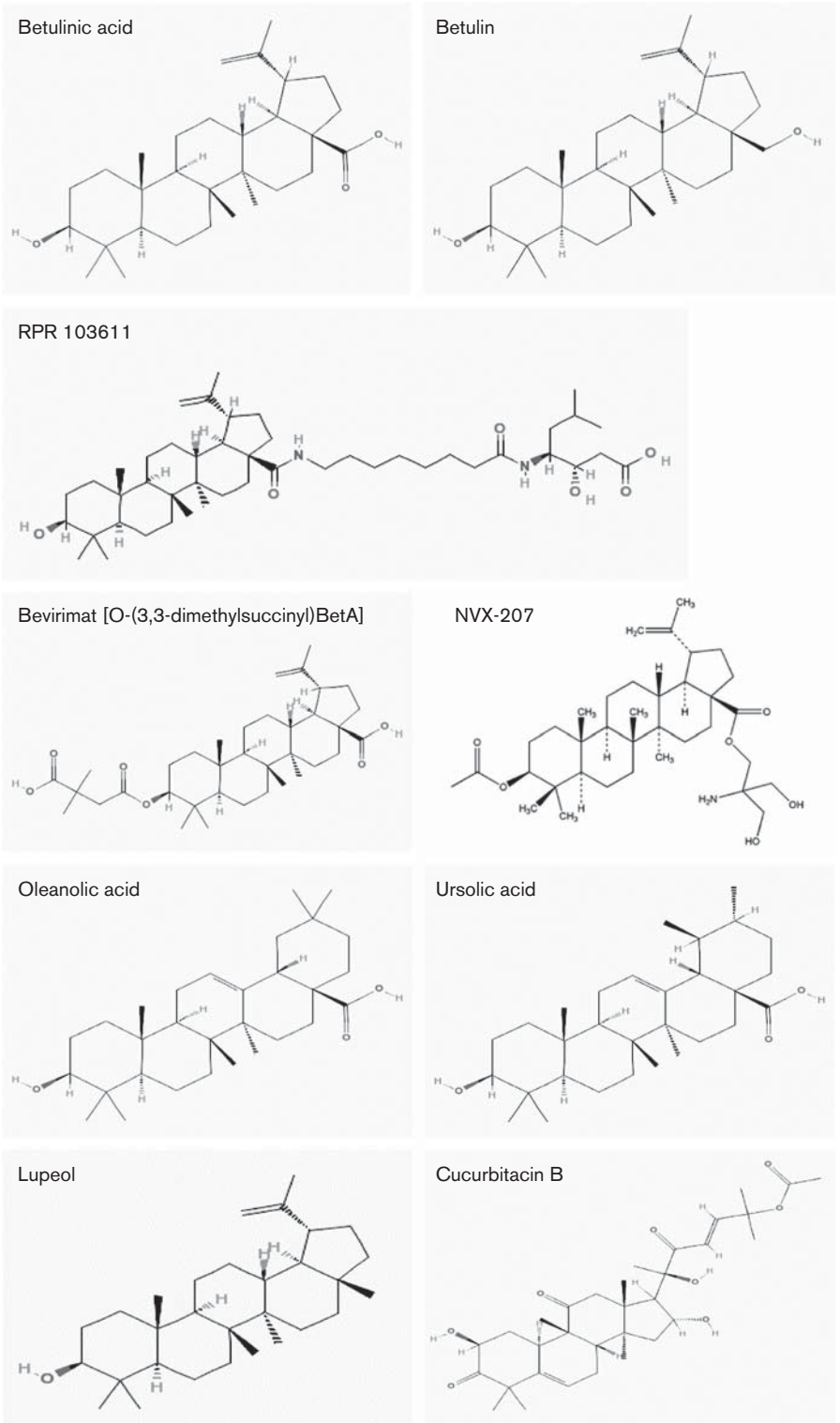
Triterpenoids

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 subclassified 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 being investigated as medicinal products for various diseases, including cancer. For example, 3β,25-epoxy-3α-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 and promoted using 12-*O*-tetradecanoylphorbol-13-acetate. 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 3β,25-epoxy-3α-hydroxylup-20(29)-en-28-oic acid resulted in an almost 50% inhibition of papilloma incidence [14]. In a different in-vivo study similar antitumor effects of lupeol (Fig. 1), another

0959-4973 © 2010 Wolters Kluwer Health | Lippincott Williams & Wilkins DOI: 10.1097/CAD.0b013e3283357c62

lupine-type triterpenoid, were observed. Preapplication of lupeol in 12-*O*-tetradecanoyl-phorbol-13-acetate-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,

Fig. 1



Structures of various triterpenoids as published on PubChem, NVX-207 as published by Willmann *et al.* [15]; BetA, betulinic acid.

strawberries, and mangoes it was proposed to have potential as a dietary antitumor agent [16]. In-vitro studies showed that lupeol possesses antitumor effects against cell lines derived from lung, prostate and pancreatic cancer, leukemia, and hepatocellular carcinomas through the induction of apoptosis [17].

In addition, oleananes (Fig. 1) are investigated for their antitumor 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 [18]. 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 being tested in phase I clinical trials [18].

From the subfamily of ursanes, ursolic acid (Fig. 1) was found to have antitumor effects by inhibiting the expression of tumor necrosis factor (TNF)-induced and NF-kappa B (NF-κB)-regulated genes, cyclin D1, cyclooxygenase, and 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 [19]. Furthermore, ursolic acid inhibited signal transducers and activators of transcription 3 (STAT3) 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 nontoxic triterpenoid inhibited proliferation and induced apoptosis in tumor cells. On account of its presence in apples, basil, prunes, and cranberries, it was suggested to have potential, not only for treatment, but also for the prevention of different cancer types including multiple myeloma [20]. Another representative of the triterpenoids with anticancer activity is cucurbitacin B (Fig. 1), which can be found in many cucurbitacaea species [21], for example, also in the stems of *Cucumis melo* (melon) [22]. Cucurbitacin B was found to have antiproliferative activity on glioblastoma multiforme cells [23], breast cancer [24], myeloid leukemia [25], pancreatic cancer [26], laryngeal squamous cell carcinoma, and other tumor cells [27]. It was reported to exert its anticancer effects through the inhibition of the JAK/STAT signaling pathway [22,26].

Finally, betulinic acid (BetA), which is a lupane-type triterpenoid, was selected from an extensive screen of 2500 plant extracts executed by the National Cancer Institute (NCI). 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 BetA [28].

Betulinic acid

Discovery and sources

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 [29,30]. 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 such as *Sarracenia flava* (Sarraceniaceae) [31] to trees and shrubs such as *Diospyros* spp. (Ebenaceae), *Inga punctata* (Fabaceae) [32], *Ziziphus* spp. (Rhamnaceae), *Vauquelinia corymbosa* (Rosaceae) [33], and *Syzygium* spp. (Myrtaceae) [30,34,35]. 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, however, through a simple oxidation process from betulin isolated from this tree [34,36]. 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 [35]. 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 [37]. 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 anticancer agent BetA had already been shown to be effective against HIV through the inhibition of replication [38]. 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 [39]. A very promising BetA derivative is PA457 (bevirimat, Fig. 1), which prevents HIV-1 virus maturation and virus release from infected cells [40]. It was well tolerated in a phase I/II clinical trial as a single-dose administration and importantly no bevirimat resistance mutations were detected in this study [41]. Other studies, however, showed mutations in a certain region of the viral protein gag causing resistance to bevirimat [42,43]. Nevertheless, it is a highly promising candidate and is 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 the optimal pharmacophores for HIV-1 and HIV-2 targeting are different [44].

BetA has also been shown to possess antibacterial activities, although the results are conflicting [30]. 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 [45]. When it was first tested as

an antimalarial drug in a murine malaria model, it was ineffective in reducing parasitemia [46]. Recent in-vivo results in mice, however, infected with *Plasmodium berghei* and treated with BetA-acetate suggest an antiplasmodial activity by the analysis of parasitemia [47]. Other biological activities of BetA include anthelmintic and anti-inflammatory effects [48,49].

Antitumor effects

In a systematic screening of 2500 plant extracts tested by the NCI, BetA was rediscovered in 1995 as a potent antimelanoma 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 to 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-G_1 peak by flow cytometry analysis in MEL-2 cells suggested that BetA-induced apoptosis. Most importantly, this study also showed 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 intraperitoneal application of 50, 250, or 500 mg/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/kg bodyweight BetA [28]. On the basis of these results it was selected for the RAID (Rapid Access to Intervention Development) program by the NCI [50].

Initially described to be specific against melanoma cells [28], 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 [51,52]. It was shown that other brain tumors such as glioma cells [53], 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 nonmalignant neuronal cells were observed [54]. In 2001, BetA was shown to induce antiproliferative effects in ovarian carcinoma (IC_{50} 1.8–4.5 μg/ml), nonsmall-cell 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 [55]. 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 antineoplastic effects of BetA were confirmed in an in-vivo ovarian carcinoma xenograft mouse model [55]. Later on, head and neck squamous cellular carcinoma cells were also discovered to be sensitive to BetA [56]. Finally, we and other investigators established the universal efficacy of BetA against tumor cells derived from colon, lung, breast, prostate, and cervix cancer [57–60], indicating that BetA has a broad efficacy against a wide range of different solid tumors. IC_{50} for most tested lung, colorectal, breast, prostate, and cervical cancer cell lines ranged from 3.8 to 16.4 μg/ml in cell death assays [59]. 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 [61]. 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 [62]. BetA also induced apoptosis in the antileukemic therapy-resistant human chronic myelogenous leukemia cell line, K-562 (derived from the blast crisis stage), without affecting the levels of Bcr-Abl [63].

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 compared with normal melanocytes as measured by growth analysis [64] and apoptosis [65]. Interestingly, normal human keratinocytes differentiated into corneocytes whereas the immortalized keratinocyte cell line, HaCaT, underwent apoptosis [65]. In addition, peripheral blood lymphocytes and human skin fibroblasts were reported to be highly resistant toward BetA [55,60]. The molecular mechanisms underlying this remarkable phenomenon remain to be elucidated. Most importantly, BetA's nontoxicity 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 anticancer 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 regard to the role of Bcl-2. The apoptosis-inducing effects of BetA have been studied quite extensively and are discussed below. Recently, however, we discovered that the cytotoxic effects of BetA do not solely rely on caspase activity [59], prompting the idea that other cell death pathways may be induced by BetA, especially in cells in which apoptosis is blocked. To unravel these pathways as well as their role in BetA-induced cell death will be an extremely challenging task

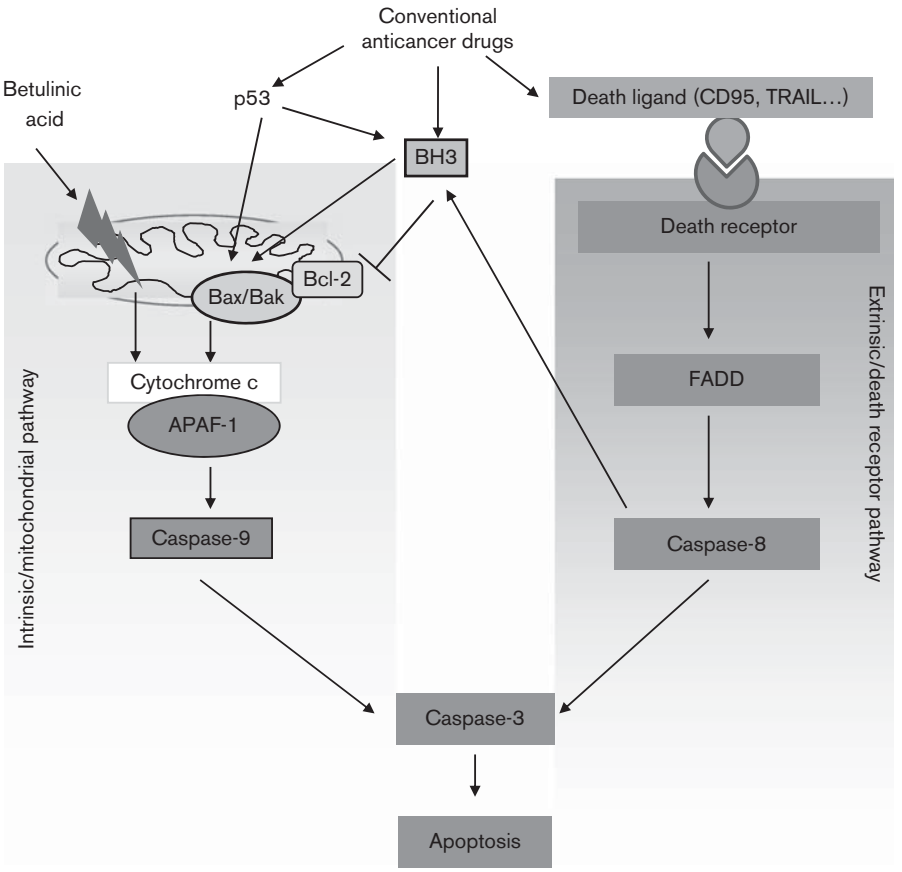
because of the numerous reported targets of BetA. These include enzymes (kinases, aminopeptidase N, acetyl-CoA acetyltransferase, topoisomerase I/II), the transcription factor NF-κB as well as cell cycle regulation and the proteasome. On account of the broad anticancer effects of BetA it is likely that even more molecular targets of this compound will be discovered in the future. It is, however, 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 TNF receptor superfamily [66–68]. This leads through the adaptor molecule, Fas-associated death domain, to a cleavage of caspase-8 and caspase-10 [69,70]. The mitochondrial pathway is regulated by the Bcl-2 family proteins consisting of prosurvival (e.g. Bcl-2, Bcl-XL or Mcl-1) and proapoptotic 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 proapoptotic (Bax and Bak) and antiapoptotic (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 [71]. In addition, p53 plays an important role in this pathway as the activation of p53 can lead to the expression of BH3-only molecules, Puma and Noxa [72], or it can directly transcriptionally or functionally activate Bax [73,74].

Induction of apoptosis with subsequent cell death is the goal of many anticancer 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

Fig. 2



Induction of apoptosis by conventional anticancer drugs and BetA: commonly used anticancer 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 through 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. BetA, betulinic acid; FADD, Fas-associated death domain.

usually target apoptosis either indirectly, by inducing cellular stress leading to the intrinsic activation of apoptosis, for example, through p53, or otherwise upstream of the mitochondria. The latter includes CD95L, TRAIL, and other death ligands, which bind to their respective death receptors, thereby triggering apoptosis. For example, the naturally occurring and widely used chemotherapeutic compound, taxol, binds to microtubule polymers, which results in the formation of depolymerization-resistant microtubules. Resistance of cancer cells toward taxol also involves, next to alterations in tubulin [75], 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 other apoptotic stimuli as well) results in translocation of Bim and neutralization of Bcl-2, thereby inducing apoptosis [76]. Deletion of Bim or overexpression of Bcl-2 in tumor cells causes resistance to taxol [77]. Other examples are DNA-damaging treatments (including γ 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 [78]. As such, treatment modalities that depend on p53-dependent apoptosis are not likely to function. Therefore, a new class of anticancer agents directly targeting mitochondria and not depending on p53, such as BetA, holds great promise in overcoming drug resistance in tumor cells (Fig. 2) [79–81].

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 [82]. 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 [53]. In ME20 melanoma cells, induction of p53 expression was not detected [64] and other studies exploring the effects of BetA on various p53 wild-type and mutant cell lines found no difference in sensitivity [55,59, 83]. Taken together these results suggest that BetA-induced apoptosis does not involve p53.

Role of the Bcl-2 family and reactive oxygen species

In SHEP neuroblastoma cells overexpression of Bcl-2 and Bcl-XL blocked BetA-induced loss of mitochondrial membrane potential, ROS hyperproduction, caspase processing, and PARP cleavage. The expression of the proapoptotic molecules, Bax and Bcl-Xs, was induced in BetA-treated cells [51]. BetA also triggered permeability transition (PT) and cytochrome c release in isolated mitochondria suggesting a direct effect on mitochondria. Mitochondria isolated from SHEP cells overexpressing Bcl-2 or Bcl-XL were resistant to BetA-induced effects [84]. 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 overexpression [85]. Consistently, in glioma cells BetA-induced ROS generation, which was blocked by Bcl-2 or the antioxidants, *N-tert*-butyl-a-phenylnitrone and *N*-acetyl-cysteine. 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 [53]. In contrast, in human melanoma cells no upregulation of Bax and Bcl-Xs was observed; however, the prosurvival molecule Mcl-1 was clearly induced under the same conditions [64]. Expression of Mcl-1 can change the balance in proapoptotic and antiapoptotic molecules and thus be crucial for BetA-induced apoptosis at least in melanoma cells. Interestingly, Bcl-2 overexpression provided only partial protection in Jurkat cells [62,86], MCF-7 [86] and melanoma cells treated with BetA [83]. These results suggest that the protective effects of prosurvival members of the Bcl-2 family are possibly cell type specific. In addition, the differential upregulation of prosurvival and proapoptotic 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 downregulation of Bcl-2 whereas Bax levels were increased, resulting in a significant change in the Bax/Bcl-2 ratio [60]. 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 [56]. Recently, we showed that BetA-induced cytochrome c release and apoptosis in Bax/Bak double deficient mouse embryonic fibroblasts and in a colon carcinoma cell line lacking functional Bax and Bak. Importantly, the level of apoptosis induced in the double deficient cells was comparable with the wild-type control cells, suggesting that BetA induces cytochrome c release and apoptosis independently of Bax and Bak. In contrast, cells were protected from BetA in the presence of cyclosporine A, an inhibitor of the PT pore [86]. This is in concordance with earlier observations in which bongkrekic acid, another inhibitor of the PT pore, was able to prevent BetA-induced cytotoxic effects [84]. Taken together, the available data in the literature indicate that BetA exerts its cytotoxic effects through a direct effect on the mitochondria that is independent of the Bcl-2 family of proteins, but instead, depends on the PT pore.

Betulinic acid and other cell death pathways

After the discovery of BetA as an anticancer agent, it was immediately established that it exerts its cytotoxic activity through the induction of apoptosis [28,52]. 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 [51]. Thus, BetA-induced apoptosis was suggested to be independent of the death receptor pathway in neuroblastoma, glioma, and melanoma cells [30], 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 [87], it is highly unlikely that BetA would exert its cytotoxic effects through this pathway.

Interestingly, BetA was found to induce cell death in the presence of the pan-caspase inhibitor zVAD.fmk in Jurkat cells. At the same time, PARP processing and DNA fragmentation were completely blocked [59]. These results suggest that the cytotoxic effects of BetA are not fully caspase dependent and that other cell death pathways are likely to be activated on treatment with BetA.

Other targets of betulinic acid

Aminopeptidase N (CD13)

Aminopeptidase N is a transmembrane peptidase that is expressed in neovessels in developing tumors whereas normal endothelial cells do not express it. As aminopeptidase N is a potent angiogenic regulator and is related to tumorigenesis [88], the potential of BetA as an inhibitor of angiogenesis was investigated. One study suggested that the antimelanoma effects of BetA are because of the inhibition of aminopeptidase N activity [89]. The results of another study, however, showed that the anti-angiogenic activity of BetA was not because of the effects on aminopeptidase N but rather through an effect on the mitochondria of endothelial cells [90]. It is therefore unclear what the significance of BetA-induced inhibition of aminopeptidase N is for tumor cell death.

Acetyl-CoA acyltransferase, diacylglycerol acyltransferase

Acetyl-CoA acyltransferase (ACAT) exists in mammalians in two isoforms and catalyzes the acylation of cholesterol to cholesteryl ester. Therefore, ACAT inhibitors are investigated for the treatment of hypercholesterolemia and atherosclerosis [91]. BetA was found to be a potent inhibitor of human ACAT1 (mitochondrial acetyl-CoA acetyltransferase) and ACAT2 (cytosolic acetoacetyl-CoA thiolase) [91]. As the anticancer effects of BetA are strongly linked to the mitochondria, it is interesting to study whether ACAT inhibition is associated with BetA-induced anticancer effects.

Diacylglycerol acyltransferase, a microsomal enzyme linked to obesity, catalyzes the terminal step in triacylgycerol synthesis and plays an important role in lipid metabolism [92]. It is inhibited by BetA [93] and in this context BetA has been also suggested to be a potential lead compound for the treatment of obesity [94]. 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 anticancer 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 [15].

Kinases

Treatment with BetA was shown to cause the activation of p38 and other proapoptotic mitogen-activated protein (MAP) kinases whereas antiapoptotic MAP kinases remained unaffected [95]. The investigators 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 [95]. Another study described the antagonizing effects of U0126, a MEK (MAP kinase kinase) inhibitor, on BetA-induced apoptosis [96]. Interestingly, BetA was also reported to transiently activate the epithelial growth factor receptor/AKT survival pathway and to enhance survivin expression, resulting in the decreased sensitivity of melanoma cells [97]. Others, however, did not detect significant changes in ERK1/2 and AKT kinase activity [60] and survivin expression was decreased in the prostate cancer line, LNCaP [57]. 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

Anticancer agents, etoposide and camptothecin, depend for their action on topoisomerase inhibition [98]. In addition, BetA has 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 religation [99,100]. 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 [101]. It is, however, 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, semisynthetic BetA analogs were discovered to possess strong topoisomerase I and IIα inhibitory effects and also exhibited stronger cytotoxic effects on cancer cells compared with BetA itself [102]. Although whether cell death depends on

topoisomerase was not studied, the investigators concluded that BetA is a useful platform for designing potent new topoisomerase inhibitors [102].

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 TNF, thereby enhancing TNF-induced apoptosis. In addition, NF-κB-regulated growth factors, such as COX-2 (cyclooxigenase 2) and matrix metalloproteinase 9, were suppressed [103,104]. 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 [105]. 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 [103] and prostate cancer [104] cell lines whereas the activation of NF-κB by BetA was found in the neuroblastoma cell line, SHEP [105]. It was also suggested that the role of NF-κB in BetA-induced apoptosis is context specific [79–81]. Furthermore, it is important to note that SHEP cells gave different results compared with cell lines derived from other tumor types when the effect of Bcl-2 overexpression 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 [57,60]. 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 through the induction of G_0/G_1 arrest, thereby inhibiting proliferation [106]. Another group found accumulation of p21 on BetA exposure in glioma cells. This, however, did not result in cell cycle arrest [53]. Similarly, BetA did not affect cell cycle distribution in an ovarian cancer line [55]. In melanoma cells, BetA-induced cell cycle arrest in the G_1 phase [107] 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 [96]. 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 anticancer 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) [57]. 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 [108]. 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 is already applied in the clinic for multiple myeloma [109]. 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 [28]. It is surprising that only a few studies addressing the in-vivo efficacy of BetA have followed since. This is likely because of 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 anticancer effect. The initial report described a method for enhancing the solubility by coprecipitating BetA with polyvinylpyrrolidone. After reconstitution, polyvinylpyrrolidone-complexed BetA was injected intraperitoneally into nude mice-bearing subcutaneous human melanoma (Mel-1 and Mel-2, see before) xenografts. A dose of 50 mg/kg body weight injected every 4 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/kg body weight (as judged by body weight) [28]. 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, and low tissue concentrations were found in the heart and the brain [50]. BetA showed antimetastatic activity by itself and in combination with vincristine in a B16F10 melanoma mouse model. The treatment dose of BetA was 10 mg/kg bodyweight per day and again, no signs of toxicity were

detected [107]. In an ovarian cancer xenograft model BetA-treated mice (100 mg/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 [55]. In all these studies BetA was applied intraperitoneally. 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/kg bodyweight orally every other day with corn oil serving as a vehicle [57]. This indicates that BetA retains its activity even after oral application. Similarly, activity is observed when the route of application is through an intravenous 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 [110]. 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 because of drug resistance toward one of the compounds resulting in either additive or synergistic antitumor effects. Moreover, such protocols generally can suffice with lower concentrations of single compounds and toxic effects for the patient can therefore be lesser. The anticancer effects of BetA have been studied in combination with several other cancer treatments. Sensitizing effects of BetA were shown *in vitro* for hyperthermia applied on human melanoma cells that were first adapted to low pH [111]. Treatment with BetA in combination with irradiation resulted in additive growth inhibition of melanoma cells. The investigators concluded that the additive effects were because of the targeting of either different pathways or different tumor cell populations [64]. In another murine melanoma cell model the combination effects of BetA and vincristine were explored *in vitro* and *in vivo*. The effect of 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 [107]. In addition, the combination of BetA with the epithelial growth factor receptor inhibitor PD153035 was found to enhance cell death of melanoma cells *in vitro* [97]. Furthermore, BetA cooperated with anticancer drugs, doxorubicin and etoposide, to induce apoptosis and to inhibit clonogenic survival in SHEP neuroblastoma cells [112]. It also cooperated with TRAIL (TNF-related apoptosis-inducing ligand) to induce apoptosis in tumor cell lines and primary tumor cells, but not in normal human fibroblasts [87]. Although these reports would suggest that 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 h) even showed antagonistic and subadditive effects [113]. In addition, combined treatment of BetA and NF-κB inhibitors was concluded to have no therapeutic benefit and could in certain tumors even be counterproductive [105]. 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 [58]. 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* [114]. 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 precursor molecule of BetA. Initially, it was described as being inactive or less active against cancer cells compared with other triterpenoids [115–117]. The results of recent reports, however, suggest that betulin also might have potential as an anticancer drug [118,119]. In particular, the combined application of betulin and cholesterol resulted in massive cell death of leukemia, lung, and cervix cancer cell lines *in vitro* [120]. It is not clear whether this is because of a conversion of betulin to BetA, but these results indicate that betulin deserves more attention as a potential anticancer reagent.

Betulinic acid derivatives

BetA holds great promise as an antitumor agent, but as mentioned has a severe drawback in its poor solubility in aqueous solutions and thus its application *in vivo*. Another nonscientific 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 anticancer 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 [116]. Modifications at C-20 did not enhance cytotoxicity in several cancer cell lines [121], 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 [122]. Hydroxylation at the C-3 position gave promising results when tested on murine melanoma cells [123] and

another chemical modification at the C-3 position (dimethylsuccinyl BetA) turned BetA from a proteasome activator into a proteasome inhibitor [108]. Yet another C-3 modification gave better antitumor results in a colon cancer xenograft mouse model when compared with BetA [110]. The ring skeleton of BetA is the platform for many other interesting modifications [99,124,125]. One novel, well-tolerated BetA derivative is NVX-207, which showed significant antitumor activity in clinical studies in canine cancer patients with treatment-resistant malignancies [15].

Future avenues

In summary it can be concluded that BetA is a promising natural compound for future treatment of cancer. It induces apoptosis through a direct effect on the mitochondria, which does not depend on Bax and Bak. The direct molecular target(s) of BetA, however, remain to be identified. The identification of the molecular target(s) is especially important as it would also allow the specific design of other new anticancer drugs (including BetA derivatives), which would not have certain drawbacks, such as poor solubility. We believe that this feature is the main difficulty in finding a formulation suitable for application in humans. For other lipophilic compounds the use of liposome formulations has proven to be successful in the clinic. Whether such a formulation would also be suitable for BetA remains to be clarified. In addition, other approaches such as the use of cyclodextrins as vehicle should be addressed. NVX-207, for example, has been formulated earlier with cyclodextrin at 10 mg/ml [15]. For finding an optimal BetA formulation extensive in-vivo studies will be required to determine antitumor effects, tolerability and pharmacokinetics. Knowing the exact molecular target(s) would also help the design of combination protocols. It would be easier to predict whether the combined effects will be additive/synergistic or antagonistic. One of the most striking features of BetA is its high efficacy against cancer cells derived from therapy-resistant or refractory tumors while being nontoxic for cells derived from nontumor tissues. To unravel the underlying principles of this remarkable phenomenon will be a major challenge for future research. As the mitochondria have been concordantly reported to be a direct target of BetA it is conceivable to find an answer in the differential metabolism of tumor cells and healthy cells. Finally, it should be noted that bevirimat, a highly promising BetA derivative, which inhibits virus maturation is currently being investigated in HIV-1 patients in phase II clinical trials. The results of another phase II study investigating the therapeutic effect of betulin-based oleogel against actinic keratosis, an in-situ squamous cell carcinoma, are also highly promising and, importantly, no severe adverse events were observed [126]. These results indicate that, in principle, betulin, BetA derivatives (and possibly BetA itself) are safe to be used in humans.

Acknowledgement

This study was supported by a grant of the Sticht-ing Nationaal Fonds tegen Kanker, Amsterdam, The Netherlands.

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