Betulinic acid and the pharmacological effects of tumor suppression (Review)

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Abstract. Betulinic acid (BA), a lupane-type pentacyclic triterpenoid saponin from tree bark, has the potential to induce the apoptosis of cancer cells without toxicity towards normal cells *in vitro* and *in vivo*. The antitumor pharmacological effects of BA consist of triggering apoptosis via the mitochondrial pathway, regulating the cell cycle and the angiogenic pathway via factors, including specificity protein transcription factors, cyclin D1 and epidermal growth factor receptor, inhibiting the signal transducer and activator of transcription 3 and nuclear factor-κB signaling pathways, preventing the invasion and metastasis of tumor cells, and affecting the expression of topoisomerase I, p53 and lamin B1. In previous years, several studies have shown its antitumor effect, initially applied to malignant melanoma, however, it also has broad efficacies against most solid types of tumor from different regions of the body. There have been few investigations in hematological malignancies, however, this direction may offer potential in such a novel field of research. In this review, the primary pharmacological effects of BA in tumors, particularly in hematological malignancies are discussed.

Contents

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1. Introduction

Betulinic acid (BA) is a lupane-type pentacyclic triterpenoid saponin (3β-hydroxy-lup-20 (29)-en-28-oic acid; MW, 456.71; Fig. 1), which exists in the bark of a variety of natural plants, principally in *Betula*. It has been investigated extensively in previous decades due to its beneficial properties, including anticancer, anti-inflammatory, anti-angiogenic, and immunomodulatory effects, its anthelmintic activity and its anti-human immunodeficiency virus effects. Its antitumor effects are higher at a reduced pH (<6.8), a characteristic of several types of tumor (1-3).

In previous decades, BA has been shown to have a marked antitumor therapeutic effect in melanoma cells and several types of solid tumor, including glioblastoma (4), lung carcinoma (5), breast carcinoma (6), colorectal carcinoma (7) and prostate carcinoma (8). In addition, the antitumor effects on hematological malignancies have been investigated in our previous studies and in those of others in previous years (1,9-11).

The reported primary mechanisms of the anticancer effects of BA treatment are shown in Fig. 2 and described below.

Promotion of apoptosis by activation of the mitochondrial pathway. BA improves the level of reactive oxygen species (ROS) production and alters the mitochondrial membrane potential gradient, followed by the release of cytochrome *c* (Cyt *c*), which causes the mitochondrial-mediated apoptosis of tumor cells via a caspase-dependent mechanism and apoptosis inducing factor (1,12,13). It has been demonstrated that there is a link between ROS and the p38 and stress-activated protein (SAP) kinase/c-Jun N-terminal kinase (JNK) in melanoma cells. This indicates that ROS act upstream of the mitogen-activated protein kinases (MAPKs) in the signaling pathway of BA (14). In addition, autophagy has been shown to occur downstream of the mitochondrial damage induced by BA (15).

Regulation of cell cycle and the angiogenic pathway via specificity protein (Sp) transcription factors, cyclin D1 and epidermal growth factor receptor (EGFR). BA can inhibit cancer cell growth and proliferation via cell cycle arrest. Drugs, including BA, can inhibit the protein expression of Sp1, Sp2 and Sp4 through the microRNA (miR)-27a-ZBTB10-Sp1 axis and slow down the aggressiveness of the tumor (16-19).

Inhibition of the signal transducer and activator of transcription 3 (STAT3) and nuclear factor (NF)-κB signaling pathways. BA can downregulate the activation of STAT3 through the upregulation of Src homology 2 domain-containing phosphatase 1 (SHP-1), and affect the STAT3/HIF-1/VEGF signal pathway (20-22). The expression of NF-κB can be inhibited by reducing the activation of inhibitor of NF-κB (IκBα) kinase (IKKβ) and phosphorylation of IκBα with BA (23).

Prevention of the invasion and metastasis. The invasion and metastasis of malignancies is prevented via epithelial-mesenchymal transition (EMT) and inhibition of topoisomerase I (24).

The aim of this review was to discuss the primary pharmacological effects of BA in solid types of tumor and in hematological malignancies, and to provide a valuable reference for future investigations in the hematological system.

2. Sources of BA

BA is a type of pentacyclic triterpene acid, which is found in the bark of several species of plant. As a natural compound, it has a wide range of biological activities, and also the characteristics of low toxicity and a high safety index. BA has attracted increasing attention over previous years due to these properties (25).

Previous studies have revealed three sources of BA. Its direct extraction from plants is the earliest and most direct source. The primary raw material used for BA extraction is *Betula* bark. The bark of *Platanus acerifolia*, *Vochysia divergen*, *Euphorbiacea*, *Ficus pandurata* Hance, and the leaves of *Vitex negundo* and *Pterospermum heterophyllum* Hance can also be used as raw material to extract BA. However, the extraction rate (up to 3.3%) is low due to the low content of BA in the bark of these plants. In order to increase the extraction efficiency, the preparation of semi-synthetic BA has been introduced. This method provides a higher rate of extraction from betulin, which is an associated natural compound and important constituent of birch bark (22-30%), which can be converted into BA in high yields through an oxidation process (26,27). Another method used to extract BA is microbial fermentation. Microbial transformation has several advantages, including mild reaction conditions, low cost and reduced pollution. Several types of microbes have been used, namely *Aspergillus oryzae* AS 3.49, *Aspergillus* sp. WZ, *Aspergillus foetidus* ZU-G1 and *Trichoderma koningii* ZJ. However, further investigations are required for the large-scale preparation of BA with the use of microbes (28).

3. Antitumor effects of BA in solid tumor types

BA was initially confirmed as a selective inhibitor of human melanoma cells (29). BA has attracted attention due to its unique anticancer activities of selective tumor growth inhibition or apoptosis, without damaging normal cells, at a concentration >100 mg/kg body weight (30). Several types of solid cancer cell have been shown to be sensitive towards BA. The following section focuses on the mechanisms underlying the effects of BA in solid tumor treatment.

Malignant melanoma. Malignant melanoma, to which individuals of European origin are vulnerable, accounted for 1.6% of new cancer cases in 2012 worldwide (31). As a consequence, it is imperative to identify an effective treatment approach. BA is a specific toxic reagent towards melanoma cells and was first used for the treatment of melanoma. Tan *et al* (14) demonstrated that treatment of UISO-Mel-1 human melanoma cells with BA leads to the activation, via phosphorylation, of pro-apoptotic MAPK proteins, P38 and SAP/JNK, the formation of ROS and the upregulation of caspase (14). Pisha *et al* (29) initially reported that BA induced the apoptosis of a number of melanoma cell lines, including MEL-1, 2, 3 and 4, with half maximal effective dose values ranging between 0.5 and 4.8 μg/ml. In addition, BA interferes with EMT-associated changes, a mechanism to antagonize invasive melanoma cells A375 at a concentration of 10 μM, whereas BA reduces A375 cell proliferation at a concentration of 50 μM (32).

Cervical cancer. BA activates the endoplasmic reticulum pathway and the ROS-mediated mitochondrial pathway to induce apoptosis of HeLa cells. Potze *et al* (33) demonstrated that BA causes cell membrane rupture, apoptosis and mitochondrial depolarization in HeLa cell lines, with an minimum effective concentration of 7.5 μg/ml, reaching a plateau at 10 μg/ml.

BA increases the levels of microtubule-associated protein 1 light chain 3 (LC3-II) more markedly in HeLa cell lines, compared with DMSO-treated control groups, and the BA-treated HeLa cell lines have a potent inducing effect on the expression of p62. BA can also inhibit the autophagic flux by increasing the degradation of long-lived proteins following 14 h medium replacement (33).

The B cell lymphoma-2 (Bcl-2) family members interact with each other to maintain mitochondrial integrity and regulate cell apoptosis. The two predominant types of Bcl-2 proteins include anti-apoptotic proteins, including Bcl-2-A1, Bcl-2, Bcl-extra large (Bcl-xL), Bcl-2-like protein 2, and myeloid cell leukemia-1, and pro-apoptotic proteins, including Bcl-2-associated death promoter, Bcl-2 homologous antagonist/killer, Bcl-2-associated X protein (Bax), BH3-interacting domain death agonist, Bcl-2-interacting killer, Bcl-2-interacting mediator of cell death, activator of apoptosis harakiri, phorbol-12-myristate-13-acetate-induced protein 1 and p53-upregulated modulator of apoptosis. BA downregulates Bcl-2 and upregulates the Bax gene in HeLa cell lines (34-36).

Breast cancer. Previous research demonstrated that Sp is overexpressed in tumors (19). Previous reports have shown that BA mediates antitumor activity by downregulating the Sp1 transcription factor. Knocking down the expression of Sp1 inhibits tumor growth and angiogenesis in xenograft models (10,19). ZBTB10 is a transcriptional repressor of Sp transcription factors, and drugs inhibit Sp transcription factors through the microRNA (miR)-27a-ZBTB10-Sp1 axis. BA induces the apoptosis of MDA-MB-231 estrogen-receptor-negative breast cancer cell lines by downregulating the mRNA and protein levels of Sp1, Sp3 and Sp4 at concentrations of 2.5-10 μM, decreasing the expression of miR-27a and increasing the levels of ZBTB10 *in vitro* and *in vivo* (16,37,38).

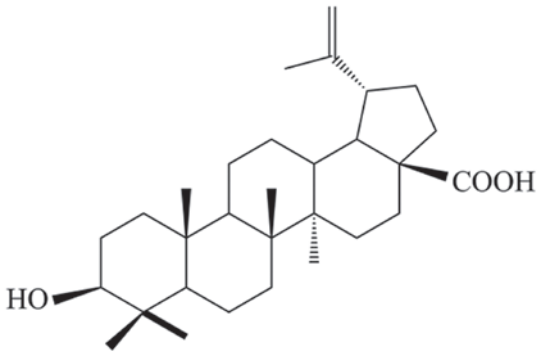

Figure 1. Chemical structure of betulinic acid ($C_{30}H_{48}O_3$; MW, 456.71).

Yin Yang 1 (YY1), an Sp-regulated gene, is a key upstream regulator of ErbB2. BA inhibits the expression of YY1 in BT474 and MDA-MB-453 cell lines. The activation of cannabinoid type 1 (CB1) and CB2 receptors, which modulate the miR-27a-ZBTB10-Sp1 axis, mediate the effects of Sp transcription factors and ErbB2 on these two cell lines (39).

P53, a well-known tumor suppressor, mediates cell cycle arrest and apoptosis (40). BA induces the apoptosis of MCF-7 and T47D breast cancer cell lines in an p53-independent apoptotic pathway with half maximal inhibitory concentration (IC_{50}) values of 12.3 and 9.8 μg/ml, respectively, following 72 h incubation (6,41).

Lung carcinoma, colorectal carcinoma and gastric adenocarcinoma. Lung cancer and colorectal cancer are considered to be major contributors to incidence and mortality rates (42,33). In a previous study in nude mice, compared with control mice, BA-treated transplanted tumors of A549 lung cancer or SW480 colon cancer cell lines grew at a slower rate, with an IC_{50} of 4.3 μg/ml in the A549 cell lines, determined using MTT (44).

BA inhibits the proliferation of colon cancer cells and xenograft tumor growth. It induces the proteasome-dependent and -independent downregulation of Sp transcription factors, including Sp1, Sp3 and Sp4, in SW480 and RKO cell lines, at concentrations of 5-10 μM. In addition, BA disrupts the expression of miR-27a and ZBTB10 mRNA in RKO cell lines (45,46). The expression levels of Sp-regulated genes, including cyclin D1, p65, EGFR and Bcl-2 also decrease. In addition, BA can markedly decrease the percentage of RKO cells in the G0/G1 and S phases, and increase the percentage in the G2/M phase (47,48).

BA mediates G2/M cell cycle arrest and downregulates the protein expression of Hiwi and cyclin B1 in the AGS human gastric adenocarcinoma cell line, with an IC_{50} of 12.99 μg/ml (49).

Vascular endothelial cell growth factor (VEGF) is a regulator of physiological and pathological angiogenesis. It is expressed at high levels in several types of solid tumor, including colon carcinoma and breast cancer. BA can decrease the expression of VEGF via Sp proteins, thus having an antiangiogenic role (50,51).

Pancreatic cancer and hepatocellular carcinoma. The lamin B1 protein, an important member of the lamin protein family (52), regulates apoptosis, proliferation, invasion and metastasis (53). The expression of lamin B1 is reduced in lung cancer, colon cancer, breast cancer, bronchial carcinoma and gastric cancer (54,55), whereas the expression of lamin B1 is increased in prostate cancer and hepatocellular carcinoma (56,57).

The expression of lamin B1 is positively correlated with the growth of cancer. Sp1, a lamin B1 downstream gene, may regulate the expression of lamin B1. However, BA suppresses the expression of lamin B1 in pancreatic cancer cells independent of the Sp1 protein *in vitro* and in xenograft models (58-60).

The upregulation of lamin B1 in hepatocellular carcinoma tumors correlates with tumor size, stage and nodule number. Elevated levels of plasma lamin B1 can predict early stage hepatocellular carcinoma with a sensitivity of 76% and a specificity of 82% (61).

Prostate cancer, bladder cancer and endometrial adenocarcinoma. The dysregulation of STAT3 is involved in tumor cell survival, proliferation, apoptosis and metastasis. BA mediates anticancer activity through inhibiting STAT3 in solid tumors. It was reported that BA may be a potent anti-angiogenic drug in prostate cancer, affecting the expression and transcription of hypoxia-indicible factor (HIF)-1α, STAT3 and VEGF, and capillary tube formation (20,22).

In endometrial adenocarcinoma cells, BA is vital in cancer development and progression. It inhibits prolidase, which catalyzes collagen degradation in the final step, and decreases the expression of α1 and α2 integrin, HIF-1, VEGF, glucose transporter-1, erythropoietin-1, carbonic anhydrase and glyceraldehyde-3-phosphate dehydrogenase (62,63).

NF-κB, a key regulator of stress-induced transcriptional activation, regulates cell survival, proliferation, apoptosis, immune responses and adaptive responses to alterations in cellular redox balance (23,64). BA inhibits the expression of NF-κB, which leads to a decrease in the activity of IKKβ and phosphorylation of IκBa in PC-3 human prostate carcinoma cells. BA treatment for 24 h results in a dose-dependent reduction in cell viability, which ranges between 2.9 and 91.2% in PC-3 cells at concentrations of 1-40 μM. Furthermore, the protein expession of cyclin D1 is lowered in a mouse model of prostate cancer treated with BA (10 mg/kg) (8).

The expression of EGFR is correlated with vascularity. BA can significantly downregulate the expression of the Sp-dependent gene, EGFR, through repression of the Sp1, Sp3 and Sp4 proteins in 253JB-V and KU7 bladder cancer cells at a concentration of 5 or 10 μM (19,65).

Head and neck carcinoma. The RET proto-oncogene, involved in recurrent chromosomal rearrangements, is found in thyroid and lung cancer. Of all papillary thyroid carcinoma (PTC) cases, ~20% are attributed to RET/PTC rearrangements (66,67).

Topoisomerases, a class of ubiquitous enzymes located in the cell nucleus, catalyze the fracture and combination of DNA strands. BA secludes to topoisomerase I in the nucleoplasm. Therefore, BA inhibits topoisomerase I DNA cleavage complex formation. Coincidentally, fragile site breakage of the RET proto-oncogene is affected by DNA topoisomerase I (68-70).

The BA derivative, compound 15, shows marked inhibition of SW1736 anaplastic thyroid cancer cell lines in a short

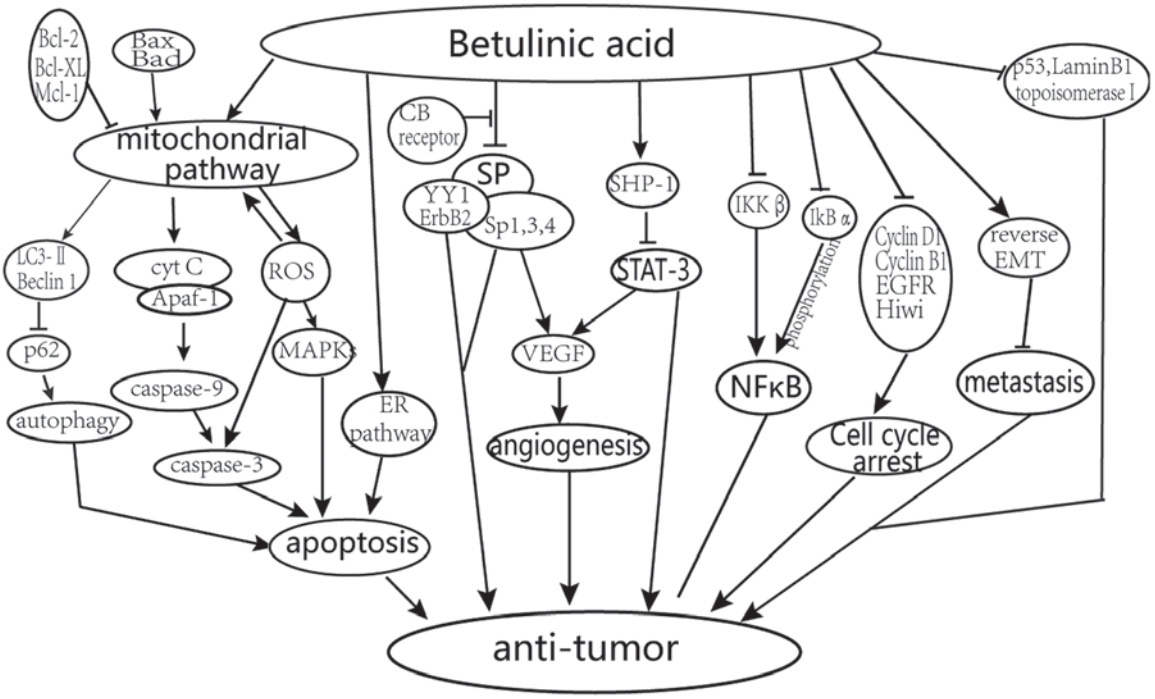

Figure 2. Diagram showing the antitumor pharmacological effects of betulinic acid.

duration with an IC_{50} of 3.54±0.66 μM (71). Compared with BA, B10, a semi-synthetic glycosylated derivative of BA, shows a higher cytotoxicity in glioma cell lines. B10 induces cell death by inducing autophagy and lysosomal permeabilization in glioblastoma cells. It induces autophagy and abrogates the autophagic flux on a panel of glioblastoma cell lines. The release of lysosomal enzymes contributes to B10-triggered cell death. B10 decreases the level of poly ADP ribose polymerase, the apoptotic protein, and survivin (72).

The phosphoinositide 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) pathway, integrating extra- and intracellular survival signals, stimulates cell growth and inhibits cell death (73,74). In addition, the PI3K/Akt signal can regulate the activity and stability of lysosomes (75). The PI3K/Akt/mTOR signaling pathway is inhibited in B10-treated (18 μM) U87MG cells by decreasing the phosphorylation of Akt, a downstream target of PI3K, which is an upstream target of mTOR. The activation of caspase-3, lysosomal permeabilization and cell death are decreased significantly when ATG7, ATG5 or BECN1 are downregulated by RNA interference (76).

4. Antitumor effects of BA in hematological malignancies

Currently, the therapeutical effect of BA against hematological malignancies predominantly focuses on multiple myeloma, acute leukemia and lymphoma. This review elaborates on the correlative functional mechanism of BA-treated cell lines.

Multiple myeloma. The U266 and MM.1S human multiple myeloma cell lines have been investigated in order to determine whether BA can modulate the STAT3 pathway. BA downregulates the activation of STAT3 (22) through the upregulation of SHP-1 (77). Thus, BA inhibits the activation of STAT3, Src kinase, janus kinase (JAK)1 and JAK2 (77). The ability of BA to inhibit STAT3 activation is abolished and BA-induced cell death is rescued when the SHP-1 gene is silenced. In multiple myeloma, the expression levels of STAT3-regulated gene products, including Bcl-extra large (Bcl-xL), Bcl-2, cyclin D1 and survivin, are downregulated by BA (77).

In our previous study, it was demonstrated that BA inhibits cell proliferation and autophagic flux, and induces apoptosis in a time-dose-dependent manner in KM3 multiple myeloma cells, which was bound up with the activation of caspase 3. These experimental results indicated that the proliferation of the KM3 cells was suppressed when the cells were treated with BA (5-25 μg/ml). The IC_{50} values at 12, 24 and 36 h were 22.29, 17.36 and 13.06 μg/ml, respectively. However, the cells were sensitized to BA-induced apoptosis when they were treated with Z-DEVD-FMK, a specific inhibitor of caspase 3. The accumulation of LC3-II and P62 in KM3 cells treated with dose-dependent BA increased, which indicated the suppression of autophagic flux. Furthermore, the expression of Beclin 1, an important inducer of autophagy, was downregulated in the KM3 cells treated with BA (78). Our previous study also confirmed that BA can induce the apoptosis of RPMI-8226 multiple myeloid cell lines via modulating the apoptosis-associated genes, Bcl-xL and caspase 3 (79). This efficiency showed a time- and dose-dependency. In the RPMI-8226 cell lines, BA also affected the cell cycle in the G1/S phase and arrested cells in the G0/G1 phase. The IC_{50} values of BA to RPMI-8226 cells at 24 and 48 h were 10.156±0.659 and 5.434±0.212 μg/ml, respectively (79).

Acute leukemia. Ehrhardt *et al* (80) found that BA induced marked apoptosis in 65% of primary pediatric acute leukemia cells and in all leukemia cell lines assessed through the mechanism of induction of Cy *c* and second mitochondria-derived activator of caspases. In all cell lines assessed, including the

SKW6, HUT 78 and CEM T-cell leukemia cell lines, the BJAB, NALM6 and BOE B-cell lines and the HL-60 myeloid cell line, the cells showed sensitivity towards BA-induced apoptosis at a concentration of 10 μg/ml (80). Kumar *et al* (81) found that the methanolic extract of *Dillenia indica* L. fruits showed significant antileukemic activity in U937, HL60 and K562 human leukemic cell lines. BA can induce the apoptosis of these leukemic cell lines with IC_{50} values of 13.73±0.89, 12.84±1.23 and 15.27±1.16 μg/ml, respectively (81). Our previous study also showed that BA inhibited the proliferation of K562 cells through the induction of cell cycle arrest, and upregulation of the protein expression levels of Bcl-2-associated X protein and caspase 3. BA was cytotoxic towards K562 cells with an IC_{50} of 21.26 μg/ml at 24 h (82). It was also found that BA is important in T lymphocytic leukemia. BA is able to inhibit the proliferation of Jurkat cells by regulating the cell cycle, with arrest of cells at the G0/G1 phase and the induction of apoptosis. The antitumor effects of BA were associated with the downregulated expression levels of cyclin D3 and Bcl-xL. The proliferation of Jurkat cells was decreased in the BA-treated group with an IC_{50} value of 70 μmol/l at 24 h (83).

Lymphoma. Our previous study in the Raji Burkitt lymphoma cell line showed that BA can induce cell cycle arrest and apoptosis via suppressing the expression of the D-type cyclin, cyclin D3. The IC_{50} values of BA at 24, 48 and 72 h were 39.44±0.65, 26.26±2.39 and 15.35±1.83 μg/ml, respectively. BA primarily caused the arrest of Raji lymphoma cell lines in the G0/G1 phase at 24 h (84).

5. Outlook

In conclusion, BA is a promising antitumor reagent. It mediates selective cell death without cytotoxicity towards normal cells and tissues. The antitumor activities described above indicate BA as a veritable candidate for clinical cancer treatment. In addition, previous studies have demonstrated that BA is involved in the treatment of solid tumors, however, there are few reports on BA-treated hematological malignancies, the elucidation of which may be of potential value in such a novel field of research, and indicates a direction for future investigations.

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