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Review

Betulinic acid: A natural product with anticancer activity

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Betulinic acid (BA) is a naturally occurring pentacyclic triterpene that exhibits a variety of biological activities including potent antitumor properties. This anticancer activity has been linked to its ability to directly trigger mitochondrial membrane permeabilization, a central event in the apoptotic process that seals the cell's fate. In contrast to the potent cytotoxicity of BA against a variety of cancer types, nonmalignant cells and normal tissue remained relatively resistant to BA, indicating a therapeutic window. Since agents that exert a direct action on mitochondria may trigger cell death under circumstances in which standard chemotherapeutics fail, there is increasing interest to develop such compounds as experimental cancer therapeutics. Thus, mitochondrion-targeted agents such as BA hold great promise as a novel approach to bypass certain forms of drug resistance in human cancers.

Keywords: Apoptosis / Betulinic acid / Cancer / Mitochondria

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1 Introduction

The multistep process of tumorigenesis is characteristically linked to apoptosis resistance. For example, cancer cells have the tendency to disable the mitochondrial (intrinsic) pathway of apoptosis by blocking signals that trigger mitochondrial perturbations. Mitochondria play a key role in determining the point-of-no-return during the process of apoptosis apart from their vital function for cellular bioenergetics. Therefore, targeting the mitochondria is a promising concept to break the resistance of human cancers to current treatment regimens. Most conventional chemotherapeutic agents induce mitochondrial membrane permeabilization in an indirect manner by activating endogenous signal transduction molecules involved in the control of mitochondrial integrity. In addition, a variety of experimental compounds with antitumor activity have been described to directly affect mitochondrial functions. Among them is betulinic acid (BA; Fig. 1), a pentacyclic triterpenoid of plant origin that exhibits potent antitumor activities. In cell-free systems as well as in intact tumor cells BA has been shown to cause mitochondrial membrane permeabilization in a direct fashion independently of p53 or CD95/CD95 ligand interaction. On theoretical grounds, compounds that directly act on mitochondrial membranes may trigger cell death under circumstances in which conventional chemotherapeutic agents fail to do so, *e.g.*, because upstream regulators of mitochondrial permeabilization are defective. Thus, mitochondrion-targeted agents such as BA may constitute a novel approach to overcome some forms of drug resistance of human cancers. This review will focus on the anticancer properties of BA with specific respect to its mitochondriotropic action.

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Abbreviations: AIF, apoptosis inducing factor; **BA,** betulinic acid; **IAPs,** inhibitor of apoptosis proteins; **ROS,** reactive oxygen species; **Smac,** second mitochondria-derived activator of caspase; **TRAIL,** tumor necrosis factor-related apoptosis-inducing ligand

2 BA: A phytochemical with antitumor activity

Natural products are the organic molecules that are produced by living tissues from higher plants, fungi, microbes, marine organisms, and animals. They exhibit a wide range of chemical diversity and biological properties. Natural resources have been used for combating human diseases for thousands of years. Over the last decade interest in natural products and their mechanisms of action has been reviving. Naturally

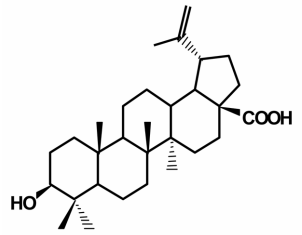

Figure 1. Structure of betulinic acid.

occurring substances play an increasing role in drug discovery and development. In fact, the majority of anticancer and anti-infectious agents are of natural origin [1].

BA is a naturally occurring pentacyclic triterpene that exhibits a variety of biological activities including antitumor properties [2]. BA (3β, hydroxy-lup-20(29)-en-28-oic acid) is widely distributed in the plant kingdom throughout the world [2]. For example, considerable amounts of BA are available in the outer bark of a variety of tree species, *e.g.*, white-barked birch trees. The reduced congener of BA, betulin (3β-lup-20(29)-en-3,28-diol), was one of the first natural products identified and isolated from plants in 1788. It is interesting to note that white birch bark, *Betula alba* which contains BA, has been used by Native Americans as a folk remedy.

3 Apoptosis: The cell's intrinsic suicide program

Apoptosis or programmed cell death is an intrinsic cellular program that occurs in various physiological and pathological situations and that is highly conserved among different species [3]. Since most anticancer therapies currently used in the clinic, for example chemotherapy, primarily kill cancer cells by triggering apoptosis, defects in apoptosis programs may result in resistance to cytotoxic therapies [4, 5].

There are two major signaling pathways that mediate apoptotic cell death: the extrinsic or receptor pathway and the intrinsic or mitochondrial pathway [5]. In most cases, triggering of apoptosis pathways eventually results in activation of caspases that exert an important function as central cell death executioners [5, 6] (Fig. 2). Caspases are synthesized as inactive proenzymes and become activated upon cleavage [6]. Once activated they cleave a large variety of substrates, both in the cytoplasm and in the nucleus, thereby causing many of the characteristic features of apoptotic cells [6]. In the extrinsic (receptor) pathway, ligation of death receptors (DRs) of the tumor necrosis factor (TNF) receptor superfamily such as CD95 (APO-1/Fas) or tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) receptors by their respective ligands or agonistic antibodies leads to activation of the initiator caspase-8 which in turn can directly cleave downstream effector caspases such as

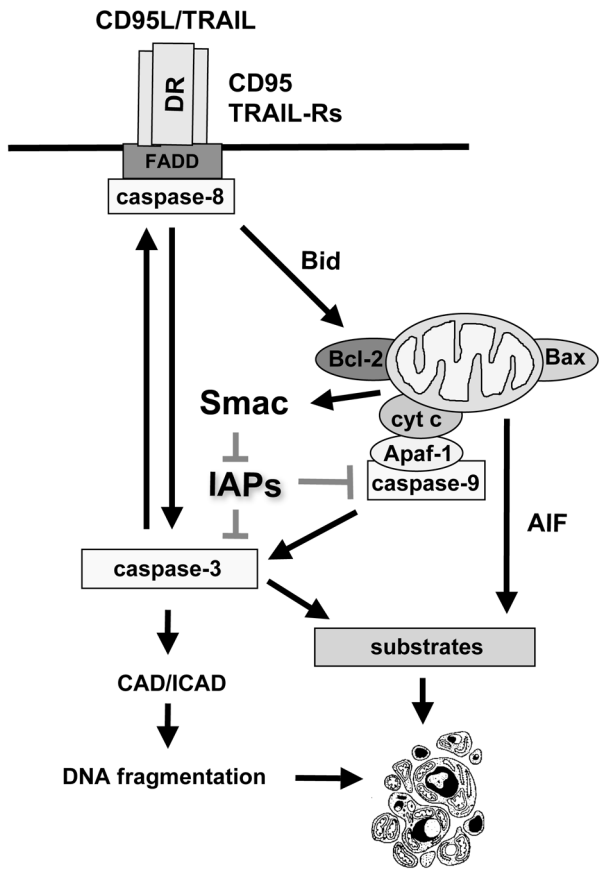

Figure 2. Apoptosis pathways. Apoptosis pathways can be initiated by ligation of death receptors (DR) such as CD95 or TRAIL receptors (TRAIL-Rs) by their respective ligands, *e.g.*, CD95 ligand (CD95L) or TRAIL, followed by receptor trimerization, recruitment of adaptor molecules (FADD) and activation of caspase-8 (receptor pathway). The mitochondrial pathway is initiated by the release of apoptogenic factors such as cytochrome *c*, Smac, or AIF from mitochondria in the cytosol. Apoptosis can be inhibited by Bcl-2 or by IAPs. Smac promotes apoptosis by neutralizing IAP-mediated inhibition of caspase-3 and -9. See text for more details.

caspase-3 [7]. Alternatively, caspase-8 may promote caspase-3 activation by cleaving Bid, which links the extrinsic to the intrinsic pathway by translocating to mitochondria to promote cytochrome *c* release. In the intrinsic or mitochondrial pathway, apoptogenic factors such as cytochrome *c*, apoptosis inducing factor (AIF), second mitochondria-derived activator of caspase (Smac)/direct inhibitor of apoptosis protein (IAP) Binding protein with Low PI (DIABLO) or Omi/high temperature requirement protein A (HtrA2) are released upon initiation of mitochondrial apoptosis from the mitochondrial intermembrane space into the cytosol [8]. Once in the cytosol, cytochrome *c* promotes caspase-3 activation through formation of the cytochrome *c*/apoptotic protease activating factor-1 (Apaf-1)/caspase-9-containing apoptosome complex [8]. Smac enhances

apoptosis by releasing effector caspases from inhibition imposed by IAPs.

4 Anticancer activity of BA

4.1 Anticancer activity of BA in vitro

The antitumor cytotoxicity of BA has been extensively studied over the last years in a large variety of cancer cell lines, primary tumor samples, and xenograft mouse models. Initial reports suggested that BA exerts selective cytotoxicity against melanoma cell lines [9]. Subsequent studies, however, reported anticancer activity also against other types of human cancers including neuroectodermal tumors (neuroblastoma, glioblastoma, medulloblastoma, Ewing tumor) as well as other malignancies, *e.g.*, head and neck, colon, breast, hepatocellular, lung, prostate, renal cell, ovarian or cervix carcinoma, and leukemia [10 – 20]. BA was not only cytotoxic against established tumor cell lines, but also against primary cancer cells isolated from tumor samples of different origin, *e.g.*, neuroblastoma, glioblastoma, and leukemia [17 – 20]. Of note, BA induced marked apoptosis in 65% of primary pediatric acute leukemia samples [17]. Moreover, BA even elicited cell death in several models of primary or acquired drug resistance [17, 18]. For example, neuroblastoma cells resistant to CD95- and doxorubicin-mediated apoptosis proved to be sensitive to treatment with BA [18]. Also, BA was cytotoxic against primary pediatric acute leukemia samples that were refractory to standard chemotherapeutic agents [17]. When compared for *in vitro* efficiency with conventionally used cytotoxic drugs, BA was more potent than nine out of ten standard therapeutics in primary childhood acute leukemia samples and especially efficient in tumor relapse [17]. This suggests that there is no cross-resistance between BA and conventional chemotherapeutics. Thus, BA may overcome certain forms of drug resistance. In addition, it is interesting to note that BA has been reported to exert preferential cytotoxicity against metastatic over nonmetastatic melanoma cell lines [21]. In contrast to the potent antitumor activity of BA on cancer cells stands the apparent lack of cytotoxicity of BA on nonmalignant cells. To this end, nontransformed cells of different origin, *e.g.*, fibroblasts, melanocytes, neuronal cells and peripheral blood lymphocytes, have been reported to be much more resistant to the cytotoxic effect of BA than cancer cells [13, 19, 20, 22]. The underlying molecular mechanisms for the differential sensitivity of neoplastic *versus* non-neoplastic cells to BA has not exactly been delineated and remains to be explored in future studies.

4.2 Anticancer activity of BA in combination protocols

Besides its anticancer properties as single agent, BA has also been incorporated in combination protocols. Importantly, combining BA with different cytotoxic stimuli resulted in additive or synergistic tumor cell killing. For example, BA was reported to act in concert with ionizing radiation in melanoma cell lines [22]. Moreover, BA was found to cooperate with various chemotherapeutic drugs, including doxorubicin, etoposide, cisplatin, taxol, and actinomycin D, to induce apoptosis and to inhibit clonogenic survival of tumor cells [23]. Also, BA showed a synergistic cytotoxic effect on melanoma cells by combinational use of vincristin [24]. Combined treatment with BA and anticancer drugs acted in concert to induce loss of mitochondrial membrane potential and the release of cytochrome *c* and Smac from mitochondria, resulting in activation of caspases and apoptosis [23]. Overexpression of Bcl-2, which blocked mitochondrial perturbations, also inhibited the cooperative effect of BA and anticancer drugs, indicating that cooperative interaction involved the mitochondrial pathway [23]. BA and anticancer drugs acted in concert to induce apoptosis even in p53 mutant cells and also in primary tumor cells, but not in human fibroblasts indicating some tumor specificity [23]. Furthermore, BA cooperated with TRAIL to induce apoptosis in tumor cells [25]. Through functional complementation, simultaneous stimulation of the mitochondrial pathway by BA and the DR pathway by TRAIL resulted in complete activation of effector caspases, apoptosis, and inhibition of clonogenic survival [25]. BA and TRAIL cooperated to trigger loss of mitochondrial membrane potential and release of cytochrome *c* and Smac from mitochondria [25]. Also, combination treatment with BA and TRAIL resulted in increased cleavage of caspase-8 and Bid indicating that activation of effector caspases may feed back in a positive amplification loop [25]. Importantly, the combination treatment with BA and TRAIL synergized to induce apoptosis in different tumor cell lines and also in primary tumor cells, but not in normal human fibroblasts indicating some tumor specificity.

These reports suggest that using BetA as sensitizer in chemotherapy-, radiotherapy-, or TRAIL-based combination regimens may be a novel strategy to enhance the efficacy of anticancer therapy.

4.3 Anticancer activity of BA in vivo

In addition to its potent antitumor activity in a variety of cancer cell lines, BA also suppressed tumor growth *in vivo* in a number of animal studies. Intraperitoneal injection of BA significantly reduced tumor burden of established melanoma xenograft in nude mice [9]. In a xenograft mouse model of ovarian cancer administration of BA significantly increased the survival time [13]. Besides *in vivo* activity as single agent, BA also acted in concert with chemotherapeutic agents such as vincristin. In an *in vivo* model of metastatic melanoma, the addition of BA to vincristine augmented suppression of the experimental lung metastasis of

melanoma cells in mice compared to animals treated with vincristin alone. These results suggest that BA is an effective supplement for enhancing the chemotherapeutic effect of vincristin on malignant metastatic melanoma. Of note, no systemic toxicities or weights loss were observed in BA-treated mice even at high systemic doses of BA [9, 13]. Pharmacokinetic studies in mice bearing melanoma xenografts demonstrated that BA was well absorbed and distributed with highest concentrations found within the tumor [26, 27]. Currently, BA is evaluated in a phase I/II clinical trial in the topical treatment of dysplastic nevi with the potential to transform into melanoma.

5 Mechanisms of action of BA

Numerous studies over the last years aimed at elucidating the molecular mechanisms of BA-mediated apoptosis. One characteristic feature of BAs cytotoxicity is its ability to selectively trigger the mitochondrial pathway of apoptosis in cancer cells.

5.1 Activation of the mitochondrial pathway by BA

Most chemotherapeutic agents and radiotherapy induce cancer cell death by triggering the mitochondrial pathway as a consequence of DNA damage or cellular stress response [28]. A pivotal initial event in the intrinsic pathway is mitochondrial membrane permeabilization [28]. During this process, both the mitochondrial outer and inner membranes are permeabilized leading to the release of soluble, potentially toxic proteins, which are normally secured in the mitochondrial interspace such as cytochrome *c*, Smac, or AIF [28]. A long list of protein factors and second messengers have been identified that can positively or negatively modulate permeabilization of the outer mitochondrial membrane [29]. Thus, factors that can directly induce mitochondrial outer membrane permeabilization (MOMP) can act as effective cytotoxic agents.

5.2 Induction of MOMP by BA

BA has been reported to induce apoptosis *via* direct mitochondrial perturbations. All of these effects have been observed in both intact cells and in cell-free systems. When added to isolated mitochondria, BA directly induces loss of mitochondrial membrane potential in a way that is not affected by the caspase inhibitor *N*-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (zVAD.fmk) and yet is inhibited by bongkrekic acid, an inhibitor of the permeability transition pore complex. In a cell-free system comprising mitochondria, cytosols, and purified nuclei, mitochondria undergoing BA-induced permeability transition mediate cytosolic caspase activation and nuclear fragmentation *via* the release of soluble factors, such as cytochrome *c* or AIF [30]. Antiapoptotic Bcl-2 family proteins such as Bcl-2 and Bcl-X_L inhibit all mitochondrial and cellular manifestations of apoptosis induced by BA, as does bongkrekic acid.

Perturbance of mitochondrial function constitutes a central coordinating event in BA-induced cell death resulting in activation of the caspase cascade and apoptosis. Mitochondria from cells, which were treated with BA, induced cleavage of both caspases-8 and -3 in cytosolic extracts. Cytochrome *c*, released from mitochondria undergoing BA-mediated permeability transition, activated caspase-3 but not caspase-8 in a cell-free system. Cleavage of caspases-3 and -8 was preceded by disturbance of mitochondrial membrane potential and by generation of reactive oxygen species (ROS). In addition, activation of caspases was restricted to cells that already had lost their mitochondrial membrane potential further suggesting that mitochondrial alterations were involved in BA-induced activation of caspases. Overexpression of Bcl-2 and Bcl- X_L conferred resistance to BA at the level of mitochondrial dysfunction, protease activation, and nuclear fragmentation indicating that these events occurred downstream of the Bcl-2- or Bcl-X_L-controlled checkpoint of apoptosis. These findings suggest that caspase-8 is activated downstream of mitochondria during BA-induced apoptosis. Activation of the caspase cascade was required for BA-triggered apoptosis, as the broad-spectrum peptide inhibitor zVAD.fmk, which blocked cleavage of caspases and poly(ADP-ribose)polymerase (PARP), also completely abrogated BA-triggered apoptosis. Interestingly, neuroblastoma cells resistant to doxorubicin-mediated apoptosis were still responsive to treatment with BA. This suggests that BA may overcome some forms of drug resistance.

There is evidence that production of ROS initiated by BA is involved in mitochondrial membrane permeabilization and cell death induction. To this end, ROS generation was detected in cancer cell lines of different origin upon treatment with BA [12, 18, 31]. Incubation with antioxidants prior to adminstration of BA rescued cells from undergoing apoptosis. ROS generation was linked to activation of proapoptotic p38 and SAP/JNK kinases with no change in the phosphorylation of ERK indicating that ROS act upstream of the MAPKs in the signaling pathway of BA [31].

5.3 Regulation of BA-induced apoptosis by Bcl-2 family proteins

Among the many signal transduction proteins that can act on mitochondria to regulate mitochondrial membrane permabilization are proteins of the Bcl-2 family. Bcl-2 family proteins comprise both antiapoptotic members, *e.g.*, Bcl-2, Bcl-X_L, Mcl-1, as well as proapoptotic molecules such as Bax, Bak, Bad, and BH3 domain only molecules that link the DR pathway to the mitochondrial pathway [32]. There are currently two models how BH3-only proteins activate Bax and Bak. According to the direct activation model [33],

putative activators such as Bim and cleaved Bid (tBid) bind directly to Bax and Bak to trigger their activation, while BH3-only proteins that act as sensitizers, *e.g.*, Bad, bind to the prosurvival Bcl-2 proteins. By comparison, the indirect activation model holds that BH3-only proteins activate Bax and Bak by engaging the multiple antiapoptotic Bcl-2 proteins that inhibit Bax and Bak [34, 35]. Bak has recently been reported to trigger apoptosis only if both Bcl-X_L and Mcl-1 are inactivated [36]. Imbalances in the ratio of antiapoptotic *versus* proapoptotic Bcl-2 proteins may tip the balance in favor of tumor cell survival instead of cell death and have been shown to drastically alter apoptosis in response to several stimuli in a number of experimental systems [32].

BA has been reported to modulate expression levels of multiple Bcl-2 family proteins. For example, treatment with BA resulted in up-regulation of the proapoptotic Bcl-2 family proteins Bax in neuroblastoma, glioblastoma and melanoma cells, whereas Bcl-X_S was found at elevated levels in BA-treated neuroblastoma cells [12, 18, 22]. Expression levels of proapoptotic proteins Bak and Bad were not altered in response to BA in melanoma cells [22, 37]. While expression levels of antiapoptotic Bcl-2 remained unchanged upon incubation with BA in neuroblastoma and squamous cell carcinoma cells, an increase in Bcl-2 protein levels was reported in glioblastoma cells [11, 12, 18]. Also, BA triggered up-regulation of another antiapoptotic Bcl-2 family proteins, namely Mcl-1, in melanoma cells, whereas no changes in Mcl-1 levels were detected in squamous cell carcinoma cells [11, 22, 37]. As fas as Bcl-X_L is concerned, no alterations in expression levels were reported upon exposure to BA in neuroblastoma, glioblastoma, or melanoma cells [12, 18, 37]. These reports indicate that BA regulates Bcl-2 family protein in a complex and probably cell type-dependant manner.

5.4 p53- and CD95-independent induction of apoptosis by BA

Another interesting mechanistic aspect is that BA-induced apoptosis does not involve accumulation of wild-type p53 protein [12, 18, 22, 38 – 40]. In addition, BA triggered apoptosis in p53 mutant cell lines at doses similar to those used in p53 wild-type cell lines and was also active in p53 deficient melanoma cells [13, 22]. These findings further support the notion that p53 is not required for BA-mediated apoptosis. However, an increase in p53 expression was reported in some melanoma cell lines suggesting that the effect of BA on p53 activation is regulated in a cell line-dependent manner [21]. Moreover, BA was reported to trigger apoptosis independent of CD95-ligand/receptor interaction and thus, independent of the extrinsic pathway of apoptosis [12, 17, 18]. Accordingly, neuroblastoma cells resistant to CD95-mediated apoptosis proved to be still sensitive to treatment with BA suggesting that BA may bypass some forms of resistance.

5.5 BA as new modulator of nuclear factor-κB (NF-κB)

Since BA can trigger production of ROS, which in turn can activate NF-κB, a key regulator of stress-induced transcriptional activation, it was also investigated whether BA may modulate NF-κB activity. These studies revealed that BA is a potent activator of NF-κB in a variety of tumor cell lines. NF-κB DNA-binding complexes induced by BA consisted of p50 and p65 subunits. Nuclear translocation of p65 was also confirmed by immunofluorescence microscopy. BA-induced NF-κB activation involved increased IKK activity and phosphorylation of IκBα at serine 32/36 followed by degradation of IκBα. Reporter assays revealed that NF-κB activated by BA is transcriptionally active. Interestingly, inhibition of BA-induced NF-κB activation by different chemical inhibitors (proteasome inhibitor, antioxidant, IKK inhibitor) attenuated BA-induced apoptosis. Importantly, specific NF-κB inhibition by transient or stable expression of IκBα super-repressor inhibited BA-induced apoptosis in some neuroblastoma cells, while transient expression of IκBα super-repressor had no influence on BA-induced apoptosis in other cell lines. These findings indicate that activation of NF-κB by BA promotes BA-induced apoptosis in a cell type-specific fashion. Thus, NF-κB inhibitors in combination with BA may have no therapeutic benefit or could even be contraproductive in certain tumors, an important aspect to consider in the design of BA-based combination protocols. By comparison, BA was also reported to inhibit activation of NF-κB and NF-κB-regulated gene expression induced by carcinogens and inflammatory stimuli, which may provide a molecular basis for the ability of BA to suppress inflammation and modulate the immune response [41]. Together, these findings suggest that the role of NF-κB in the regulation of BA-mediated apoptosis is context dependant.

6 Other signaling pathways modulated by BA

BA has also been reported to have an effect on additional signaling pathways besides those discussed in more detail in the preceding sections. For example, BA has been shown to inhibit aminopeptidase N, an enzyme involved in the regulation of angiogenesis and overexpressed in several cancers [42 – 44]. Moreover, it was demonstrated that BA blocks the catalytic activity of topoisomerase I by abrogating the interaction of the enzyme and the DNA substrate [45].

The effect of BA on cell cycle regulation appears to be context dependant, as no consistent changes in cell cycle progression and cell cycle regulatory proteins were reported. While BA was found to reduce expression of p21 protein in melanoma cells, an increase of p21 protein was observed upon treatment with BA in glioblastoma cells [12, 21]. Alterations in cell cycle progression in response to BA

were also highly dependant on individual cell lines [21]. Clearly, additional studies are required to explore the relative contribution of BA-mediated cell cycle changes to the antitumor activity of BA.

7 Other biological activities of BA

As many natural products BA exhibits a plethora of biological activities besides its anticancer properties. Probably the most important biological effect of BA apart from its cytotoxicity against cancer cells is its anti-HIV-1 activity [46 – 49]. While its mechanism of action has not been fully determined, it has been shown that some BA analogs disrupt viral fusion to the cell in a postbinding step through interaction with the viral glycoprotein gp41, whereas others disrupt assembly and budding of the HIV-1 virus, depending on the specific side-chain modifications [46, 47, 49]. Some of the highly active derivatives exhibited even greater potencies and better therapeutic indices than some current clinical anti-HIV agents [49].

Moreover, BA was shown to exhibit antiparasitic, *e.g.*, against malaria, and anti-inflammatory activities [50].

8 Conclusions

The natural compound BA is a promising novel cancer therapeutic that triggers apoptosis in cancer cells by directly engaging the mitochondrial pathway. Although BA has been demonstrated in cell-free systems to exert a direct action on mitochondria resulting in the release of apoptogenic proteins, the precise molecular target(s) of BAs antitumor activity have yet to be identified. The use of BA as anticancer agent may be especially promising in combination regimens, for example together with radiotherapy, conventional chemotherapeutics or the DR ligand TRAIL. Also, BA could be exploited in malignancies that are refractory to standard care, because of its ability to bypass some forms of drug resistance. A series of derivatives of BA have been developed that may prove to have even enhanced anticancer activity compared to BA. Because of its relative selective cytotoxicity against neoplastic cells while sparing nonmalignant cells BA holds great promise as experimental anticancer agents for the treatment of human cancers that warrants exploitation in the clinic.

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9 References

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