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Targeting mitochondrial apoptosis by betulinic acid in human cancers

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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, non-neoplastic cells as well as normal tissue remain relatively resistant to BA, thus pointing to a therapeutic window. Because agents that exert a direct action on mitochondria may bypass resistance to conventional chemotherapeutics, 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 overcome certain forms of drug resistance in human cancers.

Introduction

Natural products have been used to combat human diseases for thousands of years [1] and play an increasing role in drug discovery and development. In fact, the majority of anticancer and anti-infectious agents are of natural origin. The antitumor activity of natural products has been explained, at least in part, by their ability to trigger cell death pathways, including apoptosis in cancer cells. Apoptosis or programmed cell death is the cell's intrinsic death program that plays a pivotal role in maintaining tissue homeostasis and that is highly conserved among different animal species [2]. Because apoptosis is involved in the regulation of many physiological processes, defective apoptosis signaling may contribute to a variety of different pathological conditions. Thus, increased apoptosis is involved in degenerative processes affecting neurons, muscle or lymphoid tissues. Conversely, disabled apoptosis is one of the hallmarks of human cancer cells [3]. Thus, cancer cells have a marked tendency to disable the mitochondrial (intrinsic) pathway of apoptosis. Besides their vital function for cellular bioenergetics, mitochondria play a key role in the regulation of the point-of-no-return during apoptosis. Betulinic acid (BA) is a natural product that exhibits potent anti-tumor activities and that triggers the mitochondrial path to apoptosis [4]. Here we review the current literature on the mitochondrion-targeted actions of BA and discuss the perspective to take advantage of BA to overcome some forms of anticancer drug resistance [4].

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BA, a phytochemical with antitumor activity

BA (3β, hydroxy-lup-20(29)-en-28-oic acid) is a pentacyclic triterpenoid of the lupane class that belongs to the group of terpenes [5,6]. BA is contained in various plants throughout the plant kingdom and, hence, throughout the world (Fig. 1a) [7]. For example, considerable amounts of BA are available in the outer bark of several tree species, including white-barked birch trees. It is interesting to note that betulin (3β-lup-20(29)-en-3,28-diol), the reduced congener of BA, was one of the first natural products that was isolated from plants more than two centuries ago [8]. Further, Native Americans used the bark of white birch trees (*Betula alba*), which contains BA among other active compounds, as a folk remedy [8]. It was not until 1995, however, that BA acquired more attention, when it was identified as a specific inducer of apoptosis in melanoma cells [9]. BA exerts several biological activities, among which its antitumor properties stand out as probably the most important and best studied ones.

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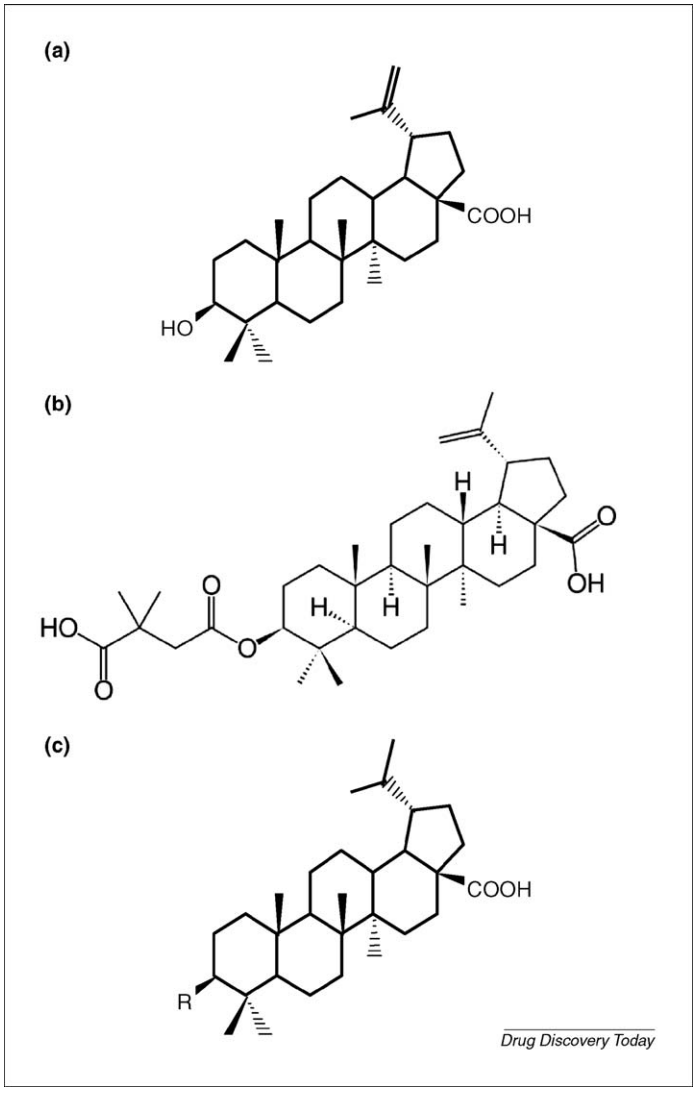

FIGURE 1

Structure of betulinic acid and derivatives. **(a)** Structure of betulinic acid. **(b)** Structure of betulinic acid derivative bevirimat [54,55]. **(c)** Structure of betulinic acid derivatives modified at C-3 position. Modifications at C-3 position are [58]: R = $NOCH_2C_6H_4NO_2$(4) (4-nitrobenzyl-oximino), $OCOC_6H_3F_2$(2,4) (2-4-difluoro-benzoyloxy), $NCHC_6H_3F_2$(2,4) (2-4-difluoro-benzylidene-amino), $NNHCOC_6H_5$ (benzoyl-hydrazono), $NNHC_6H_4F$(4) (4-fluorophenyl-hydrazono).

The mitochondrial pathway of apoptosis

Apoptosis is an intrinsic cell death program that is operative in every cell and regulated by defined signaling pathways [10]. Irrespective of the morphological features of end-stage cell death (that may be apoptotic, necrotic, autophagic or mitotic), the permeabilization of mitochondrial membranes is frequently the decisive event that delimits the frontier between survival and death. The intrinsic (mitochondrial) pathway of apoptosis is triggered upon treatment with chemotherapeutic agents or upon radiotherapy as a result of DNA damage or cellular stress responses [11] and has been specifically linked to mitochondrial outer membrane permeabilization (MOMP). In this context, mitochondrial membranes constitute the battleground on which opposing signals combat to seal the cell's fate. Local players that determine the propensity to MOMP include the pro- and antiapoptotic members of the vast Bcl-2 family, proteins from the mitochondrial permeability transition pore complex (PTPC), as well as a plethora of interacting partners including mitochondrial lipids. Intermediate metabolites, redox processes, sphingolipids, ion gradients, transcription factors, as well as kinases and phosphatases link lethal and vital signals emanating from distinct subcellular compartments to mitochondria. Thus, mitochondria integrate a variety of lethal signals. Once MOMP has been triggered, it entails the release of catabolic hydrolases and activators of such enzymes (including those of caspases) from mitochondria. These catabolic enzymes, as well as the cessation of the bioenergetic and redox functions of mitochondria, finally cause cell death, meaning that mitochondria coordinate the late stage of cellular demise. Among the soluble intermembrane proteins that are released upon MOMP, several (such as cytochrome *c* and Smac/DIABLO) contribute to the activation of caspases, a class of proteases that is overactivated in apoptosis, while other intermembrane proteins (such as AIF and endonuclease G) can translocate to the nucleus and participate in caspase-independent chromatinolysis [11]. Pharmacological induction of MOMP in tumor cells constitutes the ultimate goal of anticancer chemotherapy. This goal is often achieved indirectly, for example by the induction of DNA damage responses that activate the mitochondrial apoptotic pathway. The action of agents that provoke MOMP in a direct fashion may, however, circumvent some of the problems that typically affect tumor cells, in which upstream signals of the apoptotic signaling cascade such as DNA damage responses are frequently perturbed.

Mechanisms of action of BA

Numerous studies over the past few years have been aimed at elucidating the molecular mechanisms of BA-mediated antitumor activity. One characteristic feature of BAs cytotoxicity is its ability to trigger the mitochondrial pathway of apoptosis in cancer cells (Fig. 2).

Induction of mitochondrial outer membrane permeabilization by BA

BA has been reported to induce apoptosis via a direct effect on mitochondria. When added to isolated mitochondria in cell-free systems, BA triggered loss of mitochondrial membrane potential in a manner that was not affected by the caspase inhibitor Z-VAD-fmk, yet was inhibited by bongkrekic acid, an inhibitor of the mitochondrial permeability transition pore complex (PTPC) [12]. Also, in intact cells, BA was shown to trigger cytochrome *c* in a permeability transition pore-dependent (and caspase-independent) manner, meaning that bongkrekic acid (but not Z-VAD-fmk) inhibited cytochrome *c* release [13]. In a cell-free system comprising mitochondria, cytosol and purified nuclei, mitochondria undergoing BA-induced permeability transition mediated cytosolic caspase activation and nuclear fragmentation via the release of soluble factors, such as cytochrome *c* or AIF [12]. Anti-apoptotic Bcl-2 family proteins, such as Bcl-2 and Bcl-X_L, inhibited all mitochondrial and cellular manifestations of apoptosis induced by BA, as did bongkrekic acid [12], indicating that mitochondrial permeability transition was required for these events. From these data, we conclude that the perturbance of mitochondrial function constitutes a central coordinating event in BA-induced caspase activation and apoptotic DNA fragmentation. Mitochondria from

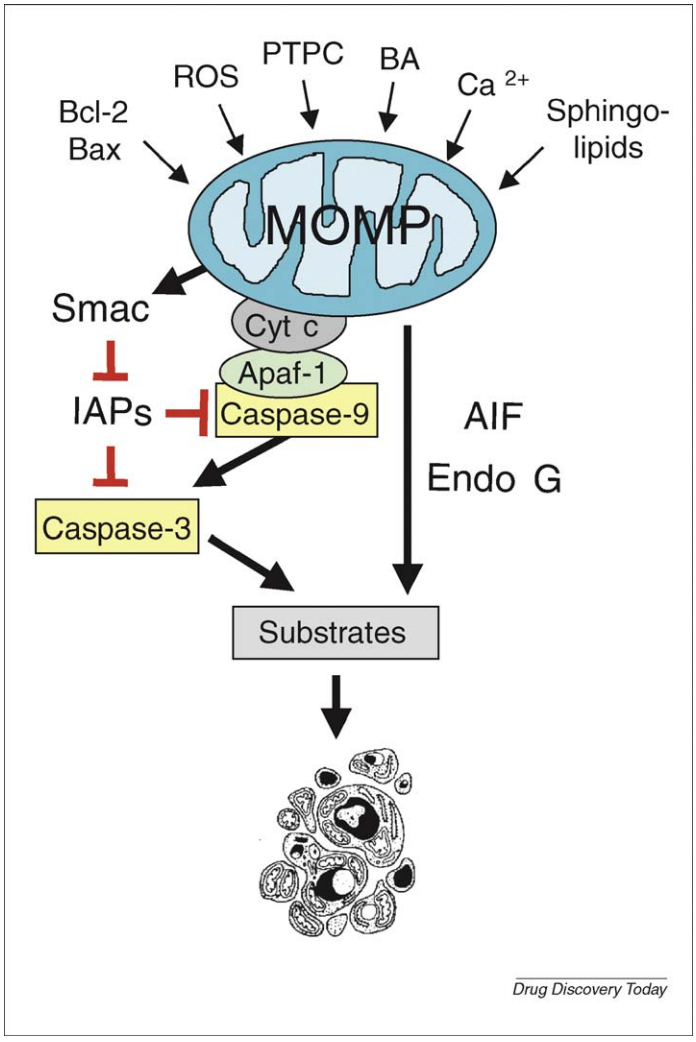

FIGURE 2

Mitochondrial pathway of apoptosis. The intrinsic (mitochondrial) pathway of apoptosis is linked to mitochondrial outer membrane permeabilization (MOMP), which is regulated by various factors including pro- and antiapoptotic Bcl-2 proteins, reactive oxygen species (ROS), proteins from the mitochondrial permeability transition pore complex (PTPC), ions, sphingolipids and BA. MOMP in turn results in the release of soluble intermembrane proteins from mitochondria into the cytosol such cytochrome *c*, Smac/DIABLO, AIF and endonuclease G. Cytochrome *c* and Smac/DIABLO promote activation of caspases, whereas AIF and endonuclease G contribute to caspase-independent chromatolysis. See text for more details.

intact cells treated with BA, induced cleavage of both caspase-8 and caspase-3 when they were purified and added to cytosolic extracts [12]. Moreover, cytochrome *c*, released from mitochondria undergoing BA-mediated permeability transition, activated caspase-3, but not caspase-8, in a cell-free system. Cleavage of caspase-3 and -8 was preceded by the disturbance of mitochondrial membrane potential and by the generation of reactive oxygen species (ROS) in intact cells treated with BA [12]. In addition, the 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-3 is activated downstream of mitochondria during BA-induced apoptosis. Activation of the caspase cascade was required for BA-triggered apoptosis, as broad-spectrum peptide inhibitors of caspases completely abrogated BA-triggered chromatin condensation. Interestingly, neuroblastoma cells resistant to doxorubicin-mediated apoptosis were still responsive to the treatment with BA [14]. This suggests that BA may overcome some forms of drug resistance.

Generation of ROS upon treatment with BA has been reported to be involved in initiating mitochondrial membrane permeabilization [15]. ROS generation was detected in several cancer cell lines of disparate origin that were treated with BA [15–17]. Incubation with antioxidants before the administration of BA suppressed apoptosis, suggesting that ROS production was involved in mediating cell death. Antioxidants also avoided the activation of proapoptotic p38 MAPK and SAP/JNK kinases (with no change in the phosphorylation of ERK), indicating that ROS act upstream of BA-elicited stress kinases [17].

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

Proteins of the Bcl-2 family are among the many signal transduction proteins that can act on mitochondria to regulate outer membrane permeabilization [18]. Bcl-2 family proteins comprise antiapoptotic members, such as Bcl-2, Bcl-X_L and Mcl-1, as well as proapoptotic molecules such as Bax, Bak and BH3-only molecules [18]. Imbalances in the ratio of antiapoptotic versus proapoptotic Bcl-2 proteins may contribute to the survival of tumor cells in adverse conditions including hypoxia and chemotherapy [18].

BA can modulate the expression levels of different Bcl-2 family proteins. For example, BA treatment resulted in upregulation of the proapoptotic Bcl-2 family protein Bax in neuroblastoma, glioblastoma and melanoma cells, whereas Bcl-X_S (a proapoptotic splice variant of Bcl-X_L) was induced in BA-treated neuroblastoma cells [15,16,19]. The expression levels of proapoptotic proteins Bak and Bad were not altered in response to BA in melanoma cells [19,20]. 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 for glioblastoma cells [15,16,21]. Also, BA triggered upregulation of Mcl-1, another antiapoptotic Bcl-2 family protein, in melanoma cells, whereas no changes in Mcl-1 levels were detected in squamous cell carcinoma cells [19–21]. As far as Bcl-X_L is concerned, no alterations in expression levels were reported upon exposure to BA in neuroblastoma, glioblastoma or melanoma cells [15,16,20]. These findings suggest that BA regulates Bcl-2 family proteins in a context-dependent manner.

Apoptosis induced by BA was not associated with accumulation of wild-type p53 protein [15,16,19,22–24]. Also, BA similarly induced apoptosis in p53 mutant and p53 wild-type cell lines and efficiently killed p53-deficient melanoma cells [19,25]. Moreover, BA triggered apoptosis independent of CD95-ligand/receptor interaction [15,16,26]. These data invalidate initial claims that BA would induce apoptosis through p53-dependent pathways [27].

Modulation of NF-κB activity by BA

BA has also been reported to activate the transcription factor nuclear factor-κB (NF-κB), a key regulator of stress-induced transcriptional

activation, in several cancer cell lines [28]. BA-induced NF-κB activation involved increased activity of the inhibitor of NF-κB (IκBα) kinase (IKK), phosphorylation of IκBα at serine 32/36, followed by the proteasome-dependent degradation of IκBα and finally translocation of the NF-κB subunit p65 from the cytoplasm to the nucleus. Reporter assays confirmed that NF-κB activated by BA was transcriptionally active. Interestingly, the inhibition of BA-induced NF-κB activation by a range of pharmacological agents (such as antioxidants, as well as inhibitors of IKK or the proteasome) also impaired BA-induced apoptosis. Importantly, specific NF-κB inhibition by transient or stable expression of the so-called IκBα super-repressor (IKSR), a nonphosphorylable mutant of IκBα, inhibited BA-induced apoptosis in some neuroblastoma cells, while transient expression of IKRS had no influence on BA-induced apoptosis in other cell lines [28]. These findings indicate that the activation of NF-κB by BA promotes BA-induced apoptosis in a cell type-specific manner. By comparison, BA was found to interfere with NF-κB activation and NF-κB-regulated gene expression triggered by carcinogens and inflammatory stimuli [29,30]. These findings may provide a molecular basis for the ability of BA to suppress inflammation and modulate the immune response. Together, these findings point to a context-dependent function of NF-κB in the regulation of BA-mediated apoptosis. It is interesting to note that CDDO-Me, a related triterpenoid compound, was also reported to inhibit NF-κB signaling [31,32].

Additional anticancer effects of BA

BA has been reported to exert antiangiogenic effects by inhibiting growth factor-induced *in vitro* angiogenesis in endothelial cells [33]. Interestingly, the antiangiogenic activity of BA was linked to its mitochondrial damaging effects [33]. Accordingly, the inhibition of mitochondrial permeability transition by pharmacological inhibitors attenuated the antiangiogenic activity of BA on endothelial cells [33]. Further, the activation of selective proteasome-dependent degradation of the transcription factors specificity protein 1 (Sp1), Sp3 and Sp4, which regulate vascular endothelial growth (VEGF) expression, has been suggested to explain the antiangiogenic properties of BA [34]. In addition, BA-induced inhibition of aminopeptidase N, an enzyme that is involved in the regulation of angiogenesis and overexpressed in several cancers, has been claimed as the mechanistic basis of antiangiogenic effects of BA [33,35,36]. Compared to BA, 20,29-dihydro-BA derivatives were found to possess better antiangiogenic properties as BA [37].

Beyond its antiangiogenic activity, BA has been suggested to exert direct effects on topoisomerases and cell cycle advancement. BA can inhibit the catalytic activity of topoisomerase I [38,39]. Via interaction with cellular topoisomerase I, BA may inhibit the interaction of topoisomerase I with oxidatively damaged DNA, thereby preventing topoisomerase I from directly participating in the apoptotic process. Furthermore, BA exerts context-dependent effects on the cell cycle. 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 [16,27]. Alterations in cell cycle progression in response to BA were also highly dependent on individual cell lines [27] and it remains elusive whether the anticancer effects of BA may be ascribed to its cell cycle effects.

Anticancer activity of BA

The antitumor cytotoxicity of BA has been extensively studied in a panel of cancer cell lines, primary tumor samples and xenograft mouse models. While initial reports suggested that BA is selectively cytotoxic against melanoma cell lines [9], anticancer activity was subsequently reported against other types of human malignancies, including neuroblastoma, glioblastoma, medulloblastoma, Ewing tumor, leukemia as well as several carcinomas, that is head and neck, colon, breast, liver, lung, prostate, renal, ovarian or cervix carcinoma [15,16,21,25,26,40–46]. While most of these results were obtained with tumor cell lines, BA was found more specifically to kill primary cancer cells isolated from tumor specimens obtained from neuroblastoma, glioblastoma and leukemia [15,26,44,45]. By comparison, normal cells of different origin have been reported to be much more resistant to BA than cancer cells pointing to some tumor selectivity [19,25,44,45].

BA was cytotoxic in different models of drug resistance, for example primary pediatric acute leukemia samples that were refractory to standard chemotherapeutic agents [15,26]. Furthermore, there is evidence that BA exerts preferential cytotoxicity against metastatic over nonmetastatic melanoma cell lines [27]. It is important to note that BA even exhibited activity against fully chemoresistant cancer cell lines: for example, doxorubicin-refractory neuroblastoma cells and colon carcinoma cells with acquired resistance to oxaliplatin, irinotecan or 5-fluorouracil [47,48]. Interestingly, the induction of cell death via mitochondrial damage was linked to its ability to break chemoresistance [48]. This suggests that BA can be used as a sensitizer in combination regimens to enhance the efficacy of anticancer therapy or to bypass some forms of drug resistance. Indeed, BA cooperated with different cytotoxic stimuli to suppress tumor growth, including ionizing radiation [19], chemotherapeutic drugs [47–50] or the death receptor ligand TRAIL [51].

Besides its potent antitumor activity *in vitro*, BA also suppressed tumor growth in several animal models of human cancer. In a xenograft mouse model of ovarian cancer administration of BA significantly increased the survival time [25]. Also, BA suppressed tumor growth in a melanoma xenograft model [9]. BA cooperated with chemotherapeutic agents such as vincristin to reduce lung metastasis in a metastatic melanoma model [49]. Of note, no systemic toxicities or weight losses were observed in BA-treated mice even at high systemic doses of BA [9,25].

Pharmacokinetic studies in mice bearing melanoma xenografts demonstrated that BA was well absorbed and distributed with highest concentrations found within the tumor [52,53]. These mouse data are compatible with results obtained on a BA derivative in humans. Phase I/II studies of 3-*o*-(3′,3′-dimethylsuccinyl) BA (bevirimat, Fig. 1b) in patients with human immunodeficiency virus (HIV) infection demonstrated that single oral doses of bevirimat were well tolerated and that plasma concentrations ranged from 8 to 58 μg/ml [54,55]. This suggests that the plasma levels of BA derivatives achieved after oral administration in humans attain the range of concentrations that exert antitumor activity *in vitro*.

BA has also been reported to act as a chemopreventive agent. BA was shown to inhibit tumor formation in a mouse model of two-stage skin carcinogenesis [56]. Indeed, BA is currently under evaluation as a topical agent in a phase I/II clinical trial for the

treatment of dysplastic nevi with the potential to transform into melanoma.

In addition to BA, a variety of BA derivatives were developed with the aim of improving their anticancer potency and pharmacokinetic properties. For example, adding a methoxycarbonyl group markedly enhanced the apoptosis-inducing activities of BA [57]. In addition, some BA derivatives showed higher plasma and tissue levels compared to BA [57]. Further, C-3 modified BA derivatives (Fig. 1c) proved to have better *in vivo* antitumor efficacy compared to BA *in vivo* against human colon cancer and also displayed favorable pharmacokinetic properties [58]. Moreover, 17-carboxylic acid modified 23-hydroxy BA ester derivatives exhibited an enhanced cytotoxic activity on five cancer cell lines *in vitro* compared to 23-hydroxy BA and the native compound both *in vitro* and *in vivo* [59].

Other biological activities of BA

As with 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 [7,60–62]. Although 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 other BA analogs disrupt assembly and budding of the HIV-1 virus, depending on the specific side-chain modifications [7,60,62]. Some of the highly active derivatives exhibited even greater potencies and better therapeutic indices than some current clinical anti-HIV agents [62]. Moreover, BA was shown to exhibit antiparasitic, for example against malaria and anti-inflammatory activities [63].

Conclusions

The natural compound BA shows potent anticancer activity through activation of the mitochondrial pathway of apoptosis in cancer cells. BA may also be used in combination protocols to enhance its antitumor activity, for example with chemo- or radiotherapy or with the death receptor ligand TRAIL. Because of its relative selective cytotoxicity against malignant compared to normal cells, BA is a promising new experimental anticancer agent for the treatment of human cancers.

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