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Review article

Betulinic acid as apoptosis activator: Molecular mechanisms, mathematical modeling and chemical modifications

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ABSTRACT

A natural product betulinic acid (BA) has gained a huge significance in the recent years for its strong cytotoxicity. Surprisingly, in spite of being an interesting cancer protecting agent on a variety of tumor cells, the normal cells and tissues are rarely affected by BA. Betulinic acid and analogues (BAs) generally exert through the mechanisms that provokes an event of direct cell death and bypass the resistance to normal chemotherapeutics. Among several cancer protecting natural products, BA is divulged as a potentially selective anti-melanoma agent and has been entered into phase II clinical trials against skin cancer. Although the major mechanism associated with its ability to induce direct cell death is mitochondrial apoptosis, there are several other mechanisms explored recently. Importantly, mathematical modeling of apoptosis has been an important tool to explore the precise mechanism involved in mitochondrial apoptosis. Thus, this review is an endeavor to sum up the molecular mechanisms underlying the action of BA and future directions to apply mathematical modeling technique to better understand the precise mechanism of BA-induced apoptosis. The last section of the review encompasses the plausible structural modifications and formulations to enhance the therapeutic efficacy of BA.

1. Introduction

3β-Hydroxy-lup-20(29)-en-28-oic acid (betulinic acid, BA, Fig. 1) is chemically a lupan-skeleton pentacyclic triterpene and has a remarkable place in natural anticancer medicine [1]. The pentacyclic triterpene nucleus of BA is comprised of six isoprene units that possess different biological activities including antitumor activity [2,3]. The antitumor activity of BA has been well documented due to its ability to trigger apoptosis in variety of tumor cell types. Apoptosis is a process of programmed cell death that occurs due to several biochemical events followed by characteristic changes in cell morphology and death. Cancer cells have a strong ability to impair the mitochondrial pathway of apoptosis [4]. Besides cellular bioenergetics, mitochondria play an imperative role in the persistent regulation of apoptosis. In particular, BA and analogues (BAs) have been known to exhibit potential antitumor action via provoking the mitochondrial pathway of apoptosis [5].

Several authors have reported their views over the mechanisms underlying the antitumor action of BAs. For example, the downstream regulation of Bcl-2 family proteins (Bcl-2 and Bcl-xl) [6], cytosolic caspase activation and nuclear fragmentation [7], inhibition of pro-apoptotic p38, MAPK and SAP/JNK kinases [8], and decreased expression of pro-apoptotic proteins and vascular endothelial growth factor (VEGF) [9].

Despite the availability of plethora of literatures signifying the awesome function of BA in cancer drug discovery, none of the previous artwork emphasized its mechanistic action in correlation with mathematical modeling of apoptosis to the best of our knowledge. This article is consequently devoted to review the explored molecular mechanisms of BA action and to direct the future needs in search of precise mechanism of BA action using mathematical modeling. Other important aspects in the study of BA cytotoxicity are its chemical modifications and various formulations, summarized in last section of the review.

2. Molecular mechanism of BA

2.1. General mechanism for anticancer activity of BA

Although the exact mechanism underlying BA-induced cytotoxicity is broadly unexplored to date, several studies have offered considerable insights into BA-induced cell death through a number of apoptotic mechanisms. Apoptosis occurs due to several biochemical events leads

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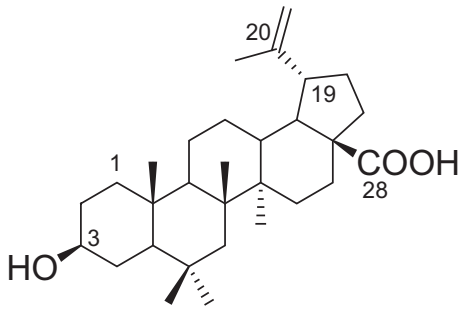

Fig. 1. Structure of betulinic acid (BA).

to characteristic morphological alterations including caspase activation, cell shrinkage, DNA fragmentation, nuclear condensation, and plasma membrane blebbing [5]. In view of this, apoptosis is a critical physiological process to limit the growth of tumor cells and thereby considered a major target for anticancer drugs [10]. The process of apoptosis may include the intrinsic *pathway* (mitochondria) as well as the *extrinsic pathway* (activation of *death receptors*). The extrinsic pathway of apoptosis is initiated by external stimuli that can stimulate TNF/Fas-receptor to activate procaspase-8 [11]. This activated caspase-8 further leads to the activation of caspase-3, -6 and -7 [12]. The activated caspase-3, -6 and -7 may cause cell death by disruption of the cytoskeleton as well as the nucleus. Except this, the intrinsic pathway of apoptosis is induced by internal stimuli which, in turn, stimulate pro-apoptotic genes in the mitochondrial outer membrane. Growing evidences showed that BA induces apoptosis in cancer cells through the activation of mitochondrial (intrinsic) pathway and not to the death receptor (extrinsic) pathway [13,14]. This evidence has been corroborated well with a study where BA was recognized to trigger the process of apoptosis in human metastatic melanoma cells (Me-45) by releasing apoptosis inducing factor (AIF) and cytochrome *c* (Cyt C) through mitochondrial membrane [15]. The pro-apoptotic Bcl-2 family proteins (Bax, Bak, Bad) are generally involved in mitochondrial membrane permeabilization to release cytochrome *c* in cytosol, where it binds to caspase-activating protein apoptotic protease activating factor-1 (Apaf-1) and procaspase-9 in order to release apoptosome [12]. The apoptosome produces the activated caspase-9, which further activates caspase-3, -6 and -7 and thereby leads to cell death [11]. Some other study proved that BA generally induces apoptosis through release of cytochrome C, AIF and Smac, caspase activation and DNA fragmentation [7,10,16]. Through caspase activation, BA also stimulates the increased production of reactive oxygen species (ROS) that is considered a stress factor involved in initiating mitochondrial membrane permeabilization [17–19]. Moreover, the calcium overload and thereby ATP depletion are other stress factors causing enhanced inner mitochondrial membrane permeability via nonspecific pores formation [20]. The plausible mechanisms of BA action in cancer prevention are illustrated in Fig. 2. Apart from this, BA has also known to be involved in activation of nuclear factor kappa B (NF-κB) that is responsible for apoptosis induction in variety of cancer cells [21]. According to Zhang et al. [14], BA stimulates apoptosis through the suppression of cyclic AMP-dependent transcription factor ATF-3 and NF-κB pathways and down-regulation of p53 gene. It was observed that BA exhibited apoptotic action in cancer cells because of improved regulation in p53 apoptotic pathways [22,23]. On the contrary, a recent study reported that BA-induced apoptosis is independent of p53-apoptotic pathway in human metastatic melanoma cells (Me-45) [15]. A study reported that pro-apoptotic mitogen-activated protein kinases (MAPKs) were found to be involved in BA-induced cytotoxicity [24] whereas some other authors revealed that the overexpression of Bcl-2 family proteins and treatment with bongkrekic acid (a permeability transition pore complex stabilizer) inhibited cytochrome C release and BA-induced apoptosis [6,7]. In addition, the inhibition of topoisomerases could also be associated with BA-induced cytotoxicity [25,26]. Later, the cytotoxicity of BA has also been explained owing to decreased expression of vascular endothelial growth (VEGF) and the anti-apoptotic protein surviving in LNCaP prostate cancer cells. This antiangiogenic and pro-apoptotic effects in LNCaP cells could probably due to the stimulation of selective proteasome-dependent targeted degradation of transcription factors specificity proteins (Sp1, Sp3, and Sp4), which generally regulate VEGF and survivin expression and highly over-expressed in tumor conditions [9].

2.2. Induction of mitochondrial outer membrane permeabilization

A chemotherapeutic agent triggers mitochondrial (intrinsic) pathway of apoptosis either via DNA damage or via producing cellular stress. This activation of apoptosis is probably initiated through the mitochondrial outer membrane permeabilization. BA triggers loss of mitochondrial membrane potential in isolated mitochondria and this loss was not restored by the caspase inhibitor Z-VAD-FMK, however restored by bongkrekic acid, an inhibitor of the permeability transition pore complex [7]. Also in intact cells, BA triggers cytochrome *c* in a caspase-independent and permeability transition pore-dependent manner, which was corroborated well with the previous finding that bongkrekic acid but not Z-VADfmk inhibited cytochrome *c* release [27]. Thus, BA generally induces apoptosis through direct enhancement of mitochondrial permeability. Further, the extreme permeability of mitochondrial outer membrane leads to the release of soluble proteins i.e. cytochrome *c*, Smac or AIF from the mitochondria to the cytosol which causes a central coordinating event of cytosolic caspase activation (caspase 3 and 8) followed by nuclear and DNA fragmentation [28]. The activation of caspases was seen basically in the cells which already had perturbed mitochondrial membrane potential further substantiating the event that BA-induced caspases activation is initiated predominantly through mitochondrial perturbation. In a manner similar to bongkrekic acid, anti-apoptotic Bcl-2 family proteins including Bcl-2 and Bcl-xl inhibit BA-induced mitochondrial disturbance, again indicating the role of mitochondrial permeability factor in apoptosis [7]. In addition, besides DNA and nuclear fragmentation, caspase activation also leads to the generation of ROS, as evidenced through the fact that some peptide inhibitors of caspase completely abrogated BA-induced apoptosis. Importantly, BA-induced ROS generation also results into the initiation of mitochondrial membrane permeabilization. This was evidenced through the detection of ROS generation in BA-treated cancer cell lines [6,7,29]. Incubation with antioxidants prior to administration of BA prevents cells to undergo in apoptosis suggesting that generation of ROS was involved in cell death. Importantly, neuroblastoma cells that were resistant to doxorubicin-induced apoptosis were found responsive to treat through BA administration [30]. This suggests that BA can overcome different types of chemotherapeutic drug resistance in cancer therapy.

2.3. Regulation of apoptosis by Bcl-2 family proteins

The Bcl-2 family is the best characterized protein family to regulate mitochondrial outer membrane permeabilization, consisting of anti-apoptotic and pro-apoptotic members. The anti-apoptotic members of this family (Bcl-2, Bcl-xs, Bcl-xl and Mcl-1) prevent apoptosis either by sequestering proforms of death-driving caspases (a complex called the apoptosome) or by preventing the release of mitochondrial apoptogenic factors including cytochrome *c* and AIF into the cytoplasm. After entering the cytoplasm, cytochrome *c* and AIF directly activate caspases that cleave a set of cellular proteins to cause apoptotic changes. In contrast, pro-apoptotic members of this family (Bax, Bak and Bad) trigger the release of caspases from death antagonists through heterodimerization and also by inducing the release of mitochondrial apoptogenic factors into the cytoplasm by acting on mitochondrial permeability transition pore, thereby leads to caspase activation. Importantly,