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## **Lupan-Skeleton Pentacyclic Triterpenes with Activity against Skin Cancer: Preclinical Trials Evolution**

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### **Abstract**

Skin cancer is an increasingly frequent pathology, with a dangerous high percentage of malignant melanoma. The use of synthetic chemotherapy raises the problem of severe adverse effects and the development of resistance to treatment. Therefore, the use of natural therapies became the focus of numerous research groups due to their high efficacy and lower systemic adverse effects. Among natural products evaluated as therapeutic agents against skin cancer, betulinic acid was emphasized as a highly selective anti-melanoma agent and is currently undergoing phase II clinical trials as topical application. Several other pentacyclic triterpenes exhibit antiproliferative activities. This chapter aims to present the latest main discoveries in the class of pentacyclic triterpenes with antitumor effect and the evolution of their preclinical trials. Furthermore, it includes reports on plant sources containing pentacyclic triterpenes, as well as the main possibilities of their water solubilization and cancer cell targeting. A review on recent data regarding mechanisms of action at cellular and molecular levels complements information on the outstanding medicinal potential of these compounds.

**Keywords:** pentacyclic triterpenes, betulinic acid, lupane, preclinic, mechanism of action

## 1. Introduction

Skin cancer represents one of the most frequent cancers with an increasing incidence over the past decades [1]. Malignant melanoma, squamous cell carcinoma and basal cell carcinoma represent 98% of all skin cancers [2]. Malignant melanoma determines a higher mortality compared to nonmelanoma skin cancers, being responsible of 75% of skin cancer deaths [2, 3]. Sun exposure is one of the major risk factors, but it influences differently the types of skin cancer. Squamous cell carcinoma is more often related to chronic sun exposure, while malignant melanoma is caused by intermittent sun exposure and overexposure in childhood [4].

Nonmelanoma skin cancer is considered to have the highest incidence of all cancers and occurs more frequently in people with white skin [5]. However, 232,000 new cases of malignant melanoma were diagnosed in 2012, with the highest incidence in Australia. The number of deaths due to this type of skin cancer was 55,000 worldwide in the same year [6]. Malignant melanoma cases tripled in the last 30 years in the United States and Europe [1]. According to World Health Organization [7], each year occur 132,000 cases of melanoma skin cancers worldwide. The increasing incidence is associated with an increase of treatment costs. This aspect underscores the important role of prevention and early detection efforts for this type of cancer [8].

Even though numerous efforts were made for finding effective treatments in melanoma, prognosis for these patients remains unsatisfactory. The standard treatment in early stages is represented by surgical excision, followed by an adjuvant therapy or enrollment in a clinical trial [9]. An early detection of melanoma and a proper treatment increase the chances for cure. Surgery, chemotherapy, immunotherapy or radiation therapy can be used for the treatment [10]. Interferon- $\alpha$  (IFN- $\alpha$ ) is used as an adjuvant therapy in patients with high-risk cutaneous melanoma, improving mainly disease-free survival but also overall survival, though not without side effects [11]. An improvement of survival in patients with stage III melanoma has been noticed for ipilimumab therapy. This monoclonal antibody increases the immune response and is approved for treatment in advanced melanoma, but also causes gastrointestinal, endocrine and hepatic adverse effects [12]. New drugs have also been used in the treatment of patients with inoperable or stage IV cutaneous malignant melanoma. The BRAF inhibitors vemurafenib and dabrafenib, the anti-PD1 (programmed death 1) antibodies pembrolizumab and nivolumab or the MEK (mitogen-activated protein kinase) inhibitors trametinib and cobimetinib are new agents proposed in melanoma therapy [13]. Associations of BRAF inhibitors (dabrafenib) and MEK inhibitors (trametinib) have also been evaluated in order to improve overall survival and to delay the appearance of drug resistance in patients with metastatic melanoma with BRAF mutations [14].

In nonmelanoma skin cancer, the therapy is different depending on the severity of the tumor. Standard excision or Mohs micrographic surgery (MMS), radiotherapy, photodynamic therapy (PDT), cryosurgery and topical treatment with imiquimod or 5-fluorouracil are employed in the management of this type of skin cancer [15].

Despite the numerous studies and the advances in targeted therapy and immunotherapy, the treatment options in melanoma are limited [9]. The main inconveniences of chemotherapeutic agents currently used in skin cancer are the severe side effects and the multi-drug resistance [16].

Due to these disadvantages of conventional therapies, new alternatives have been investigated in order to find compounds that can serve for the synthesis of new drugs [16]. Plant-derived compounds are intensively studied as anticancer agents, many studies being performed to evaluate their properties in different types of cancer, including skin cancer [17].

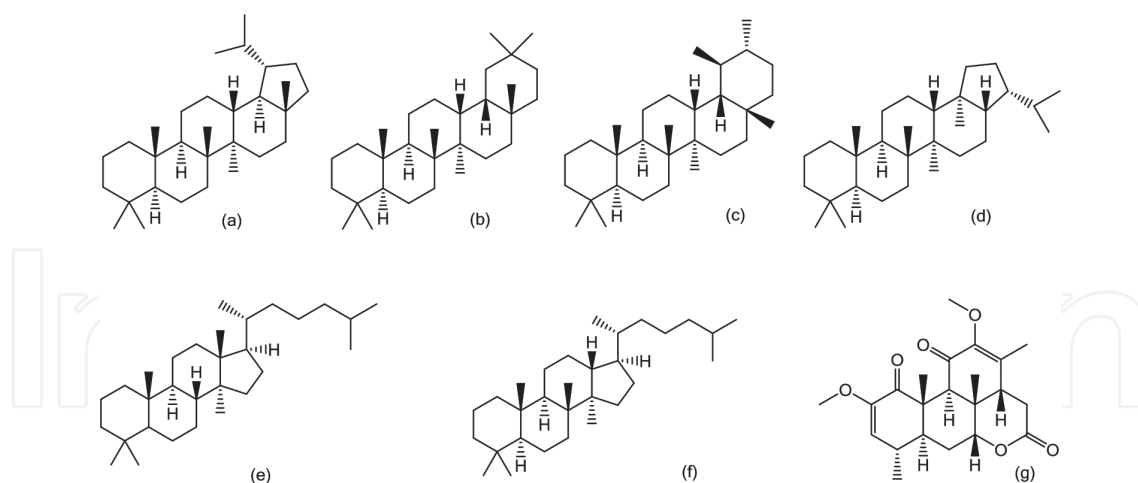
## 2. Plant sources of pentacyclic triterpenes with lupane scaffold

Plants have gained over the time an important place in the prevention and treatment of various medical conditions. Extracts from plants were obtained since ancient times following simple procedures and used as teas, potions and ointments in an attempt to alleviate pain and to cure diseases. Natural sources of drugs remain an important branch in pharmaceutical drug discovery and therapeutic implementation. Combinatorial chemistry as an initial source of information was unable to offer the expected amount of final products, but is considered a tool for preliminary analysis of new drugs even on cancer treatment. Several groups of researchers provided routes to refine and improve the skeleton of natural compound and to prepare novel active agents [18]. Links between natural sources, synthetic chemistry and knowledge about genetic analysis of microbes are new trends in preclinical evaluations [18, 19]. Drugs derived from nature may be fall in one of the following categories: natural product botanical, derived from natural product or made by total synthesis but with the specification that the pharmacophore is in relation with a natural product [18].

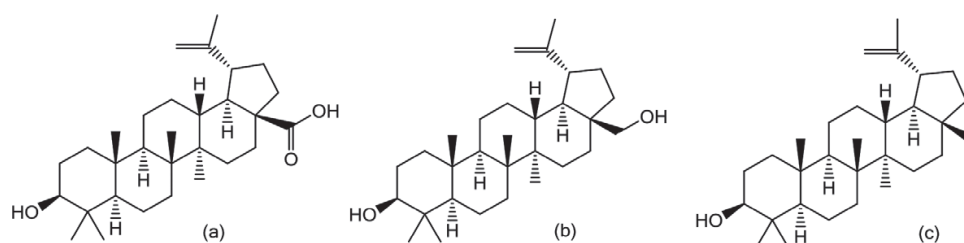
During the last decades, natural remedies are engaged in an unprecedented evolution aimed at an increased efficacy. The development of sophisticated technologies in the fields of phytochemistry, drug formulation and pharmacology, as well as the focus on the mechanism of action on a cellular and molecular level, enables the obtainment of highly efficient drugs from plants.

One of the numerous categories of plant phytochemicals are triterpenes (**Figures 1–3**). So far, over 20,000 triterpenes have been isolated from the plant kingdom. They include a variety of structural subtypes: squalene, lanostane, dammarane, tetranortriterpenoids, lupane, oleanane, ursane, hopane and other [20, 21]. Pentacyclic triterpenes, their natural sources and biological effects are presented in **Table 1**.

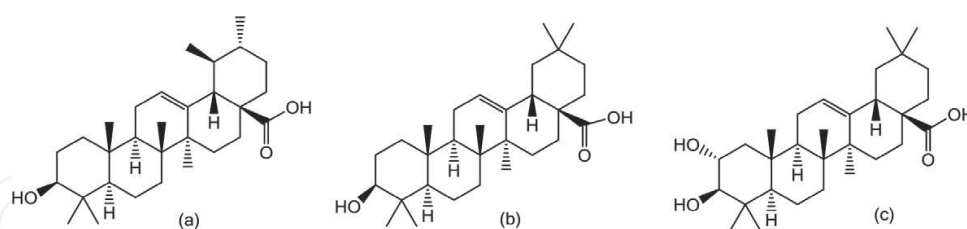
Among plant sources containing lupan-skeleton pentacyclic triterpenes (**Figure 2**), birch bark has received particular attention due to its high content in these substances, its well-proven application and uses over the time [41]. Currently, it is acknowledged that the outer birch bark is a rich source of pentacyclic triterpenes, which include: betulin (B lup-20 (29)-ene-3 $\beta$ , 28-diol), betulinic acid (BA, 3 $\beta$  acid, hydroxy-lup-20 (29)-en-28-oic) and lupeol



**Figure 1.** Triterpene structures (a) lupane; (b) oleanane; (c) ursane; (d) hopane; (e) lanostane; (f) dammarane and (g) quassin.



**Figure 2.** Lupane skeleton triterpenes (a) betulinic acid; (b) betulin and (c) lupeol.



**Figure 3.** Other triterpenes (a) ursolic acid; (b) oleanolic acid and (c) maslinic acid.

(L, Lup-20 (29)-en-3-ol). The development of birch bark extracts, their applications and bioactivity has comprehensively been reviewed [42]. By analyzing birch bark extract, it was shown that betulin is present in the highest amount, while betulinic acid content is lower. However, it is possible that plants from different geographical regions present a variable content in pentacyclic triterpene, which requires a rigorous analysis of the content [43]. Differences between barks of birch species regard the content in: betulin, betulinic acid, betulinic aldehyde, lupeol, oleanolic acid, oleanolic acid 3-acetate, betulin 3-cafeate, erythrodiol and other.

Substance	Plant (family)	Plant part	Study/effect	Reference
BA	<i>Ziziphus mauritiana</i> Lam. (Rhamnaceae)	Stem bark	<i>In vitro</i> —inhibitory effect on (MEL-1, -2, -3, -4) cells; apoptotic effect on MEL-2 cells <i>In vivo</i> —antitumor effect on athymic mice injected with MEL-2 cells	[22]
BE, LU	<i>Betula x caerulea</i> Blanch., <i>Betula cordifolia</i> Regel, <i>Betula papyrifera</i> Marsh., <i>Betula populvolia</i> Marsh. (Betulaceae)	Bark	N/A	[23]
BA, LU, BE, UA, OA	<i>Syzygium formosanum</i> Hay. Mori (Myrtaceae)	Leaves	N/A	[24]
BE, BA, UA	<i>Diospyros leucomelas</i> Poir. (Ebenaceae)	Leaves	<i>In vivo</i> —anti-inflammatory activity on Swiss mice, for induced ear edema and induced paw edema	[25]
OA	<i>Rosa canina</i> L. (Rosaceae)	Rose hip, powder	<i>In vitro</i> —immunomodulatory activity on Mono Mac 6, obtained when a mixture of OA, BA and UA was used	[26]
BA	<i>Rosmarinus officinalis</i> L. (Labiatae)	Stems and leaves	<i>In vivo</i> —antidepressant-like effect in the TST, for Swiss mice; anti-immobility effect	[27]
BE, BA	<i>Betula pendula</i> Roth, syn. <i>Betula verrucosa</i> (Betulaceae)	Bark	<i>In vitro</i> —cytotoxic effect in EPG85-257 and EPP85-181 cells line	[28]
A, BA, BE, LU, UA, OA	<i>Ligustrum pricei</i> Hayata, <i>Ligustrum sinense</i> Lour., <i>Ligustrum lucidum</i> W.T.Aiton (Oleaceae)	Leaves	<i>In vivo</i> —analgesic and anti-inflammatory effect on Sprague Dawley rats	[29]
OA, BA	<i>Viscum album</i> L. (Santalaceae) – harvested from <i>Malus domestica</i> Borkh.	Sprout	<i>In vitro</i> —cytotoxic and apoptotic effect on B16.F10 cells	[30]
OA, BA	<i>Viscum album</i> L. (Santalaceae) – harvested from <i>Malus domestica</i> Borkh.	Sprout	<i>In vivo</i> —antiapoptotic, antiproliferative effect on C57BL/6NCrL mice injected with B16.F10	[31]
BE, UA	<i>Myrica cerifera</i> L. (Myricaceae)	Bark	<i>In vitro</i> —cytotoxic activity against HL60, A549 and SK-BR-3 cell lines	[32]
LU	<i>Taraxacum</i> sp. Dandelion (Asteraceae)	Root	<i>In vitro</i> —cytostatic, not cytotoxic effect on B16 2F2 cells; inhibition of cells proliferation by differentiation	[33]
LU	<i>Lactuca indica</i> L. (Asteraceae)	N/A	<i>In vivo</i> —prevents local tumor progression, distant metastasis in dogs with COMM Dogs: two miniature Dachshunds, two Beagles, two miniature Schnauzers, one Golden Retriever, one Labrador Retriever, one American Cocker Spaniel, one Cavalier King Charles Spaniel and 1 mixed-breed dog	[34]
LU	<i>Lactuca indica</i> L. (Asteraceae)	N/A	<i>In vivo</i> —tumor growth suppression and induced cell cycle arrest in C57BL/6 mice injected with B16 2F2 cells	[35]

Substance	Plant (family)	Plant part	Study/effect	Reference
LU	<i>Bombax ceiba</i> L. (Malvaceae)	Stem Bark	<i>In vitro</i> —antiangiogenic effect on SK-MEL-2, A549 and B16-F10 cell lines	[36]
BA, OA	<i>Paeonia rockii</i> ssp. <i>rockii</i> T.Hong & J.J.Li (Paeoniaceae)	Root	<i>In vitro</i> —antiapoptotic effect induced selectively in the M-14 cell line	[37]
UA	<i>Salvia officinalis</i> L. (Lamiaceae)	N/A	<i>In vivo</i> —antiprotease and antimetastatic effects on C57BL/6N mice injected with B16 cells	[38]
BA	<i>Avicennia officinalis</i> L. (Acanthaceae)	Leaves	<i>In vivo</i> —anti-inflammatory effect on rats	[39]
BA, UA, MA	<i>Bridelia cambodiana</i> Gagnep. (Phyllanthaceae)	Whole plant	<i>In vitro</i> —cytotoxic effect against HL60 and LCC cell lines	[40]

BA (betulinic acid); BE (betulin); LU (lupeol); UA (ursolic acid); OA (oleanolic acid); MA (maslinic acid); N/A (not applicable); MEL-1, -2, -3, -4 (human melanoma cell line); Mono Mac 6 (human monocytic cell line); TST (tail suspension test); EPG85-257 (human gastric carcinoma cell line); EPP85-181 (human pancreatic carcinoma cell line); B16.F10 (murine melanoma cell line); HL60 (human promyelocytic leukemia cell line); A549 (human lung carcinoma cell line); SK-BR-3 (human breast cancer cell line); B16 2F2 (mouse melanoma derived subclone with high differentiation capability); COMM (canine oral malignant melanoma); SK-MEL-2 (human melanoma cell line); M-14 (human melanoma cell line); B16 (mouse melanoma cell line derived from spontaneous skin tumor); LLC (mouse lewis lung carcinoma).

**Table 1.** Bioactivity of various plant products containing pentacyclic triterpenes.

### 3. Obtainment of lupane-skeleton triterpenes with efficacy in skin cancer

Pentacyclic triterpenes from plants are secondary metabolites with high lipophilicity. Therefore, they are mainly located in hydrophobic histological structures. In the cork of trees, which represents the outer tissue of the secondary bark, triterpenes are associated with suberin; a well-known example is birch bark [44]. Triterpenes are as well components of cuticular and epicuticular waxes covering leaves [45] and fruits [46].

Betulin was isolated for the first time in the 1788 by Lowitz [47] from birch cork. The elucidation of its structure was performed by only in 1953 by Guider et al. [48]. Additional plant sources for betulin include hornbeam (*Carpinus betulus* L) and hazel (*Corylus avellana* L.), plants which are phylogenetically closely related to birch [49]. Betulinic acid, a triterpene of major therapeutic relevance, was isolated under the name of “graciolon” from *Gratiola officinalis* [50] and recognized as such only 40 years later [51]. “Platanolic acid,” isolated from *Platanus acerifolia* bark [52], proved later to be betulinic acid as well [53]. Furthermore, betulinic acid could be obtained from an alcoholic extract of *Cornus florida* L. bark [54]. Ko and co-workers [55] used mistletoe (*Viscum album*) to obtain an ethanol extract enriched in triterpenes, including betulinic acid and botulin.

The obtainment of triterpenes from the plant matrices employs as a first step extraction with organic solvents such as methanol or ethanol [56]. Other solvents are chloroform, dichloromethane, ethyl acetate, petroleum ether or various mixtures thereof, in accordance with the low polarity of these phytocompounds. Recovery procedures may include Soxhlet extraction,

maceration and ultrasound-assisted processes [57]. In order to progressively enrich/isolate triterpenes, the usual phytochemical approaches are employed: partition among solvents of increasing polarity, column chromatography on silica gel, countercurrent chromatography and preparative chromatography. Triterpene acids are extracted after alkalization with sodium hydroxide [57] or calcium hydroxide [58]. Pure betulin was prepared from a crude mixture using a chromatographic column with silica gel as a stationary phase and a mixture of hexane and ethyl acetate as eluent, followed by recrystallization from 75% ethyl alcohol [59]. An effective preparation of crystalline betulin (99% purity) from birch bark is clearly described in a recent work, following the steps to remove betulonic acid and lupeol. Additionally, the authors demonstrate the obvious relationship between the cytotoxic activity of betulin and its purity [58]. The analytic determination of triterpenes in samples is performed by reverse-phase HPTLC and gas chromatography, coupled with the detection using mass spectrometry detection, is a widely used method for analysis of betulin and other triterpenes in samples. High-performance thin-layer chromatography (HPTLC) is a valuable straightforward tool for the visualization of impurities [60].

Betulonic acid received high attention due to its properties to inhibit the growth of cancer cell lines, without being cytotoxic to normal cells. In plant materials used as sources of triterpenes such as birch, the content in betulonic acid is much lower than that in betulin. For this reason, various attempts have been made to obtain betulonic acid, using betulin as a starting point. In the study of Melnikova and co-workers [61], the most intense catalytic activity was noticed for aluminum salts, which also have a selective activity. The reaction proceeds via the intermediate betulonic acid, purified by recrystallization. Betulonic acid is reduced with  $\text{NaBH}_4$  (THF or isopropanol) at room temperature to obtain a mixture of  $3\alpha$ -betulonic acid and betulonic acid- $3\beta$  [61].

Enzymatic transformations, privileged for their simplicity, eco-friendliness and safety, are currently a mainstay in the obtainment of drugs. In an enzymatic approach, the fungus *Armillaria luteo-virens* Sacc ZJUQH100-6 was employed in the biotransformation of betulin into betulonic acid [62]. Optimization of the obtainment was monitored by variation of parameters like pH, glucose, betulin content, addition of tween 80 and stage of inoculation; the presence of the surfactant had a significant impact on the yield of the biotransformation.

While betulin is readily available from birch and other plants, its anticancer activity is only moderate. Being an accessible starting point for derivatizations, betulin has been the subject of many researches, aiming to obtain compounds with enhanced anticancer activities [63]. The acetylated derivatives were tested for antiproliferative effect on several cell lines: colorectal adenocarcinoma, leukemia and breast cancer. By esterifying betulin with propionic acid in dichloromethane solution in the presence of dicyclohexylcarbodiimide and 4-dimethylaminopyridine, derivatives: 28-O-propynoylbetulin and 3,28-A, IB, dipropynoylbetulin were obtained. Column chromatography was employed in order to obtain the pure components with a yield of 60% in the case of the first derivative and 12% in the second. The reaction of betulin with propargyl chloroformate and 3-butyl-1-yl chloroformate in benzene, in the presence of pyridine, resulted in the formation of a mixture of 28-O-propargyloxycarbonylbetulin monoesters and 28-O- (3-butynyloxycarbonyl) toxin and di-3,28-A sheep-di (pro-pargyloxycarbonyl)



3.28-A toxin and sheep-di (3-butyn-yloxy-carbonyl) toxin. The resulting mixture was separated by column chromatography; thus, pure components were obtained in a 64–69% yield for monoesters and 23–27% in the case of diesters [64].

#### 4. Advanced formulation of lupane triterpenes

The most challenging aspect of the biomedical use of lupane triterpenes is their low water solubility which subsequently causes poor bioavailability [65]; so far, several delivery systems have been developed in order to achieve superior pharmacokinetic outcomes. The current subchapter aims to review the most recent and promising delivery options for betulin, betulinic acid and lupeol.

The first step in the attempt to modulate the aqueous solubility of an insoluble compound relies in its convenient derivatization with water-soluble partners; such an attempt was conducted by Drag-Zalesinska and co-workers [66], who prepared mono- and diesters of betulin and betulinic acid with amino acids. All esters revealed higher water solubilities and significant cytotoxic activity via apoptosis induction; the type of ester as well as the type of the amino acid side chain strongly influences the biological effect of the respective compounds [66]. C(2)-propargyl-substituted pentacyclic triterpenoids conjugated with 1,2,3-triazole glucopyranosides were synthesized via “click” chemistry in order to achieve optimized water solubility as well as pharmacokinetic and pharmacological properties [67].

Cyclodextrin (CD) complexation represents an attractive solution to increase the aqueous solubility of numerous compounds; through their hydrophobic interior and hydrophilic surface, cyclodextrins are able to accommodate various lipophilic guest molecules which can thus be water-solubilized. According to molecular studies [68], the bulky structures of betulinic acid and betulin fit best inside the cavity of  $\gamma$ -CD and its semisynthetic derivatives, such as hydroxypropyl- $\gamma$ -CD (HPGCD). As a result of HPGCD complexation, a 14-fold increased water solubility was reported for BA accompanied by superior biological activity [69], *i.e.*, strong antiangiogenic and antitumor effect [70, 71]. Similar outcomes were achieved in terms of anti-melanoma activity tested on B16 cell line (murine melanoma) [72]. Fontanay et al. conducted a study on the inclusion of hydroxy pentacyclic triterpenoid acids, including BA, inside native  $\gamma$ -CD [73]; the physicochemical analysis revealed the formation of a 1:1 complex with a significantly improved aqueous solubility.

$\beta$ -CD derivatives also served as complexation partners for BA, and its inclusion in the cyclodextrin hydrophilic matrix led to significantly improved dissolution rate and, subsequently, antiproliferative *in vitro* activity against MCF7 (breast cancer) cell line [74]. The same tumor cell line was involved in the study of the biological activity of betulinic acid accommodated inside native  $\beta$ -CD [75]; a dose-dependent antiproliferative activity was reported, through mitochondria-mediated apoptosis induction and G2/M cell cycle arrest.

An important parameter in the cyclodextrin inclusion process is the stability constant of the final complex, its value giving the measure of the potential use of the complex as biologically active agent; significantly high stability constants were achieved for both betulin and

betulinic acid in complex with newly synthesized hydrophilic  $\gamma$ -CD derivatives [76]. Such a high stability constant characterizes a strong interaction between the host- and the guest molecule, thus enabling the delivery of the active drug at the target site in the absence of systemic adverse effects. Both complexes, with betulin and betulinic acid, respectively, were *in vitro* and *in vivo* tested, revealing moderate *in vitro* antiproliferative activity; however, *in vivo* results on murine models showed a significant decline in tumor size and volume [77, 78].

The inclusion of betulin inside HPGCD led to optimized outcomes in terms of bioavailability and antiproliferative activity [79, 80]; similar results were reported for betulin complexation with hydrophilic  $\beta$ -CD derivatives that caused stronger inhibitory activity against MCF7 (breast cancer) cell line than pure betulin [74].

Lupeol was also subjected to inclusion inside  $\gamma$ -CD by kneading in a 1:2 molar ratio; the complex revealed optimized antiproliferative and antiangiogenic activities compared to the pure drug [81].

The use of triterpenes as mixtures such as total extract of birch outer bark may trigger simultaneously various mechanisms of apoptosis induction and therefore result in an additive or synergistic effect. Hertrampf et al. [82] used HPBCD as solubilizer for birch total extract; a series of dilutions were prepared using the main ingredient, betulin, as a reference to calculate concentrations. The study reported the multivalent cytotoxic activity of the birch bark at lower concentrations than previously used presumably due to a higher bioavailability of triterpenes provided by cyclodextrin solubilization; moreover, a synergistic effect was suggested. Triterpene-rich mistletoe extract (6.9% BA) was solubilized by Strüh et al. [30] by using HPBCD and tested against B16F10 melanoma cell line; a dose-dependent reduction of cellular ATP was reported accompanied by high cytotoxicity due to DNA fragmentation. The research was continued by *in vivo* studies on C57BL/6 mice bearing B16F10 subcutaneous melanoma, revealing an increased antitumor effect and a prolonged mice survival [31].

An alternative research direction was the preparation of cyclodextrin conjugates instead of inclusion complexes; “click chemistry” was involved in the synthesis of triazole-bridged conjugates between  $\beta$ -CD and pentacyclic triterpenes [83]. All bioconjugates showed higher hydrophilicity than the parent compound, and several conjugates displayed significant cytotoxicity on various cancer cell lines; in addition, the cyclodextrin conjugation led to the disappearance of haemolytic toxicity. The authors continued their research by synthesizing  $\alpha$ -CD conjugates with several pentacyclic triterpenes including BA [84]; all conjugates exhibited lower hydrophobicity than the parent molecules accompanied by significant anti-HCV (hepatitis C virus) entry activity.

An excellent review was published in 2016 by Lima et al. [85], describing the main attempts to use cyclodextrins as nanocarriers for various terpenes; the authors concluded that cyclodextrins are feasible tools in improving the pharmacological profile of terpenes, limited mainly by the scarce pharmacokinetic and clinical studies.

Liposomes are small vesicles displaying one or more phospholipidic layers and an aqueous core [86] that may incorporate both lipophilic and hydrophilic compounds [87, 88]. Betulinic acid was trapped inside large liposomes by Mullauer et al. [89] and administered to mice

bearing experimental models of colon (SW480) and lung (A549) cancer; no systemic adverse effects were reported following parenteral (i.v.) and oral administration. Similar studies reported liposomal and proliposomal formulations with BA with 95% yield of the incorporation process [90]. Phospholipidic nanosomes prepared by means of supercritical fluids were used to entrap BA in order to increase its efficacy as antiviral agent [91, 92]. Several betulin derivatives such as 28-acetylenic derivatives [93] and pyrazoles and 1,2,3-triazole derivatives [94] were synthesized and formulated as liposomes; the nanoformulations exhibited strong apoptotic activity due to both higher biological effect of the active compound and optimized delivery. PEG-ylated BA liposomes were obtained by Liu et al. in 2016, entrapping BA in the lipid bilayer of the liposomes by the ethanol injection technique [95]; the hydrophilic outer PEG layer ensured improved sustained release and antitumor effect compared to free BA or BA liposomes.

Another attractive option in drug delivery is the use of micro- and nanoemulsions; a nanoemulsion containing BA was prepared using flax-seed oil as lipophilic phase and the high-pressure homogenization method [96]; the *in vivo* testing on the chorioallantoic membrane (CAM assay) revealed a significant antiangiogenic activity. The same procedure was applied for betulin nanoemulsion, followed by *in vivo* testing by CAM assay and experimental murine cancer model; the study reported strong anti-inflammatory, antiproliferative and antiangiogenic activities of the incorporated betulin as well as its potential benefits in inhibiting metastasis [97]. An oil-in-water nanoemulsion with BA was prepared through the use of phospholipase-catalyzed modified phosphatidylcholine as emulsifier in an ultrasound device; various factors such as composition, ultrasound amplitude, temperature and pH significantly influenced nanoparticle size and stability [98].

A different approach consists in the administration of betulin via the nasal route; in order to avoid mouth sedimentation of betulin particles, the solvent exchange method was used to limit particle sizes to nanoscale, thus leading to higher bioavailability of betulin in the lower respiratory tract [99].

Water solubility may be increased through grinding with hydrophilic polymers (i.e., polyvinylpyrrolidone, polyethylene glycol, arabinogalactan) [100, 101]; solid dispersions of BA with various hydrophilic polymers (i.e., Soluplus, HPMCAS-HF, Kollidon VA64, Kollidon K90, Eudragit RLPO) in 1:4 (w/w) ratio were prepared and analyzed by Yu et al. [102] in 2014, revealing a great potential to increase BA water solubility. Moreover, hydrophilic bioconjugates can be synthesized between active drugs and hydrophilic polymers. BA-monomethoxy polyethylene glycol (mPEG) conjugate was synthesized by covalent bonding of the carboxyl moiety of BA and the amine groups of mPEG [103]; the conjugate exhibited cytotoxicity through cell apoptosis on hepatic cancer cells (Hep3B, Huh7) as well as *in vivo* antitumor efficacy in Ehrlich ascites tumor (EAT) model while lacking any sign of biochemical and histological toxicity. A step further was represented by the use of multiarm-PEGs as conjugation partner which offer a high density of functional groups; through the formation of an ester bond, BA was linked to eight-arm PEG (8arm-PEG) and then to a targeting molecule (folate) followed by the self-assembly into nanoparticles [104]. A second anticancer drug, hydroxycamptothecin, was added by nanoprecipitation; the ensemble achieved a dramatically increased cytotoxicity, prolonged blood circulation, enhanced tumor targeting and lower systemic toxicity than the

free drugs; in addition, a synergistic antitumor efficacy was reported [104]. BA also shows the ability to self-assemble into nano- and microfibers with antileukemic efficacy and cytoprotective activity as well [105].

Biodegradable polymeric nanospheres based on poly(lactide-co-glycolide)-poly(ethylene glycol) (PLGA-PEG) were prepared by nanoprecipitation to incorporate 40% BA [106]; the study reported an increased cytotoxicity and lower IC<sub>50</sub> value compared to the pure drug. PLGA was used as building material by interfacial deposition for nanocapsules that efficiently entrapped lupeol [107]. Polymer matrixes can be involved in regional chemotherapy, an approach that avoids systemic adverse effects [108] and allows the controlled release of the pure drug; betulin was incorporated as model compound in such a matrix (poly(3,4-ethylenedioxythiophene), its release being conducted by passive or active mode. The novel formulation exhibited efficient cytotoxic activity against KB and MCF7 cancer cell lines.

BA conjugates with carboxyl-functionalized single-walled carbon nanotubes were synthesized via  $\pi$ - $\pi$  stacking interaction, leading to a 20% loading of the active drug [109]; following physicochemical and biological analysis, the authors reported the controlled, prolonged release of the drug, with no sign of toxicity on normal fibroblasts and significant cytotoxicity against A549 (lung cancer) cell line. The research continued by coating the nanotubes with four biopolymers: tween 20, tween 80, polyethylene glycol and chitosan in order to further improve biocompatibility [110]; the procedure induced sustained and prolonged release compared to the uncoated nanotubes, while cytotoxicity depended on the chosen biopolymer.

Metallic nanoparticles were also used as nanocarriers for pentacyclic triterpenes; as an example, magnetic nanoparticles coated with chitosan were loaded with BA and exhibited a pseudo-second-order kinetic model release of the active drug [111]; the nanoparticles were cytotoxic on MCF7 cells in a dose-dependent manner while lacking toxicity against normal mouse fibroblast cells. Silver nanoparticles coated with BA were involved in the *in vitro* testing on a panel of cancer cell lines, including A375 (murine melanoma) [112]; the new formulation revealed strong antiproliferative and antimigratory activity, in particular against melanoma cells.

## 5. Innovative approaches in preclinical evaluations of pentacyclic triterpenes of the lupane series in skin cancer

Preclinical trials are important in the initial evaluation of new drugs, formulations or specific new design of pathology. They are complex processes with an uncertain ending, as just a reduced percent of evaluations lead to a market product. It is estimated that around 90% of tested drugs are not launched to the market [113]. Despite intense of basic research, the actual delivery of accepted drugs is scarce.

The classical route of a tested compound in preclinical trials includes *in vitro* tests followed by *in vivo* tests on animals [113]. These tests may be conceived in a variety of ways and are

constantly improved and diversified. The mainstay of preclinical tests regarding a potential efficacy against skin cancer types is *in vitro* tests using different types of cell lines. In this regard, lupane triterpenes were tested on human melanoma (G361, SK-MEL-28, MEL-2, SK-MEL2, A375), mouse melanoma (B16-F1, B16 2F2) and human skin epidermoid carcinoma A431. In case of betulin, the latter showed a particularly high sensitivity (with a IC50 value below 10 $\mu$ M), while various types of human melanoma cell lines may display a high variation range of the sensitivity to betulin, with differences in IC50 values of one order of magnitude, from 12.4 to over 250  $\mu$ M [114]. For betulinic acid, IC50 was 154  $\mu$ M when tested on A375 melanoma [115], and 70  $\mu$ M when tested on B16-F10 murine melanoma [116]. Information of particular relevance for the actual clinical utilization as anticancer agent comes from comparative data on the cytotoxicity against normal cells and cancer cell lines. Betulin, for example, is more cytotoxic against cancerous cells than nontumoral ones [114]. Further steps in preclinical evaluation are the investigation of the mechanism of action; lupeol, betulin, betulinic acid and their semi-synthetic derivatives have so far shown significant effects on apoptosis and cell cycle regulation [117, 118]. As inflammation is an important player in the pathogenesis of cancer, the anti-inflammatory effects and mechanisms are as well explored to give a correct picture of the antitumoral potential [117]. Furthermore, it is important to explore the antimigratory potential of natural products, as it has a seminal importance for malignant melanoma—a cancer type with a high invasiveness [119]. A global approach to relevant preclinical tests regarding triterpenes should thus be multi-component. In this regard, our workgroup has established an efficient battery of tests for aimed at establishing the potential of natural triterpenes with anticancer/anti-inflammatory activity as agents against skin cancer. The module of preclinical evaluations includes as follows:

- **Step 1:** *in vitro* tests on normal cells (e.g., HaCat) comparing with specific pathological tests; evaluation of cytotoxic activity;
- **Step 2:** *in vitro* evaluations concerning the impact on apoptosis, and observations of specific markers via DAPI/HOPI staining, and evaluation of Annexin V, caspases and other cellular markers [120];
- **Step 3:** *in vivo* embryonated egg membrane assay for toxicological evaluation (HET CAM assay) and investigations of the potential to affect angiogenesis; future aspects include cultivation of cancer cells on embryonated eggs and direct research of the therapeutic potential;
- **Step 4:** *in vivo* tests including a large number of experimental protocols: photochemical model, inoculation of murine cells, xenograft of human pathological cells on adequate mouse hosts and correlations with therapeutical surveillance. Furthermore, histopathological evaluations and immuno-histochemical assays are correlated with innovative approaches like RAMAN skin evaluation, noninvasive methods for skin quality and surface damage characterization.

Additional determinations could require selection of cells from a primary experimental tumor, cultivation of cells and evaluation of compounds, PET animal observations and other methods applied for a detailed pathologic surveillance of drugs.

## 6. Pentacyclic triterpenes: mechanism of action at cellular and molecular level

Apoptosis is a programmed cell death consisting in morphological changes including cell shrinkage, nuclear condensation, chromosomal DNA fragmentation, plasma membrane blebbing and caspase activation [121]. In this regard, apoptosis is considered a crucial physiological process in tumor clearance, being a major target for anticancer drugs [122]. The molecular mechanisms of apoptosis can include extrinsic and intrinsic pathways. The extrinsic pathway of apoptosis is initiated by external signals, which can activate TNF/Fas-receptor, which in turn activates procaspase-8 [123]. The activated caspase-8 is involved into the caspase-3, -6 and -7 cascade activation [124]. Caspases are important cellular enzymes synthesized as inactive zymogens, which can be activated into their active tetrameric forms by various apoptotic signals [124]. The activation of caspase-3, -6 and -7 leads to cell death not only by breaking down the cytoskeleton, but also the nucleus.

The intrinsic pathway of apoptosis, known as the mitochondrial pathway, is initiated by internal stimuli which can activate the proapoptotic genes from the outer membrane of mitochondria. Bcl-2 family proteins (Bax, Bak, Bcl-xs) are important proapoptotic genes involved in permeabilization of the mitochondrial membrane in order to release cytochrome c in cytosol, where it binds to the caspase-activating protein apoptotic protease activating factor-1 (APAF-1) and with the procaspase-9, transforming into an apoptosome [124]. The apoptosome releases the activated form of caspase-9, which is also involved into the caspase-3, -6 and -7 activations, which lead to cell death [123].

Previous evidences showed that the pentacyclic triterpenes, especially betulin, betulinic acid, lupeol and ursolic acid, have induced apoptosis in different types of cancer cells via activation of the mitochondrial pathway and not to the death receptor pathway (extrinsic way) [125, 126]. These data have been supported by Drag-Zalesinska et al. [118] study in which betulin and betulinic acid proved to induce apoptosis in human metastatic melanoma cells (Me-45) by releasing cytochrome c or the apoptosis inducing factor (AIF) through the mitochondrial membrane. Liu et al. [122] have also demonstrated that betulinic acid, as well as betulin, could kill CNE2 cells through the mitochondrial pathway. Betulinic acid induced the DNA fragmentation, caspase activation and cytochrome c release but independent of Bax proteins [115, 122]. Moreover, betulinic acid has been also involved in activation of nuclear factor kappa B (NF- $\kappa$ B) responsible for apoptosis in various types of cancer cells [127].

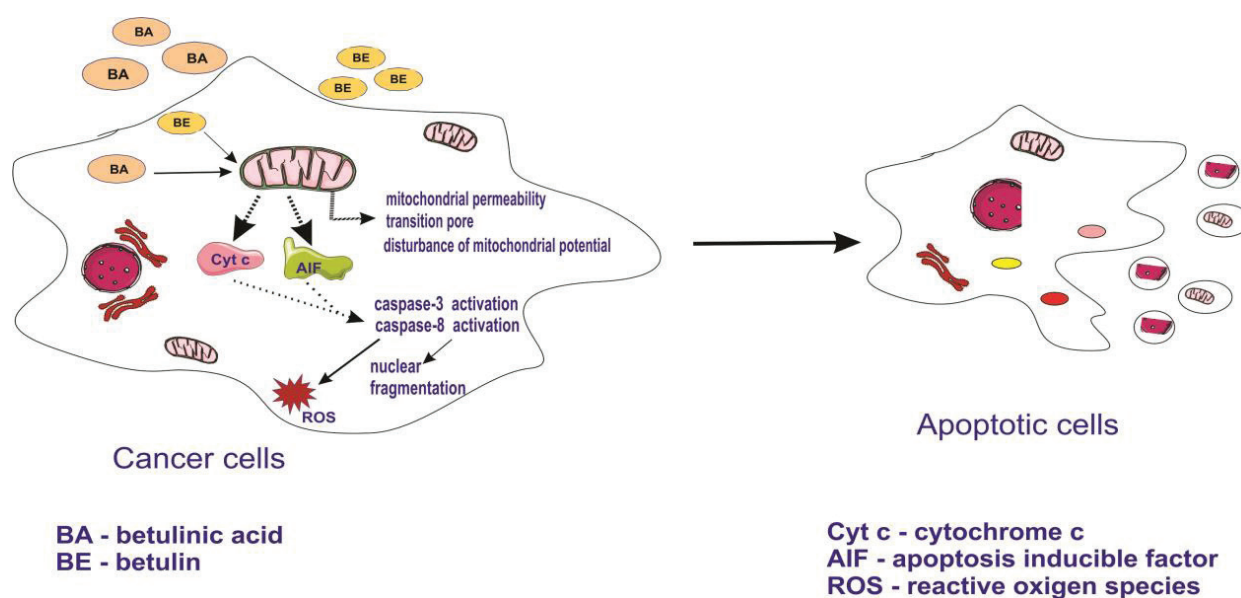
The increased production of reactive oxygen species (ROS) caused by betulin and betulinic acid stimulation [128, 129] has been considered a stress factor involved in the depolarization of mitochondrial membrane [130]. Furthermore, both calcium overload and ATP depletion were additional stress factors responsible for increasing the permeability of the inner mitochondrial membrane through formation of nonspecific pores [116]. For instance, the dimethylaminopyridine triterpenoid derivatives have also caused the depolarization of the mitochondrial membrane in situ, in order to increase the permeability transition pore [131].

Unlike the previous data, the study of Şoica et al. [77] on B164A5 murine melanoma cells and on a mouse melanoma model showed that BE and its derivatives had no effect on caspase-2 regulation, the apoptotic mechanism of betulin being suggested to be probably through the transformation of BE into betulinic acid inside the cells.

According to the study of Muceniece et al. [132] *in vitro*, betulin had a mimetic effect on melanocortin (MC) receptor, especially on MC-1 subtype. This observation has been also supported by Şoica et al. [77] study in which betulin had revealed strong inhibitory effects on B64A5 murine melanoma cells, by binding to the melanocortin receptors. Betulin has been not involved by itself in stimulation of cAMP generation, but it acted as a weak antagonist on alpha-melanocyte-stimulating hormone (alpha-MSH)-induced cAMP accumulation in B16-F1 mouse melanoma cells [132].

*In vitro* and *in vivo* studies have also revealed that the birch bark extract and betulin have significantly increased the expression of PARP-1 in melanoma cells [118], exhibiting interferon-inducing activity [133].

According to Zhang et al. [126] study, betulinic acid induced apoptosis by suppressing the cyclic AMP-dependent transcription factor ATF-3 and NF- $\kappa$ B pathways and decreasing the expression of topoisomerase I, p53 and lamin B1. On one hand, earlier studies indicated that betulinic acid had induced apoptosis of cells due to the p53 pathways [134]. This conclusion has been also supported by Tiwari et al. [135] study, in which BA proved a dose-dependent apoptotic effect on both p53 mutant and wild-type cells probably because of its involvement in p53-independent apoptotic pathway. On the other hand, a recent study has shown that the apoptotic effect of betulinic acid in human metastatic melanoma cells (Me-45) had been independent of p53-apoptotic pathway [118]. The presumable mechanisms of action of betulinic acid and betulin in skin cancer are depicted in **Figure 4**.



**Figure 4.** The mechanism of action of betulinic acid and betulin in skin cancer.

Lupeol is a complex multitarget phytochemical, being involved in controlling IL-1 receptor-associated kinase-mediated toll-like receptor 4 (IRAK-TLR4), Bcl-2 family, nuclear factor kappa B (NF- $\kappa$ B), phosphatidylinositol-3-kinase (PI3-K)/Akt and Wnt/ $\beta$ -catenin signaling pathways [136]. According to the Tarapore et al. study, the anticarcinogenic effect of lupeol has been related to the Wnt/ $\beta$ -catenin signaling pathway. That study has revealed that lupeol caused a dose-dependent decrease in Wnt target genes in Mel 1011 cells. Moreover, there has been also observed a decrease of nuclear  $\beta$ -catenin expression, associated with an enhancement of plasmatic  $\beta$ -catenin expression in melanoma cells (Mel 928 and Mel 1241). Consequently, lupeol has been involved in blocking the movement of  $\beta$ -catenin between cytoplasm and nucleus [137].

An *in vivo* study on Swiss Albino mice showed that lupeol exerted apoptotic effects through the enhancement of bax and caspase-3 genes expression and downregulation of bcl-2 anti-apoptotic genes [138].

Unlike botulin and betulinic acid, lupeol has also induced apoptosis via extrinsic pathway by enhancing the expression of FADD protein and Fas receptors [127].

Ursolic acid has strongly increased the IR-induced apoptotic effect in various types of cancer cells, likely DU145, CT26 and B16F10, playing a major role in DNA fragmentation, mitochondrial dysfunction and apoptotic marker modulation [139]. Moreover, ursolic acid has induced apoptosis in M4Beu cells human melanoma through intrinsic pathway by enhancing the caspase-3 activity in a dose-dependent manner, correlated with a low caspase-9 activity [140]. Ursolic acid has also proved to act as an inhibitor of the endogenous reverse transcriptase (RT) activity in the following tumor cells: melanoma (A375), glioblastoma (U87) and thyroid anaplastic carcinoma (ARO), as well as on nontransformed human fibroblast cell line (WI-38), exhibiting strong antiproliferative effects [141].

The mechanism of apoptosis induced by pentacyclic triterpenes is not fully understood, although, according to the previous studies, we can conclude that these triterpenes exhibited strong apoptotic effects, especially via intrinsic pathway, being involved in increasing the permeability of inner mitochondrial membrane, activation of caspase-9 and 3, as well as cell death.

## 7. Conclusion

Pentacyclic triterpenes represent an important issue in the field of antiskin cancer formulations; nowadays, the researches focus on the development of nanoformulations that provide multiple advantages over the classical pharmaceutical formulations, including the possibility of being decorated with targeting moieties that significantly improve the antiproliferative activity of the loaded active drug. Different mechanisms of action have been identified so far at cellular and molecular level, in particular for betulinic acid; however, future studies are needed in order to fully comprehend the intimate details of the anticancer treatment with pentacyclic triterpenes and formulations thereof.



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