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The Potential of Triterpenoids in the Treatment of Melanoma

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1. Introduction

Malignant melanoma can be currently allocated within the list of the most serious diseases with high mortality, every year, over 50,000 patients with this diagnosis die worldwide (Pirard & Vries, 2007). In addition, its incidence (200,000 per year worldwide) (Mathers *et al.*, 2001) is still increasing due to deteriorating environmental factors (increased exposure to UV light, unhealthy lifestyle, pollution etc.). Chemotherapy is one of the most important therapeutic modalities of this diagnosis however, despite significant advances in the field of contemporary medicine and a broad portfolio of medicaments in use, the treatment success rate is still insufficient. For these reasons, scientific and pharmaceutical communities have intensively investigated new compounds that could be applied as new drugs for this devastating disease.
Triterpenes - substances abundant in natural sources belong to a group of isoprenoids, compounds made of 6 isoprene building units. They have a wide spectrum of pharmacological activities (Dzubak *et al.*, 2006), of which the most noteworthy are: antiviral, anti-inflammatory, antiulcerogenic, antimicrobial, anticariogenic and most importantly anticancer activity. Betulinic acid (1) and many other triterpenes have been known over a century, however, until recently their selective cytotoxic activity against human melanoma cells was undiscovered (Cichewicz & Kouzi, 2004; Eiznhamer & Xu, 2004; Salvador, 2010). The extract of *Vauquelinia corymbosa* (Fig. 1) showed significant cytotoxic activity against a cell line of lymphocytic leukemia P-388 in the work of Trumbull by 1976 and betulinic acid (1) along with uvaol and ursolic acid were supposed to be responsible for it (Trumbull *et al.*, 1976). Despite that, the fundamental study of selective cytotoxic activity of acid 1 against human melanoma cells MEL-1 with IC_{50} values in the range between 0.5 - 1.5 μg/mL was

reported by Pisha and his team (Pisha *et al.*, 1995) incredible 19 years after Trumbull's paper. A large number of natural triterpenes have been isolated and many more semi-synthetic compounds have been prepared from them since then. Plenty of these derivatives also showed cytotoxic activity against melanoma cells (Salvador, 2010).
In this chapter (**Isolated pentacyclic triterpenes** and **Semi-synthetic triterpenoids**), there will be a summary of representatives from both natural and semi-synthetic lupane triterpenoids with cytotoxic activity against melanoma cells that were studied during the past fifteen years. Current knowledge of the mechanisms of action of the leading representatives, results and aspects of *in vivo* tests and clinical trials will be discussed here. Betulinic acid (1) will be given the most attention as it is historically the first known triterpene with activity against melanoma. Betulinic acid (1) is currently in the second phase of clinical trials for treatment of dysplastic nevus and therefore, it has a high potential to be used in future clinical practice.

Fig. 1. *Vauquelinia corymbosa* (Photo: Dr. Carlos Gerardo Velazco Macías)

2. Anticancer effects of triterpenoids used for treatment of melanoma

To date, several hundred triterpenoid compounds with significant *in vitro* cytotoxic activity against a variety of cancer cell lines have been found (Salvador, 2010) however; few of them were screened against human melanoma lines such as MEL-1, -2, -3, -4. Most of the triterpenes that actually were tested and showed interesting activity against melanoma cell

lines were derivatives of lupane. That is why this chapter will be focused on lupane triterpenoids with anti-melanoma activity and they will be divided into two groups 1) isolated pentacyclic triterpenoids, meaning they were isolated from a natural source and 2) semi-synthetic triterpenoids that were obtained by modification of natural compounds using chemical reactions or biotransformations.

2.1 Isolated pentacyclic triterpenes

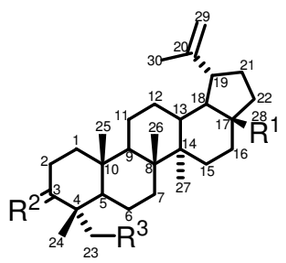
Natural appearance and properties of betulinic acid (1)

Betulinic acid (1) is a natural triterpene with extraordinarily high activity against human melanoma and is found throughout the plant kingdom (e.g. genus *Betulla, Ziziphus, Syzigium, Diospiros, Paeonia*) (Cichewicz & Kouzi, 2004). When isolated as a pure compound, this natural pentacyclic triterpene appears to be snow-white crystalline odorless and tasteless powder that is almost insoluble in water (Cichewicz & Kouzi, 2004; Glassby, 1982; Simonsen & Ross, 1957).

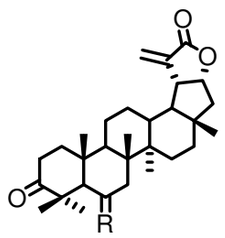
Fig. 2. *Gratiola officinalis* (photo: http://botanika.wendys.cz);

Fig. 3. *Platanus acerifolia* (photo: www.shutterstock.com);

Fig. 4. *Ziziphus Mauritiana* – bark (photo: http://en.wikipedia.org)



1: $R^1 = CO_2H$, R^2 = β-OH, α-H, R^3 = H
2: $R^1 = CO_2H$, R^2 = β-OH, α-H, R^3 = OH
3: $R^1 = CH_2OH$, R^2 = β-OH, α-H, R^3 = H
4: $R^1 = COH$, R^2 = β-OH, α-H, R^3 = H
5: $R^1 = CO_2H$, R^2 = O, R^3 = H



6: R = H, H
7: R = O

Fig. 5.

The first isolation of the compound currently known as betulinic acid (1) was described by Retzlaff in 1902 (Retzlaff, 1902; Simonsen & Ross, 1957), who extracted the yet unknown substance from *Gratiola officinalis* (Fig. 2) which he called "graciolon". In 1925, betulinic acid (1) was independently obtained from the bark of plane trees *Platanus acerifolia* (Fig. 3) by Zellner and Ziffer (Simonsen & Ross, 1957; Zellner & Ziffer, 1925) using either extraction or sublimation. The authors named it after its source "platanolic acid" and did not recognize its true identity. Similarly, the same compound was isolated from *Florida Cornus* (Fig. 6) during the dissertation research of Soliman who, in 1939 first called it "cornolic acid". However, soon he and his co-workers identified it correctly as betulinic acid (1) (Robertson *et al.*, 1939). In 1944, Barton and Jones used physical constants to correctly identify "graciolon" as betulinic acid (1) (Barton & Jones, 1944) and in 1948, Bruckner and co-workers identified (Bruckner *et al.*, 1948) "platanolic acid" as betulinic acid (1) as well. Demonstrating, that betulinic acid (1) is widely spread throughout nature and that its identification was often accompanied with many problems during the 20th century.

Acid 1 is accessible from plane tree and birch bark on an industrial scale since both types of bark contain several percent of it. In addition to the extraction procedures (Cichewicz & Kouzi, 2004; Simonsen & Ross, 1957; Urban *et al.*, 2004), several industrial processes were developed to manufacture (Csuk *et al.*, 2006; Krasutsky *et al.*, 2003, 2006; Pezzuto *et al.*, 2007) betulinic acid (1) from betulin (3), a triterpene far more abundant (up to 30 %) in birch bark (e.g. *Betula pendula*; Fig. 7) than the acid, which makes it far more profitable process. This is especially useful in northern European countries, Russia, Canada, where paper mills mostly use birches to produce cellulose pulp and where over 40 tons of birch bark (Krasutsky, 2005, 2006; Sarek, 2008) is produced daily, with 6 tons a day being burned as a cheap fuel (5-7 USD per ton; 7-11 MJ/kg). Using birch bark as a cheap source of betulin can provide the industry with a less expensive source of betulinic acid (Csuk *et al.*, 2006; Krasutsky, 2003, 2006; Pezzuto, 2007). With these procedures, there should not be a significant problem with manufacturing betulinic acid (1) on a multi ton scale. The best known industrial process to convert betulin (3) into betulinic acid (1) is based on conversion of it into aldehyde 4 (Csuk *et al.*, 2006; Krasutsky *et al.*, 2006a, 2006b, 2006c) followed by oxidation (Csuk *et al.*, 2006; Krasutsky *et al.*, 2006) of aldehyde 4 to betulinic acid (1). Older patented procedures (Pezzuto *et al.*, 2007) that use oxidation of betulin (3) with Jones reagent into betulonic acid (5) followed by selective reduction are not preferable because they use toxic Cr(VI) compounds in large quantities and also a 3α-epimeric product originates as a by-product (Pezzuto, 2007) which makes the isolation and purification far too expensive.

Fig. 6. *Florida cornus* – bark (photo: http://en.wikipedia.org);

Fig. 7. Betula pendula (photo: www.shutterstock.com)

Antitumor activities of acid 1 against human melanoma cells

Despite acid 1 having been isolated in 1902, its cytotoxic effects were not found until the examination of cytotoxicity of various *Vaquelinia corymbosa* (Fig. 1) extracts against lymphocytic leukemia cells P-388 by Trumbull in 1976 (Trumbull *et al.*, 1976). Approximately 19 years later, Pisha and his co-workers (Pisha *et al.*, 1995) published their findings of selective cytotoxic effects of betulinic acid (1) extracted from stem bark of *Ziziphus Mauritian*a (Lamnaceae, Fig. 4) against human melanoma cells Mel-1, -2, -3, -4. Furthermore, this work investigated the mechanism of action by flow-cytometry (Pisha *et al.*, 1995), and it was found that acid 1 induced selective apoptosis of tumor cells Mel-2, which remained in G0/G1 phase. Moreover, induction of apoptosis was evident from the emergence of sub-G1 apoptotic peak (Ap) in DNA histograms and its increase between 56-72 h after application. Direct perturbation of the mitochondria by betulinic acid (1) was observed by (Fulda *et al.*, 1998) and this type of activity was also found when the cancer cells were treated by various triterpene scaffolds without limitation to the melanoma cells. Despite extensive research, the molecular target of betulinic acid (1) has not yet been identified. Only from some published pathway alterations like Bcl-2 and NF-kB modulation and antiangiogenic activity were some speculations about the target able to be made (Selzer *et al.*, 2000, 2002). There is a plethora of information about the activity of potent derivatives of betulinic acid (1) (e.g. NVX-207, PA-457) in *in vitro* and *in vivo* models but it seems to be a reality, that small changes in the chemical structure could lead to significant differences in specificity and mechanisms of action (Keller *et al.*, 2001; Suh *et al.*, 1999; Willmann *et al.*, 2009).

Another advantage of using acid 1 in cancer therapy is its very low toxicity in a Hippocratic screens at doses of 200 and 400 mg per kg body weight in animal models (Pisha *et al.*, 1995). In a study published by Pisha, intraperitoneal application of acid 1 to mice in six doses of 500 mg/kg every fourth day and six doses of 250 mg/kg every third day were accepted without any signs of toxicity (Cichewicz & Kouzi, 2004). In vitro studies showed that the only symptom of high doses of acid 1 was an increase of intracellular-free calcium, and this increase was only associated with a small decrease in cell viability (Cichewicz & Kouzi, 2004). Very low toxicity and significant antitumor activity give acid 1 a very favorable therapeutic index (Cichewicz & Kouzi, 2004). These findings thrust common triterpenes - betulinic acid (1) - to the center of interest within scientific groups studying antitumor activities and mechanisms of action, and pharmaceutical companies interested in chemotherapeutics (e.g. BMS).

Other anticancer activities and studies

Acid 1 has presented promising antitumor activity against a variety of tumor cell lines such as malignant brain-tumor (Fulda *et al*, 1999), neuroectodermal (Zuco *et al.*, 2002), human chronic myelogenous leukemia (CML) 562 (Gopal *et al.*, 2005) and many other cell lines derived from the most prevalent human cancer types (lung, colorectal, breast, prostate and cervical cancer (Kessler *et al.*, 2007; Zuco, 2002). Furthermore selective cytotoxicity against tumor cell lines in comparison with normal cells has been confirmed (Hata *et al.*, 2002).

Pulsatilic acid (2), a compound isolated from *Pulsatilla chinensis* is another example of a triterpene highly cytotoxic against mouse melanoma cell line B16 (Bi *et al.*, 2007; Liu *et al*, 2004; Zhou *et al.*, 2007). Pulsatilic acid (2) is a little less active than acid 1 (53 μmol/L for acid 1 and 83 μmol/L for acid 2) (Bi *at al.*, 2007).

The last type of natural pentacyclic triterpenes active against melanoma are ochraceolides, compounds with modified E-ring. Ochraceolides A (6) and C (7) were isolated together with

other lupane derivatives from *Kokoona ochracea* and their activity against line UISO-MEL-2 was approximately 15 μmol/L (Ngassapa *et al.*, 1991).

Synergisms of acid 1 with cytostatics and other effects.

Animal and cell based studies (Sawada *et al.*, 2004; Selzer *et al.*, 2000; Wachsberger *et al.*, 2002) have focused on investigating the synergic anticancer activities of various doses of acid 1 and vincristine (Fig. 8), radio therapy, lowering pH, or hyperthermia. A significant increase in the efficiency of radio therapy against human melanoma cells was demonstrated when combined with dossing of betulinic acid (1). Response of melanoma cells to betulinic acid (1) alone or in combination with irradiation was studied by Selzer and co-workers (Selzer *et al.*, 2000). Analysis of tumor cell survival following the treatment by acid 1 combined with an application of ionizing γ irradiation (dose of 2 Gy, within the range used in clinical therapy) indicates that betulinic acid (1) and irradiation have an additive effect on the inhibition of colony-forming ability. It is possible that acid 1 can target tumor cells that are resistant to ionizing radiation.

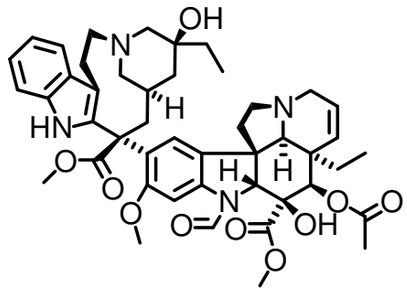


Fig. 8. Vincristine

In another study, Japanese authors looked at the influence of combination of betulinic acid (1) and vincristine on mice melanoma B16F *in vitro* and *in vivo* (Sawada *et al.*, 2004). Based on fast apoptosis of those melanoma cells, the authors suggested an existence of a synergic effect between acid 1 and vincristine, they found, that each substance causes inhibition of cell cycle in different phases: betulinic acid (1) in G1 phase and vincristine in G2/M phase (Sawada *et al.*, 2004). An application of both compounds *in vivo* increased suppression of lung metastasis of melanoma cells in a model C57BL/6 in mice, in comparison to vincristine only. Authors proclaimed that acid 1 is a suitable complement to the chemotherapy of malignant melanoma (Sawada *et al.*, 2004).
Wachsberger *et al* reported an influence of antitumor activity of compound 1 together with application of hyperthermia and lower pH in human melanoma cells DB-1 (Wachsberger *et al.*, 2002). They found that percentage of survived tumor cells during the combined application of acid 1 and hyperthermia of 42°C for two hours decreased with respect of used pH 7.3, 6.7 and 6.3 from 5 %, 9 %, to 2 %.

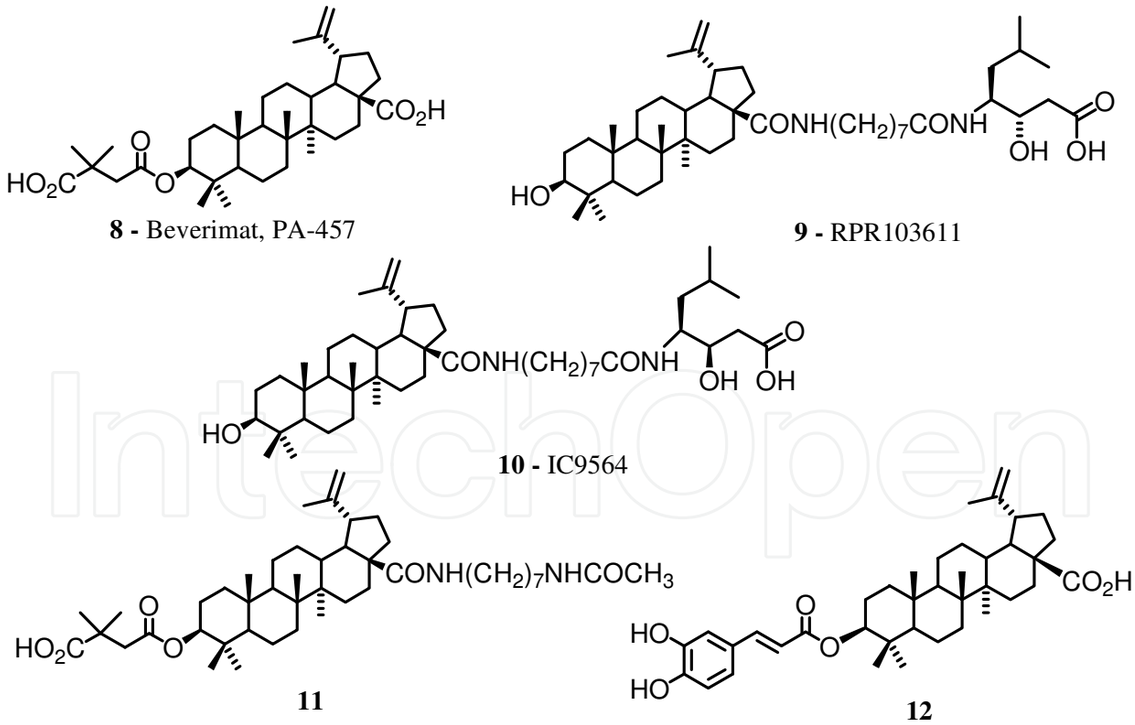
In vivo studies and the clinical trials of betulinic acid (1)

Based on promising *in vitro* and *in vivo* activity of betulinic acid (1) in melanocarcinoma cells and tumors in animal models, human clinical trials were induced. Some considerations were taken in regard to actinic keratosis (AKs), which represents the initial intraepidermal manifestation of keratinocyte abnormal transformation that may potentially progress to small cell carcinoma. In a randomised trial, Huyke (Huyke *et al.*, 2009) treated 45 patients

(with <10 AKs each) either with a topical betulin-based oleogel twice daily, or cryotherapy, or a combination of the two. Treatment with betulin-based oleogel was well-tolerated and showed efficacy in treating AKs. Continued controlled studies on larger sample sizes investigating the use of betulinic acid for the treatment of nonmelanoma skin cancer are warranted. Phase I and II clinical trials evaluating the topical application of betulinic acid (1) in the treatment of dysplastic nevi with moderate to severe dysplasia are currently ongoing (www.clinicalstrials.gov). Clinical trials with acid (1) have not been completed to date.

Overview of other biological activities of acid 1

It should be stated that betulinic acid (1) also has other interesting biological activities, especially, anti-HIV (Fujioka *et al.*, 1994; Mayaux *et al.*, 1994) and anti-inflammatory (Huang *et al.*, 1995; Mukherjee *et al.*, 1997; Recio *et al.*, 1995a, 1995b; Yasukawa *et al.*, 1991). The anti-HIV activity of betulinic acid (1) and its derivatives was first described by two independent research groups (Fujioka *et al.*, 1994; Mayaux *et al.*, 1994). Since then, several researchers prepared, published and often patented a large number of novel derivatives (Jacob *et al.*, 2010; Salzwedel *et al.*, 2010) with significant activity against HIV (EC_{50} < 1 nM). Among these derivatives, three main groups are worth mentioning. First 3β-*O*-acyl derivatives of betulinic acid that inhibit the maturation of HIV virus (Kashiwada *et al.*, 1996). 3β-*O*-3´,3´-dimethylsuccinylbetulinic acid (8), also known as PA-457 or bevirimat is the most important representative of this group.



8 - Beverimat, PA-457

9 - RPR103611

10 - IC9564

11

12

Fig. 9.

The companies Panacos (Salzwedel *et al.*, 2010) and Myriad (Jacob *et al.*, 2010) found bevirimat to be active against HIV in vivo which sent the compound to phase IIa of clinical trials under registry number NCT01026727 (US National Institute of Health). Not only does

Bevirimat (Jacob *et al.*, 2010; Kashiwada *et al.*, 1996; Salzwedel *et al.*, 2010) show very high anti-HIV activity (EC_{50} ~ 0.35 nM) but it also has a very low toxicity (typical for betulinic acid derivatives) which together puts the value of its therapeutic index over 20,000. Another advantage of bevirimat is that to date no resistance to it has been recorded. The second group of anti-HIV active compounds are 28-amides (Evers *et al.*, 1996; Soler *et al.*, 1996; Sun *et al.*, 2002) of betulinic acid that block the entry of the virion into cells. The most important amide derivatives (Holz-Smith *et al.*, 2001; Sun *et al.*, 2002) are compounds 9 and 10 also known as RPR103611 and IC9564. The third group is represented by compounds that combine both of the previously mentioned mechanizms of activity (Huang *et al.*, 2006) because they contain a combination of both pharmacophors – acyl group in the position 3β and 28-amide group. Those compounds were discovered most recently and the most active is ([(N-[3β-*O*-(3´,3´-dimethylsuccinyl)lup-20(29)-en-28-oyl]-7-aminoheptyl)-carbamoyl]methane) (11) which showed activity about 20 times higher than Bevirimat in *in vitro* tests (Huang *et al.*, 2006).

Yasukawa was the first to describe anti-inflammatory activity of betulinic acid (1) (Yasukawa *et al.*, 1991) and relevant inhibition effect against TPA –induced inflammation was found at concentration of 5 μM (Huang *et al.*, 1995; Mukherjee *et al.*, 1997; Recio *et al.*, 1995a, 1995b). Promising anti-inflammatory activity has also been found in 3β–*O*–caffeoyl betulinic acid (12) by Fuchino group (Fuchino *et al.*, 1995, 1996, 1998). This compound is one of the components of birch bark. Last but not least, betulinic acid (1) is commonly used in the cosmetic industry. It is usually used in concentrations of 50-500 mg per gram to prevent and help treat UV-induced skin cancer, to reduce signs of cellulitide and to stimulate collagen synthesis in skin-care products. It's also used to prevent sunlight-caused signs of aging, wrinkles, and blotches and to improve skin homogeneity and pigmentations. (Bradbury *et al.*, 1997a, 1997b, 1997c)

2.2 Semi-synthetic triterpenoids with activity against melanoma

The very poor solubility of acid 1 in water based media (< 1 μmol/L) (Cichewicz & Kouzi, 2004; Symon *et al.*, 2005), high hydrophobicity (*log P*) (Srivastava *et al.*, 2002) and unsuitable pK parameters (absorption, distribution, metabolism and elimination), along with strong antitumor potential (Pisha *et al.*, 1995) and low acute toxicity (Cichewicz & Kouzi, 2004) motivated a number of scientific institutions to synthesize derivatives, pro-drugs and formulations of betulinic acid (1) that could retain the activity and low toxicity, but improve the afore mentioned properties. The following semi-synthetic derivatives might be divided into three groups: simple derivatives of natural triterpenes (2.2.1), derivatives obtained by biotransformation procedures of betulinic acid (1) (2.2.2) and triterpenoids with modification of lupane skeleton (2.2.3).

2.2.1 Derivatives of natural triterpenes (acids 1, 2 and betulin (3))

Several functional groups can be used for the derivatization of acid 1, such as the secondary hydroxyl group in the position 3β, a neopentyl 28-carboxyl moiety in the location 17 and finally, a double bond located between carbons 20 and 29. As both hydroxyl and carboxyllic groups are sterically hindered, common derivatization processes usually don't work very well or fail completely. A typical example is the esterification of 28-carboxylic group. The reaction does not work with alcohols at all; acid catalysis or use of DCC does not help either.

Using alkyl bromides or iodides and alkali carbonates or DBU as the basis in DMF are the most effective conditions to introduce an ester to 28-carboxyl group. Analogously, simple alkali hydrolysis of alkyl betulinates does not work and to release a free acid from its ester, it is necessary to apply drastic conditions such as reflux with potassium hydroxide in ethylene glycol or nucleophilic deprotection using lithium iodide in collidines under reflux. For the purposes of this chapter, the compounds that are modified at more than one site of the skeleton are classified according to significance of the modification. Also, many cytotoxic compounds with no data about their activity on melanoma lines are not included in this chapter.

Derivatization of betulinic (1) and pulsatillic acid (2) in position 28

Cytotoxicity of various types of esters of betulinic acid (1) is very well researched. In the past, researchers found that the cytotoxicity of betulinic acid decreases by forming alkyl or aryl esters from 28-carboxylic group. (Kim *et al.*, 1998, 2001; Kvasnica *et al.*, 2005; Urban *et al.*, 2004, 2005). One of the explanations of this fact is that free carboxyl group is responsible for the activity and the hydrolysis of its esters is extremely difficult and requires extreme conditions which are caused by sterical hinderance of the neopentyl-type group. Curiously, difficulties with the hydrolysis were not observed at similar β,γ-unsaturated esters (*e.g.* compound 13, Fig. 10) (Sarek *et al.*, 2005) which suggests that a different reaction mechanism might exist to cleave them, so those difficulties are probably specific for alkyl and aryl esters and for acids with unmodified E-ring. Very detailed studies of the dependency of cytotoxicity on the type of the ester in position 28 of betulinic acid (1) have been published in literature (Kvasnica *et al.*, 2005; Urban *et al.*, 2005) The authors demonstrated that methyl, ethyl, and benzyl esters are an order of magnitude less active than the free acid 1.

A different situation was observed at substituted alkyl esters of acid 1 and 2 (Bi *et al.*, 2007; Urban *et al.*, 2005). Urban *et al.* compared the cytotoxicity of methyl-, pivaloyloxymethyl-, and acetoxymethyl- esters of betulinic and betulonic acid 15 - 22 on broad scale of cancer cell lines. They found that while methyl- and pivaloyloxymethyl- group significantly decrease the activity, the Acm-esters were either as active as the starting acids or better. The Acm group is therefore a suitable derivatization group that could possibly be useful to synthesize prodrugs. Another good property of Acm is that it increases hydrophilicity.

A completely new type of esters was published by Effenberger *et al.* who synthesized esters from a variety of monoterpenes, sesquiterpenes, and betulinic acid with carboxylic and hydroxyethylene derivatives of thymoquinone (Effenberger *et al.*, 2010). However, 28-esteric derivative 23 was about 7 times less active against the cell line 518A2 than the free acid 1 (Effenberger *et al.*, 2010). A SARS study done by Bi *et al.* who investigated the activity of alkenyl, alkynyl and hydroxyalkyl esters of pulsatillic acid 24 - 29 against several tumor cell lines. The authors included *in vivo* experiments using the most active allyl ester 24 and the starting acid 2 on mice in H22 and B16 models. The *in vitro* experiments showed that all prepared esters were more active than the starting acid 2 against line B16 and also that 6-hydroxyethylester was only slightly better than the acid 2 on the same line. *In vivo* experiments brought even more exciting results when they showed that allyl pulsatillate 24 (applied as intraperitoneal DMSO solution) caused a larger reduction of the mass of a tumor than the free acid 2. Its activity was actually comparable to cyclophosphamide which is a commercially used cytostatic.

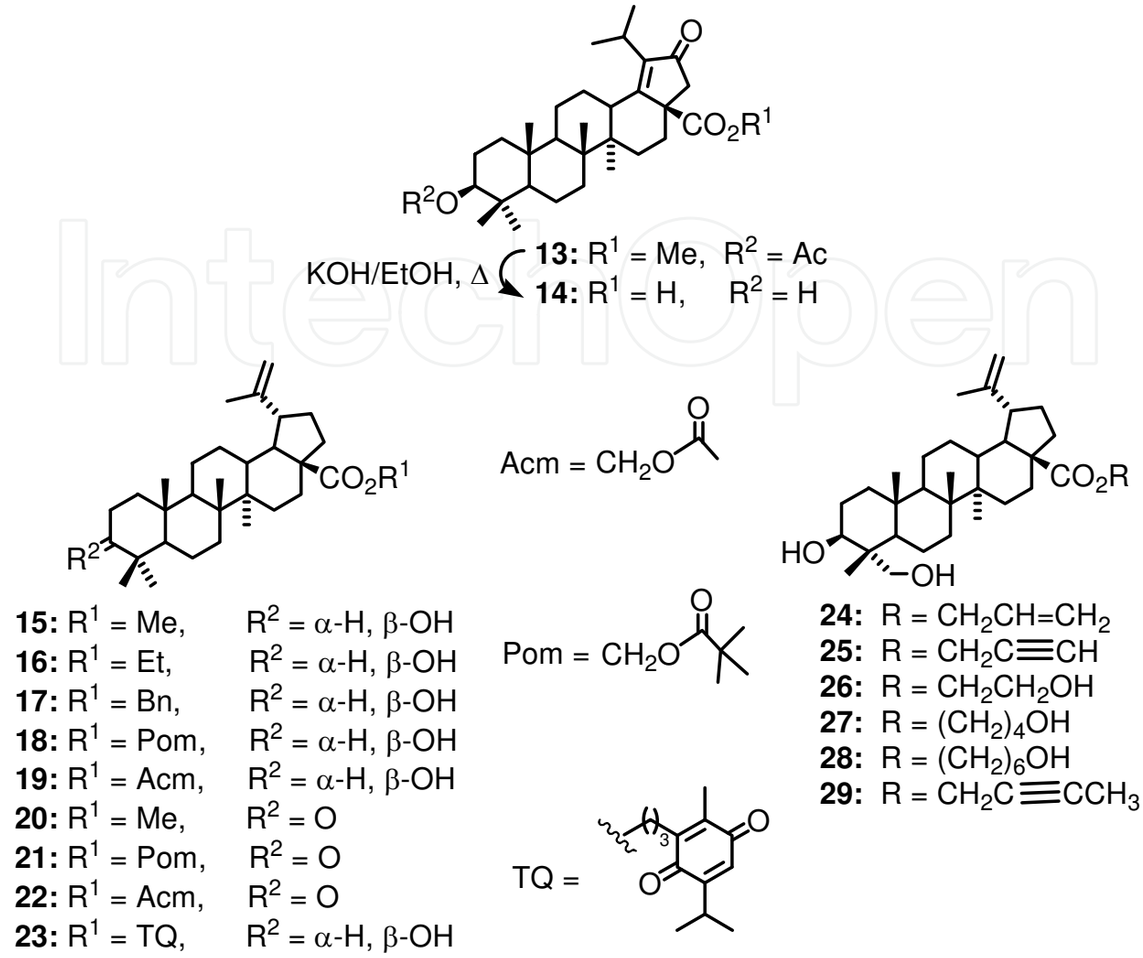


Fig. 10.

Willmann *et al.* brought even more promising results using the compound 30 - NVX-207 that is an ester of acetylbetulinic acid with tris(hydroxymethyl)aminomethane (TRIS). The compound was highly active *in vitro* against 15 cancer cell lines including human melanoma 518A2 and the activity was between 2.6 μmol/L on 518A2 and 5.6 μmol/L on fibrosarcoma HAT 1080. Those initial experiments were followed by a phase I/II animal clinical study on canine patients with spontaneous currently untreatable tumors using intralesional and surrounding tissue application of NVX-207. Five dogs bearing tumors of different histogenetic origine (squamous cell carcinoma, soft tissue sarcoma, mammary carcinoma and adenocarcinoma of the scent gland of the paw) were treated. The complete response to the therapy was observed in two dogs, almost complete remission (90% reduction), stable disease (50% response) and 30% percent reduction was observed in the remaining cases. No systemic side effects and minor local side effects (mild local discomfort) after infiltration of the tumors were observed (Willmann *et al.*, 2009).

Another large group of activated esters are esters with sugars, sometimes improperly called glycosides. The cytotoxicity of these compounds has not been studied thoroughly and the few examples in literature (Gauthier *et al.*, 2009a, 2009b; Pakulski *et al.*, 2009) do not show activity against melanoma cell lines. The only conclusion that can be made is that esters

where a terpene is connected to a single saccharide unit are far less active than the free acid. An example of this is - β-D-glucopyranosyl betulinate (31) (Gauthier *et al.*, 2009a, 2009b) which has activity lower than the activity of betulinic acid (1) by an order of magnitude. Despite this, a study which researched the cytotoxicity of bidesmosidic saponines showed that the saccharide component bound as an ester is an important part of the pharmacophore because its removal caused significant drop of activity. The study contained pulsatillic glycosides 32 isolated from *Schefflera rotundifolia* (Cioffi *et al.*, 2003).

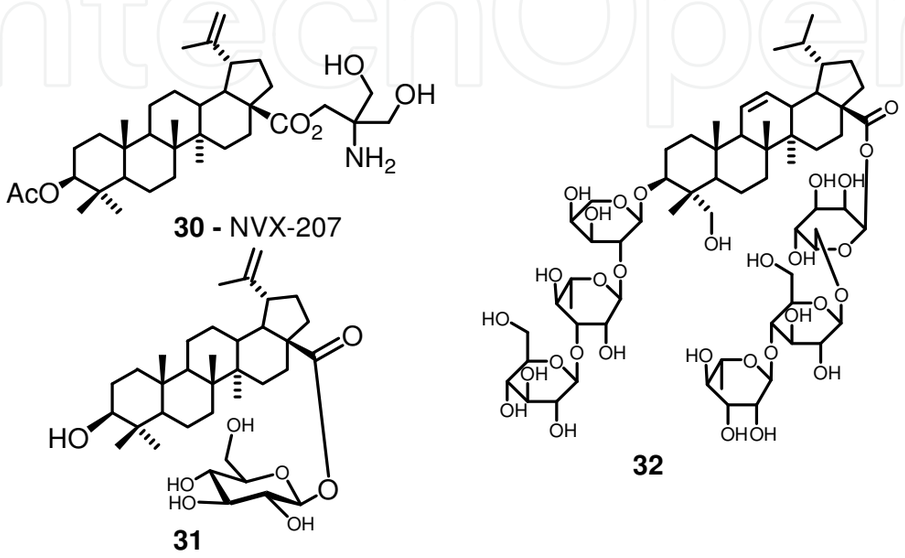
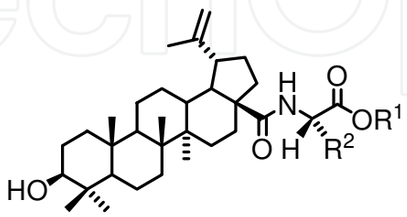


Fig. 11.

Amides represent another type of carboxylic acid derivative. Their antitumor activity is usually high and their hydrophilicity makes their use in *in vivo* tests very easy (Jeong *et al.*, 1999; Willmann *et al.*, 2009). Jeong published a very complex SARS study about the cytotoxic activity of conjugates of betulinic acid (1) with natural amino acids along with a toxicity study on fibroblasts and basic studies of solubility. The best results against MEL-2 cell line were found using conjugates of betulinic acid with alanine - 33 and 35; although the compounds were somewhat toxic against fibroblasts. While the conjugates of betulinic acid with valine 34, 37 and glycine 36 were less active than those with alanine 33 and 35; they were completely non toxic. The glycine conjugate 36 was also the most hydrophilic of the studied compounds. The authors tested the solubility of the conjugates with a very simple method. They prepared solutions of each studied compound in DMSO and kept diluting those solutions with water until they started to precipitate. While the most active alanine derivative 33 could be diluted up to 50 times, the glycine conjugate 36 could be diluted 100 times. Despite the fact, that the cytotoxicity of the glycine conjugate 36 was the same as the activity of free betulinic acid (1) (4.2 μmol/L), the conjugate 36 is far more soluble in water and that makes it a very suitable candidate for *in vivo* testing.

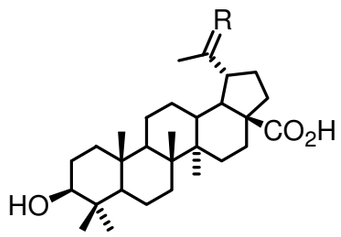
Another type of derivative that showed cytotoxicity against melanoma lines was quaternary ammonium salts (Biedermann *et al.*, 2010; Sarek *et al.*, 2008). The strong antitumor activity of quaternary ammonium salts of four terpenic acids (betulinic (1), dihydrobetulinic (38), platanic (39) and 21-oxoacid 14) on a panel of 11 cancer cell lines has been demonstrated in the literature (Sarek *et al.*, 2008). The salts were obtained by a two step reaction of a terpenic

acid with 1,2-dibromoethane followed by a quaternization with a corresponding amine (trimethylamine, triethylamine, pyridine). The best cytotoxicity was obtained with quaternary salts of triethylamine - compound 40 and 41. The dihydrobetulinic salt 41 is currently being tested on melanoma lines and has shown activity between 1.6 μmol/L against the line UAAC 62 and 14.6 μmol/L against the line M14 (see Tab. 4). Salts 40 and 41 are also very hydrophilic which makes it easy to dissolve them (55 mg/mL of vehiculum) in media based on water and moreover, their oral accessibility makes them ideal candidates for *in vivo* testing on animals.

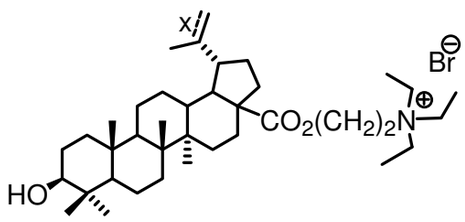


33: R^1 = H, R^2 = Me
34: R^1 = Me, R^2 = $CH(CH_3)_2$
35: R^1 = Me, R^2 = Me
36: R^1 = H, R^2 = H
37: R^1 = H, R^2 = $CH(CH_3)_2$

Fig. 12.



38: R = CH_3,H
39: R = O,



40: x = double bond
41: x = single bond

Fig. 13.

The final group of carboxylic derivatives consists of compounds with aldehydic or hydroxymethyl group in position 17 because they can be prepared by simple reduction step from carboxylic acids. Those compounds are actually more easily synthesized from betulin (3) than from betulinic acid (1). It was shown that the carbonyl group (aldehyde or carboxylic acid) is essential for cytotoxicity because while aldehydes are active, 28-hydroxyderivatives are generally not. This fact was well documented in the work (Hata *et al*., 2003) in which betulinal (4) had activity 10.6 μmol/L, and acid 1 6.5 μmol/L against melanoma cell line SK-MEL-2. However, if another part of the molecule of 28-hydroxyderivatives is modified, the activity may increase. The best examples are glycosides of betulin (3) that are very active on melanoma B-16F, especially when position 3β was glycosylated. Curiously, 28-*O*-glycosides were inactive (Gauthier *et al*., 2006). Among β-D-glucoside, α-D-arabinoside, and α-L-rhamnoside 42 – 44 (Fig. 13), the last listed was the most

active against B16-F1 cell line (18 μmol/L, which is just slightly worse than acid 1 with an activity of 16 μmol/L) (Gauthier *et al.*, 2006). There are no results for bisdesmosidic saponines on melanoma cell lines, however, work of Gauthier describes similar trend on another lines (most active is betulin-3β,28-*O*-bis(α-L-rhamnoside) 45 (Gauthier *et al.*, 2009a).

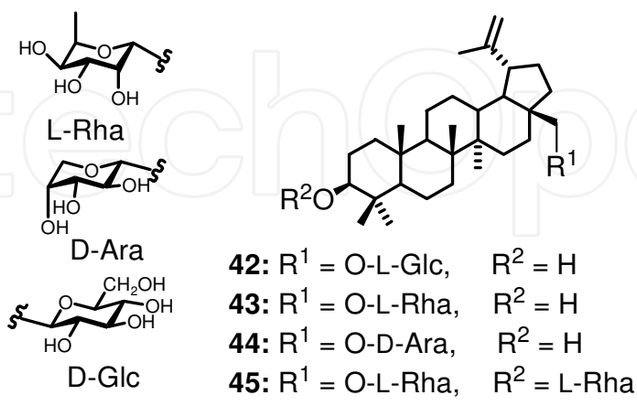


Fig. 14.

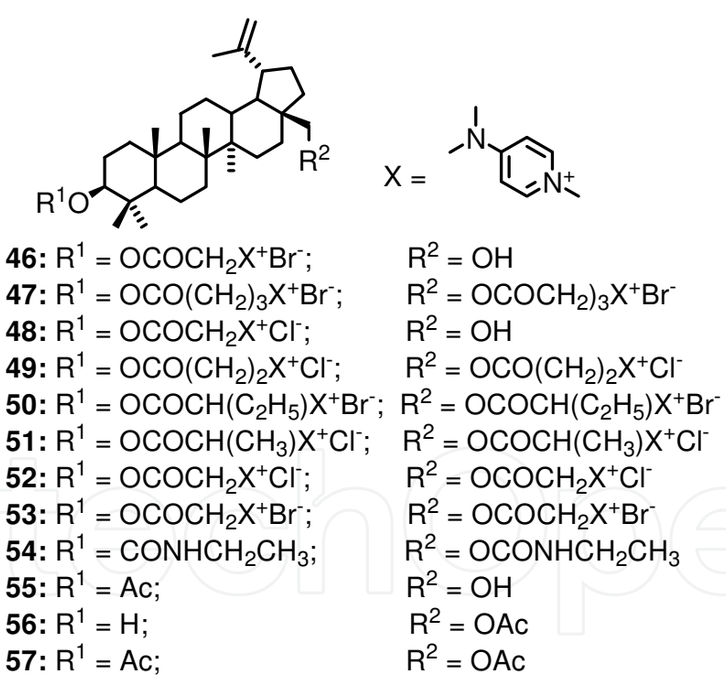


Fig. 15.

Dimethylaminopyridine quaternary ammonium derivatives of betulin 46 – 53 showed strong activities of 0.3 - 2.6 μmol/L on cell lines WM3211 a WM793 and again, the 3β,28-bisfunctional derivatives were more active than the monofunctional (Holy *et al.*, 2010). Also bis(carbamoyl) derivatives had strong activity against 518A2 when bis(ethylcarbamate) 54 was almost as active as betulinic acid (1) (about 8 μmol/L). Common acyl derivatives such as 55 - 57 acetates cause a complete extinction of any cytotoxic activity (Gauthier *et al.*, 2006).

Derivatization of betulinic acid (1) and betulin (3) in position 3β

This heterogeneous group of derivatives contains C-3 glycosides, 3β-*O*-acyl derivatives, 3-oxo compounds and their derivatives (ketones, oximes), and products of reduction of nitrogen derivatives (3β-amines). As already mentioned in the previous chapter, antitumor activity of triterpenoid saponins is relatively well explored (Gauthier *et al.*, 2006, 2009a, 2009b; Pakulski *et al.*, 2009; Thibeault *et al.*, 2007) however there is not much data for the melanoma line (Gauthier *et al.*, 2006; Pakulski *et al.*, 2009).

The situation with betulinic acid 3β-glycosides reminds the situation with esters of betulinic acid (1) with sugars. 3β-*O*-α-L-rhamnopyranoside 59 is the most active glycoside with activity of 3.9 μmol/L against metastatic murine melanoma line B-16F1. Glycoside 59 is approximately 4 times more active than acid 1 itself (Gauthier *et al.*, 2006). Approximately the same activity as betulinic acid (1) was found in case of its 3β-*O*-α-D-arabinopyranoside 60, while 3β-*O*-β-D-glucopyranoside 58 was 2 times less active than the 3β-*O*-α-L-rhamnopyranoside 59 (Gauthier *et al.*, 2006). In the same work, an inverse relation for glycosides of methyl betulinate (15) is also documented; 3β-*O*-β-glucopyranoside 58 was the most active from them. (Gauthier *et al.*, 2006)

(Pakulski *et al.*, 2009) investigated the antitumor efficacy of mannopyranosides and 3,6-branched trimannopyranosides, which were stereoselectively prepared from the natural triterepenes – betulin (3), and betulinic acid (1). Although it is a synthetically attractive method, the mannosylation did not bring any significant effect against melanoma. 3β-*O*-α-D-mannopyranoside 61 and 28-*O*-α-D-mannopyranoside 62 showed very poor results against malign melanoma line G 361 and trimannopyranosides were not active as well.

2-Deoxygalactosides and 2-deoxyglucosides (Sarek *et al.*, 2009), prepared from a large group of highly oxidized lupane hydroxyderivatives by stereospecific additions of corresponding glycals, have significant antitumor activity. The above mentioned 2-deoxyglucosides were effective against a wide range of cancer cell lines, including MDR (Sarek *et al.*, 2009). Recently, we tested the anticancer effectiveness of derivative 64 against melanoma lines where activity reached approximately 20 μmol/L (Tab. 4). This group of saponins is promising because peracetylated analogues have an order of magnitude better activity than free 2-deoxyglycosides (Sarek *et al.*, 2009). 2-deoxyglycosides are very hydrophilic in character which makes them readily soluble in aqueous media and easily applicable in *in vivo* conditions, e.g. compound 64 up to 68 mg/mL (Sarek *et al.*, 2007). It was shown on mice that 2-deoxyglycosides are orally bioavailable which is possibly result of their hydrophilicity (Sarek *et al.*, 2007), and that makes them promising candidates for anticancer drugs. An example of a natural highly active glycoside of betulinic acid is 3β-*O*-α-L-arabinopyranoside 63 isolated from *Schefflera rotundifolia*, which has an activity of 0.55 μmol/L against mouse fibrosarcoma line and also shows a combination of glycoside and saccharide ester in molecule.

Acyl derivatives of betulinic acid (1) and betulin (3) are a large group of semisynthetic derivatives known especially for their antiviral effects while their cytotoxicity is much lower than that of hydroxyderivatives. This fact dramatically improves their therapeutic index for testing anti-HIV activity (Krasutsky *et al.*, 2006; Fujioka *et al.*, 1994, Mayaux *et al.*, 1994). A comparison of activity of betulinic (1) and dihydrobetulinic acid (38) with their acetates is described in (Kim *et al.*, 2001; Mukherjee *et al.*, 2004). Tests with SK-MEL-2 and M14-MEL lines showed that the acetylation of hydroxyderivatives causes either a small decrease or no change in cytotoxicity (Kim *et al.*, 2001). This fact is in good agreement with the work, where

the influence of the 3β-*O*-acetyl group on cytotoxicity of broader group of triterpenoides 65 – 67 was studied (Mukherjee *et al.*, 2004; Sarek *et al.*, 2005). In the work of Chien *et al.*, the cytotoxicity of natural 3β-*O*-acyl derivatives of betulinic acid 68 – 71 isolated from *Strychnos vanprukii Craib* was investigated against MEL2 line. Cytotoxicity of feruoyl 68, 69 and coumaroyl 70, 71 derivatives was approximately 3 times worse than the free acid 1 (Chien *et al.*, 2004).

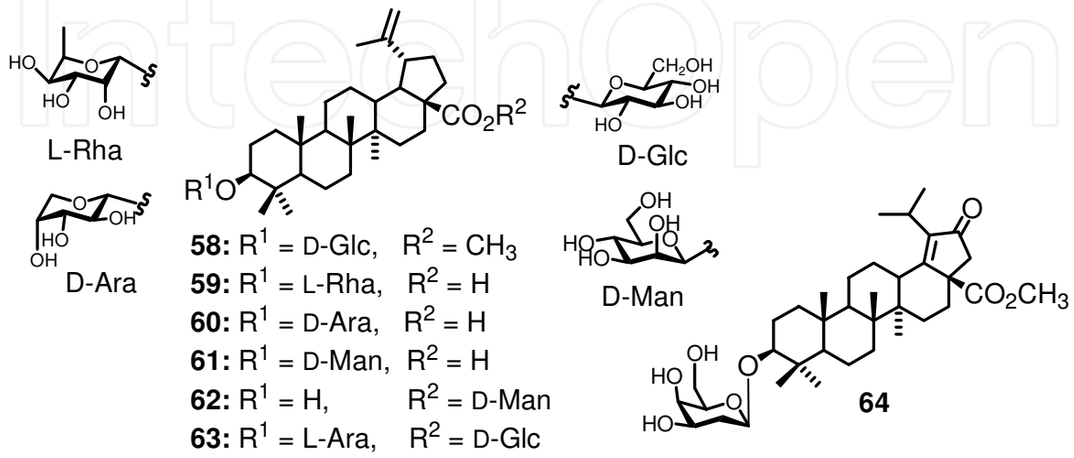


Fig. 16.

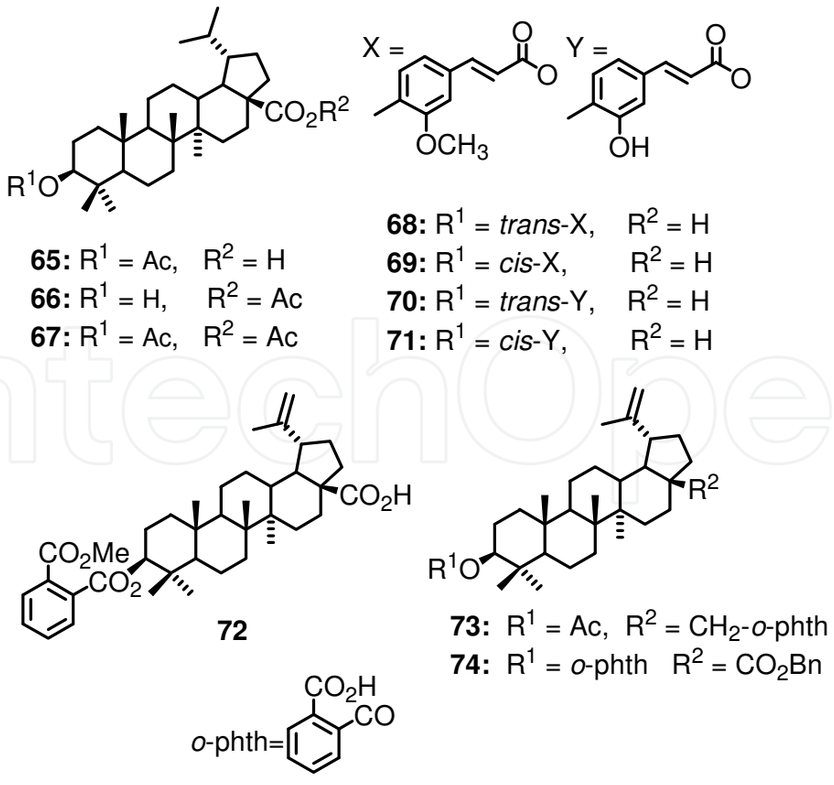
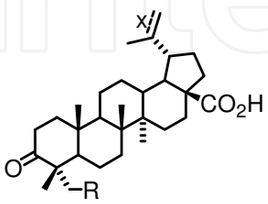


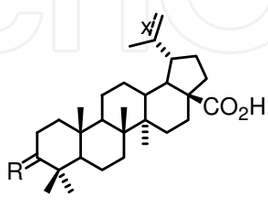
Fig. 17.

The last type of 3β-*O*-acyl derivatives of betulinic acid (1) with described cytotoxicity against the human melanoma line SK MEL2 is represented by phthalates 72 - 74 prepared by (Kvasnica *et al.*, 2005). A SARS study (Kvasnica *et al.*, 2005) of cytotoxicity of hemiphthalates and phthalates of betulinic acid (1) and betulin (3) and their esters against SK MEL2 line shows that the more lipophilic the phthalate ester is, the more active the hemiphthalate is and thus the difference in the activities of the ester is higher. The best activity was reached by derivatives 72 and 74 which was 10 times more active than benzyl betulinate (17) (Fig. 9) itself and a little more active than the free acid 1. Another convenient feature of hemiphthalates is their high hydrophilicity allowing their easy dissolution in aqueous media and their use in *in vivo* tests (Krasutsky *et al.*, 2006; Sarek *et al.*, 2007).

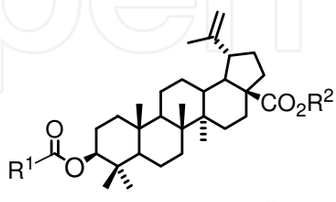
The final group consists of derivatives of 3-oxo acids 5, 75, 76 and their mostly nitrogenous analogues 77 - 81. It is known that oxidation of the 3β-hydroxy group to the oxo group increases the cytotoxicity against the human melanoma line MEL-2. (Kim *et al.*, 1998) While in the pair of betulinic acid (1) - betulonic acid (5) the change is relatively insignificant (1.2 vs. 0.9 μmol/L), the difference is almost an order of magnitude (5.8 vs. 0.7 μmol/L) large in the case of dihydrobetulinic acid (38) - dihydrobetulonic acid (75), which makes dihydrobetulonic acid (75) about twice more effective against MEL-2 than betulinic acid (1) itself (Kim *et al.*, 1998). Analogous results were obtained in cytotoxicity tests of oxidation products of pulsatillic acid (2) (Liu *et al.*, 2004; Zhou *et al.*, 2007). Pulsatillonic acid (76) showed higher activity against B16 line than the original pulsatillic acid (2) (Liu *et al.*, 2004; Zhou *et al.*, 2007). Oxime derivatives of both acids 77 and 80 showed the cytotoxicity against MEL-2 an order of magnitude lower than oxo acids 5 and 75, methyloxime analogues 78 and 81 were two orders of magnitude less active (Kim *et al.*, 1998). In contrast, the 3β-amino analogue 79 obtained by reductive ammination of betulonic acid (5) showed anti-MEL-2 activity comparable with betulinic acid (1) (Kim *et al.*, 1998). The nitrogenous derivatives of betulinic acid (1) in position 3β can also include carbamoyl derivatives 82 – 84 synthesized according to the literature (Kommera *et al.*, 2010). While the 3β-phenylcarbamoyl derivative of betulinic acid - 83 - was active against 518A2, 3β-ethyl carbamates 82, 84 showed activity comparable to or better than betulinic acid (1). Strong antitumor activity against a radial-phase WM3211 and a vertical-phase melanoma WM793 was also found in the case of the quaternary salt 85 with DMAP, with activity 2.6 respectively 5.7 μmol/L.



75: R = H, x = single b.
76: R = OH, x = double b



77: R = NOH, x = double b.
78: R = $NOCH_3$, x = double b.
79: R = β-NH_2, α-H; x = double b.
80: R = NOH, x = single b.
81: R = $NOCH_3$, x = single b.



82: R^1 = $NHCH_3CH_2$, R^2 = H
83: R^1 = NHPh, R^2 = H
84: R^1 = $NHCH_3CH_2$, R^2 = CH_3
85: R^1 = $CH_2X^+Br^-$, R^2 = H
where X see Fig. 15

Fig. 18.

Modification of skeleton of betulinic acid (1) on the isopropenyl side chain

An addition of hydrogen, respectively hydrogenation of 20(29)-double bond, represents the simplest modification on the isopropenyl side chain as well as a simple way to isotopically label the terpenic skeleton with either deuterium or tritium. Although the hydrogenation of a terminal double bond seems easy to be done, experiments show the contrary, because 20(29)-double bond is disubstituted. Under the standard conditions – using hydrogen on Pd/C, it is possible to achieve a selective debenzylation of benzyl betulinate (17), without 20(29)-double bond to be hydrogenated (Kvasnica *et al.*, 2005). As mentioned above, dihydrobetulinic acid (38) is approximately four times less active than betulinic acid (1) against the line MEL-2, whereas the cytotoxicity of oxidized forms, such as dihydrobetulonic (75) and betulonic acid (5) is almost identical. These data show that 20(29) dihydroderivatives are comparably active to their unsaturated analogs (Biedermann *et al.*, 2010; Sarek *et al.*, 2007).

Among the other explored addition reaction to 20(29)-double bond, there is the addition of halogene (Mukherjee *et al.*, 1997) or dihalocarbene (Symon *et al.*, 2005). In the study of Mukherjee's *et al.*, cytotoxic activity (except melanoma lines) of the products of addition of bromine to betulonic (5), betulinic (1), and acetylbetulinic (65) acids (e.g. 86 – 87, Fig. 19) was explored and the best results were achieved using 20,29–dibromo-3β-*O*-acetylbetulinic acid (87) which activity was about the same as betulinic acid (1). A Russian team tested the activity of formerly prepared addition adducts 88 and 89 of dichloro- and dibromocarbene to betulinic acid (1) against human melanoma cells Colo 38 and Bro.92. The best results were achieved with a dichloroderivative 88 with the activity on Bro line slightly better than the activity of acid 1, nevertheless the authors claimed that the high lipophilicity of synthesized derivatives complicated *in vitro* tests, therefore further tests of the derivatization of these non-polar compounds were suggested (Symon *et al.*, 2005).

Another option to modify the isopropenyl side chain is substitution, respectively allylic oxidation. Introducing alcohol moieties into the location 30 might be carried out either by allylic bromination using NBS followed by nucleofilic substitution (Kim *et al.*, 2001) or allylic oxidation followed with reduction (Biedermann *et al.*, 2005). The most unique compound among these oxygenated derivatives is the unsaturated aldehyde 90, synthesized by allylic oxidation of acid 1 by selenium dioxide. Aldehyde 90 disposes of strong multispectral antitumor activity, including melanoma lines B16, B16F, SK-MEL2 a MEL-3 (9.3, 12.4, 17.7 and 3.7 μmol/L; also see Tab. 4), and except for B16F, it is several times stronger than acid 1 (Sarek *et al.*, 2003). Corresponding allylic alcohol 91 and its methyl ether 92 had a similar acitivity of 20 μmol/L on SK-MEL2 lines (Kim *et al.*, 2001). 3,30-Diamino derivatives synthesized by (Mar *et al.*, 2010) showed strong proapoptotic effect against panel of seven tumors lines, include SK-MEL-2. Derivative 93 was reported to have two times better cytotoxic effect against SK-MEL2 than acid 1 itself and furthermore this compound is highly hydrophilic and therefore it is easily applicable for *in vivo* tests (Mar *et al.*, 2010).

Finally, compounds formed by oxidative cleavage of the double bond 20(29) should be mentioned (Kim *et al.*, 2001). Evaluated anti SK-MEL2 activity of platanic acid (39) (and its derivatives), obtained by the oxidative cleavage of the double bond using ruthenium tetraoxide (which provide mostly products expected from ozonolysis under standard conditions) was published in Kim's study. Platanic acid (39), alcohol 94 and oxime 95 did not show significant cytotoxicity on SK-MEL2 (Kim *et al.*, 2001).

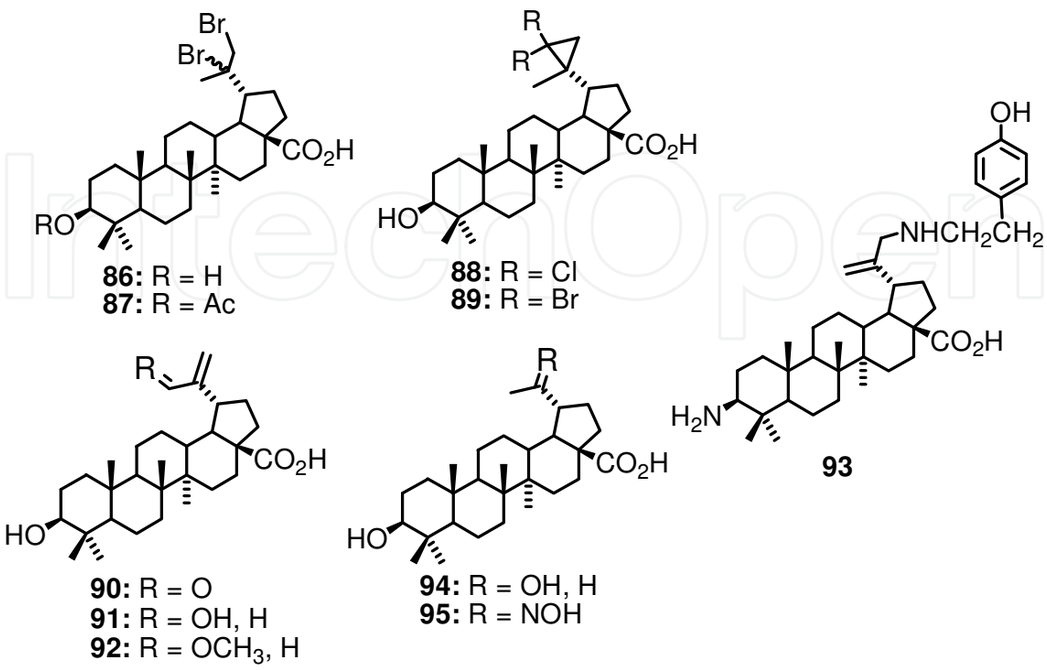


Fig. 19.

2.2.2 Derivatives obtained by biotransformation procedures of betulinic acid (1)

Several papers have described the biotransformation (Chatterjee *et al.*, 1999, 2000; Kouzi *et al.*, 2000) of betulinic acid (1) and the biological activity of those biotransformation products 96 - 102. In Fig. 20, the biotransformation products of different bacterial strains are clearly shown.

The bacterial biotransformation usually produces compounds that would be very difficult to obtain using classical chemical approaches. An example of such compounds is a group of hydroxyderivatives with hydroxyl groups in positions 1, 6, 7, 11 and 15, sometimes the hydroxylation is accompanied by an oxidation in position 3 or an esterification in position 28 by the glucopyranosyl residue. Monohydroxyderivatives 99, 100, as well as betulonic acid (5) showed an order of magnitude better activity during cytotoxicity screening against the Mel-2 cancer line (pleural fluid) than betulinic acid (1) itself, whereas their activity against the Mel-1 cancer line (lymph node) was an order of magnitude worse. 1,7-Dihydroxyderivative 102, 6,7-dihydroxyderivative 98, and 7,15-dihydroxyderivative 96 as well as 7-hydroxyderivative 97 (Kouzi *et al.*, 2000) or glucopyranosyl ester 101 did not show any significant activity against either Mel-1 or Mel-2 cancer lines.

These SARS studies of biotransformation products of acid 1 show that the hydroxylation of the lupane skeleton has a positive impact on antimelanoma activity only if it does not occur in position 7 or 15. Inactivity of β-D-glucopyranosyl ester 101 is possibly caused by the difficulties with hydrolysis of this ester function.

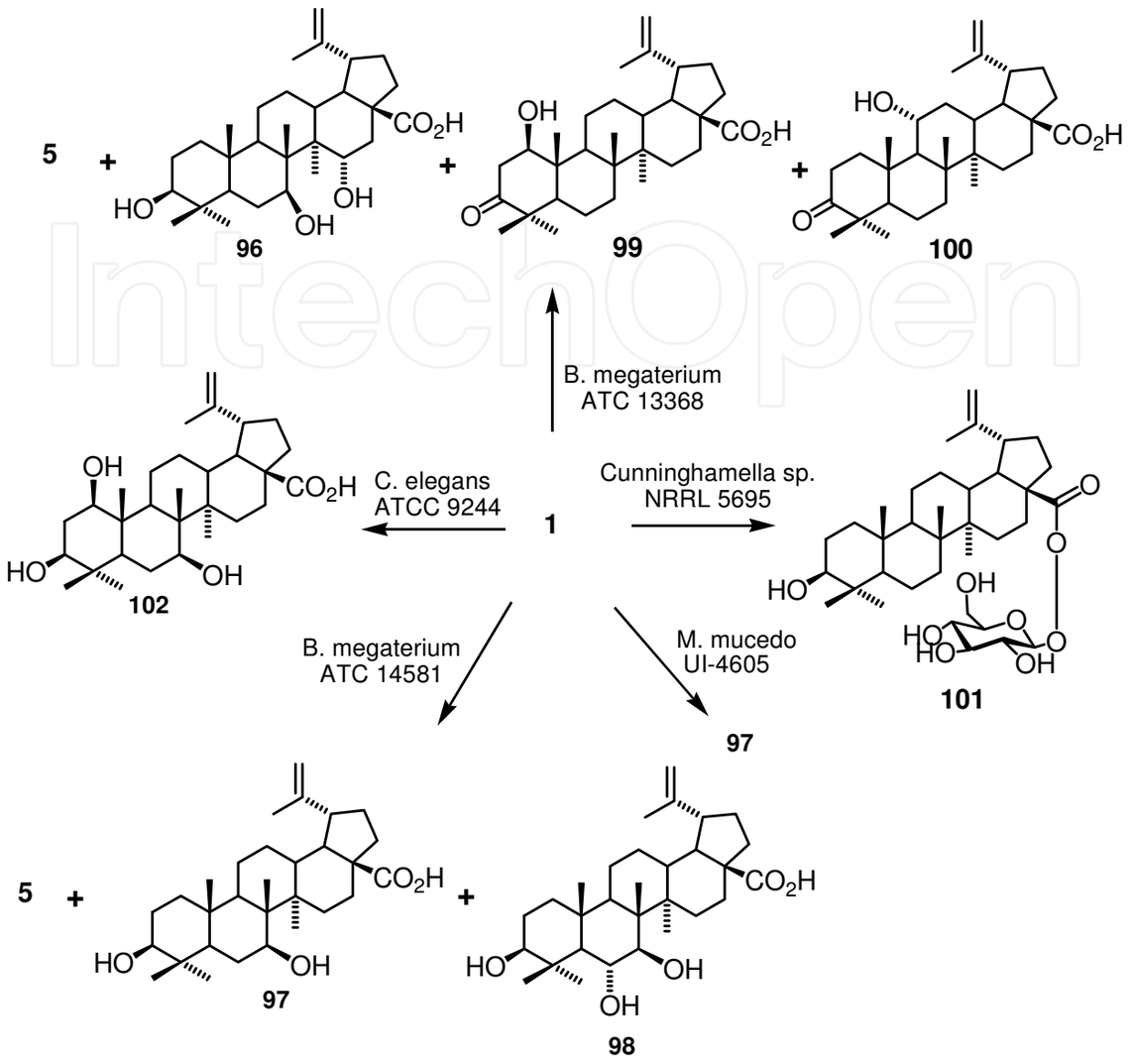


Fig. 20. Biotransformation of betulinic acid (1)

2.2.3 Triterpeoids with modification of lupane skeleton (at ring A or E)

Oxidative modification and cleavage on A-ring of betulinic acid (1)

A detailed study of the cytotoxicity of the products of oxidation of betulinic acid (1) on A-ring was published in paper (Urban *et al.*, 2004). The authors firstly oxidized betulinic acid (1) using chromium(VI) oxide to obtain betulonic acid (5) that was further oxidized with air or oxygen in the presence of potassium tert-butoxide in tert-butylalcohol. That way, either diosphenol 103 or lactol 105 was obtained. Diosphenol 103 was then oxidized with hydrogen peroxide in basic conditions to give A-seco triacid 106, reduction of this triacid afforded A-seco triol 107 and dehydratation of the same triacid gave a 7-membered anhydride 108 (Urban *et al.*, 2004). Curiously, diosphenol 103 only occurs in its enolfom which had not been known before as some literature displays it as a diketone structure that actually does not exist. Derivatives of diosphenol 103, A-seco triol 107 and 7-membered anhydride 108 were the most cytotoxic active compounds from this group. Their

cytotoxicity was firstly measured on five cancer cell lines (none of them melanoma) and the active derivatives of diosphenol 103 was then tested on three more lines including human melanoma SK-Mel2. More recent tests of the diosphenol derivatives 104 against three melanoma lines in groups of Sarek and Hajduch resulted in activity about 7-10 times worse than betulinic acid (1). Tab 4.

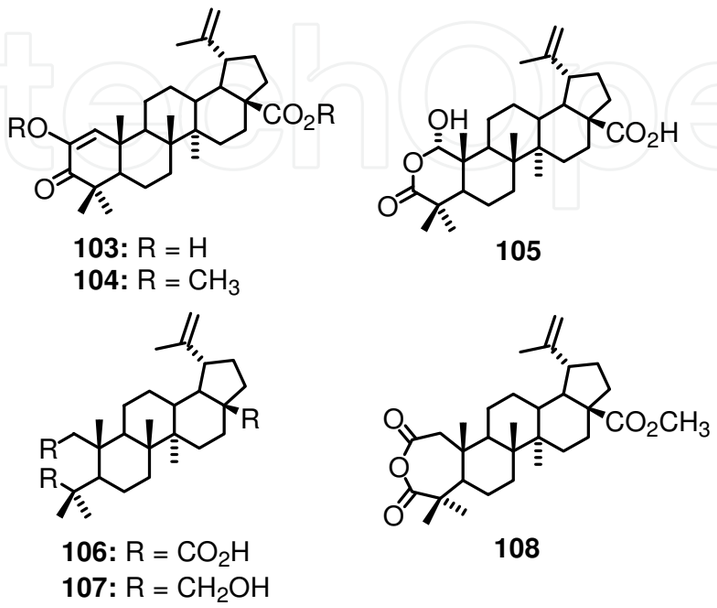


Fig. 21.

Condensation reactions at A-ring

This special chapter will discuss aldehydes and β-ketoesters, the products of Claisen condensation of triterpenoid 3-oxoderivatives with formates and carbonates. Both aldehydes and β-ketoesters are in their enolforms probably due to the boat conformation of the A-ring. Those β-dicarbonyl compounds are a good starting material for synthesis of various heterocycles and for many other substitution reactions. A very complex evaluation of activity of A-homo derivatives was published in work (You *et al.*, 2003). Probably the most interesting was 109 a cytotoxic lupane analogue of a promising anticancer drug CDDO, mainly because it's structural motif seems to be the universal pharmacophor of the cytotoxicity in pentacyclic triterpenes (Suh *et al.*, 1999; You *et al.*, 2003). This derivative is the most active compound from a group of three very promising structures – chloroderivative 110, aldehyde 111, and nitrile 109, which activities against melanoma cell lines SK-MEL-2 a B16-F10 were an order of magnitude better than the activity of betulinic acid (1) (You *et al.*, 2003). These compounds are actually also an exemption from the rule that the 28-alkyl esters are inactive because prepared methyl esters are almost as active as free acids (You *et al.*, 2003).

Heterocyclic derivatives are another type of compounds with homologous carbon atoms bound to the A-ring of the terpenic skeleton (Kumar *et al.*, 2008; Urban *et al.*, 2007; You *et al.*, 2003). You *et al.* published results of the testing of cytotoxic activity of four isoxazole derivatives 112 – 115, prepared by a condensation of aldehydes with hydroxylamine against cell lines SK-MEL-2 a B16-F10. The isoxazoles were strongly cytotoxic only when 28-carboxylic acid was free (compounds 112 and 114). In an article (Urban *et al.*, 2007), a

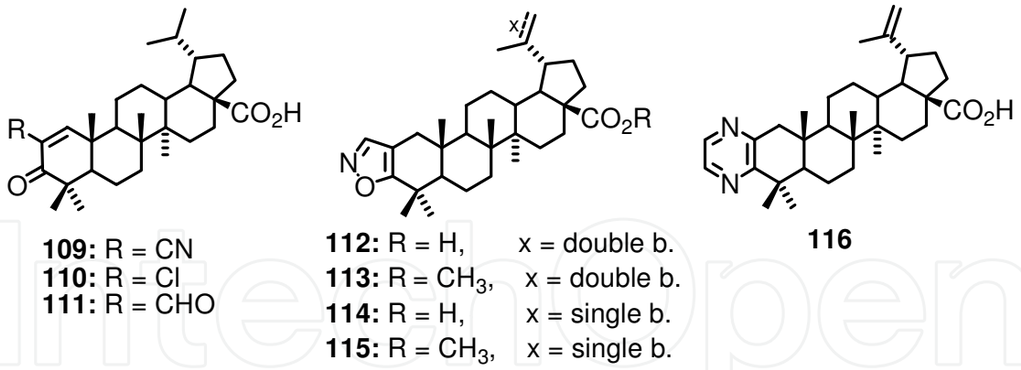


Fig. 22.

synthesis of triterpenoid pyrazines from betulonic (5) and dihydrobetulonic acid (75) and their cytotoxic activity on 9 cancer cell lines including SK-MEL2 was published. The compounds were obtained by a reaction of 3-oxoacids with ethylenediamine, followed by aromatization of the intermediate dihydropyrazine (Urban *et al*., 2007). Despite the fact that these pyrazines (e.g. 116) were more cytotoxic against many cancer cell lines than betulinic acid (1), they were four fold less active against the melanoma cell line SK-MEL2. A conclusion is ambiguous, a 2,3 annealing of a pyrazine cycle to the lupane skeleton increases the cytotoxic activity against some cancer cell lines while vanishes the activity against others, such as melanoma (Urban *et al*., 2007). Analogous quinoxalines (benzopyrazines) were inactive at all. Urban *et al.* also confirmed that there is a strong dependence of the cytotoxicity on the type of ester at 28 carboxylic group. As mentioned before, the Acm esters were generally as active as free acids. Another kind of heterocycles were described in work (Kumar *et al*., 2008), who prepared and performed a large SARS study with 2,3-annealed indole derivatives of betulinic (1) and betulonic acid (5), prepared by Fisher reaction. The article contains over 30 indole derivatives that were tested on a panel of 8 cancer cell lines, however, not including melanoma. Despite that, a general conclusion can be made that an introduction of the indol heterocycle to a terpene improves the cytotoxicity when both N-1 and C-28 positions remain free. An exception from this assumption is the most active derivative 117 (Kumar *et al*., 2008) which is conjugated with glycine through C-28 but that is not surprising with respect to high activity of compound 36.

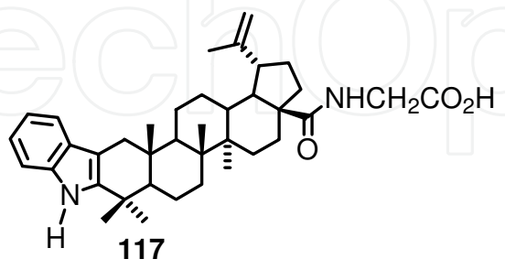


Fig. 23.

Modification of the ring E

First semi-synthetic anticancer lupane and 18,19-seco lupane derivatives with modified ring E (referred to as Betulinines) to be published were compounds derived from 18-lupene that contained either intact E-ring and oxygen functional groups in the positions 21 and 22,

or cleaved E-ring and 18,19-diketone system (Sarek *et al.*, 2003). The group of Betulinines contains several compounds with significant antitumor activity against wide range of cancer cell lines, including MDR and melanoma lines (SK-MEL-2 MEL-3, B-16, B-16F), diketones 118, and 119 and triketone 126 were among the best. Diketone 118, which is on the other tumor lines much more active than the acid 1, has shown activity in melanoma lines about three times worse than acid 1; Tab. 2, 4 (Sarek *et al.*, 2003). Compounds with pyrazine and quinoxaline heterocycle condensed to the E-ring were not as active as compounds 118, 119, and 126 (Urban *et al.*, 2007). Last but not least, we should mention quaternary ammonium salt 120 was the most active.

Taraxastane derivatives - heterobetulinic acid (121) and heterobetulonic acid (122) should be formally included in this chapter. The acid 121 was firstly isolated from *Calyptranthes pallens*, (Lobo-Echeverri *et al.*, 2005) but it is also available synthetically from betulin (3) in five steps (Bradbury & Mingjun, 2007), which include a rearrangement of lupane skeleton to taraxastane. Heterobetulinic acid (121) showed antitumor activity against melanoma lines M14 UACC62 and SK MEL 5 comparable to or slightly better than that of betulinic acid (1), Tab. 4.

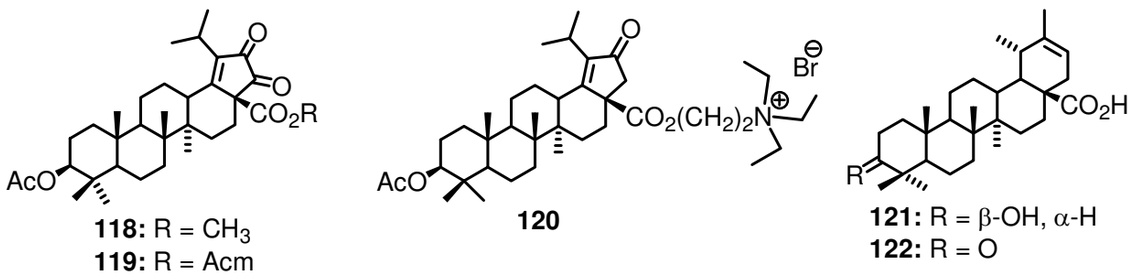


Fig. 24.

Des-E lupane derivatives

Des-E lupane derivatives represent the ultimate synthetic step in the oxidative cleavage/degradation of the ring E of the 21,22-dioxolupane compounds using ruthenium(VIII) oxide. (Sarek *et al.*, 2003) described several des-E-lupane compounds, most important are the γ-keto and β-keto acids 123, 124 and methyleneketone 125. They are also named Betulinines. β-Keto acid 124 (also called JS8) is the most promising anticancer drug within this group, based on the values of cytotoxic activity against more than 30 lines including MDR (the cytotoxicity was 1.28 μmol/L for MEL-3, 4.3 μmol/L for M14, and 1.16 μmol/L for SK-MEL-2; Tab. 4) (Sarek *et al.*, 2003). It is essential to mention that, according to data from flow cytometry, JS8 causes selective apoptosis of tumor cells with rate comparable to the commercial cytostatic diterpenic drug paclitaxel. It was found that JS8 has a unique and new mechanism of action with primary target located in mitochondria. Compound JS8 is also very interesting from the chemical point of view, because it is a β-keto acid that are known to be extremely unstable because they tend to decarboxylate spontaneously. Despite that, JS8 is relatively stable compound when stored at reduced temperature and it is probably due to a significant steric hindrance of the labile carboxyl group. As a tetracyclic pentanorlupane derivative, JS8 is very hydrophilic in comparison to the full-sized triterpenes, and so it is readily soluble in aqueous media (maximum solubility is 68 mg/mL) (Sarek *et al.*, 2007). In conclusion, JS8 is highly antitumor compound, it has favorable solubility for the *in vivo* tests;

its structure, activity and manufacturing procedure has been patented (Fisher *et al.*, 2003) and therefore it has a great chance to become an anticancer drug. Methyleneketone 125, one of the two degradation products of JS8, has similarly strong antitumor properties (activity against M14 was 4.3 μmol/L and against SK-MEL 5 was 3.4 μmol/L; Tab. 4) (Sarek *et al.*, 2007).

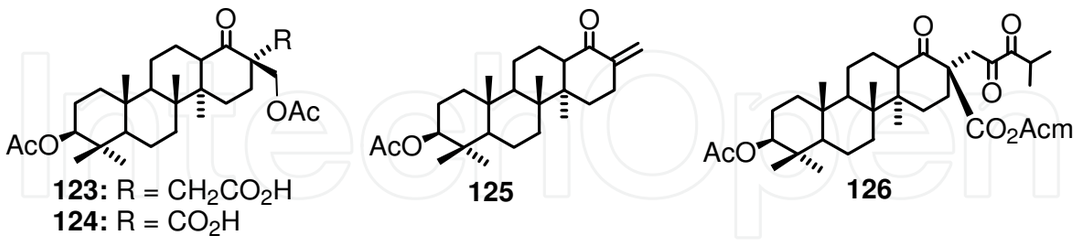


Fig. 25.

3. Conclusions and future directions

Despite the fact that betulinic acid (**1**) was first isolated in 1902, it took another forty years to solve its structure and to identify that several compounds obtained from natural sources by other research groups were identical to it. Trumbull *et al.* was the first to discover the interesting cytotoxic activity of betulinic acid (**1**) in 1976 when he studied a chloroform extract of *Vauquelinia corymbosa* against lymphocytic leukemia cells anti-P-388. He suggested that uvaol, ursolic acid, and betulinic acid (**1**) are the components responsible for the cytotoxicity. After that, it took another 19 years until Pisha *et al.* confirmed that the activity is caused by betulinic acid (**1**) and these authors were first to report that the cytotoxicity is selective against human melanoma cells. This fact sparked intensive research and gave the oncologists hope for the birth of a new drug to combat the insidious disease with steadily increasing incidence.

During early 90th of the last century, the scientists stood at the starting line – they had a natural substance with a quite complicated structure and available only in limited quantities from precious natural sources, however, the compound had strong and selective activity against melanoma and very low toxicity. The main disadvantage seemed to be its unfavorable pharmacokinetic parameters. After solving the difficulties with the availability of acid **1** by developing its synthetic procedure from betulin (**3**), the use of betulinic acid (**1**) was given a real dimension. As a result, several hundred semi-synthetic lupane analogues were published up today, derivatives of betulinic acid (**1**), pulsatillic acid (2), and betulin (**3**), many of those showed significant anticancer effects. They are more hydrophilic and their pharmacokinetic profile is improved. Some of them were tested *in vitro* on activity against human or murine melanoma cells and three of them were tested *in vivo*.

There are several tens of derivatives with promising activity against melanoma cells *in vitro*; the most significant are artificially modified compounds as pulsatillates **24 - 29**; NVX-207 (**30**); conjugates of acid **1** with amino acids **33**, **36**, **37**; quaternary ammonium salts **40**, **41** and **46 – 53**, **85**; some glycosides (the best showed to be: **32**, **58**, **59** and **64**); hemiphtalates **72**, **74**; carbamate **54**; compounds with modified isopropenyl chain (acid **90**, **93**, methoxyderivative **92** and alcohol **91**); compounds with modified A-ring (most active were compounds that have an EWG group in the location 2 of the 3-oxo lupane skeleton or the EWG with a multiple bond in conjugation with 3-oxo group – the best are **109 – 112**, **114** and **117**); compounds with modified E-ring where active was diketone **118** and heterobetulinic acid (**121**); and finally, the des-E-derivatives JS8 (**124**) and methyleneketone **125**.

Betulinic acid (1) itself is now in phase II of clinical trials against dysplastic nevus, compound NVX-207 (**30**) was tested on dogs with particularly good results and pulsatillate **24** was successfully tested on the mice models. These *in vivo* data together with current industrial availability of betulinic acid (**1**) and a variety of its active derivatives with improved hydrophilicity and a reasonable solubility in water-based media gives this group of natural compounds high potential to become a new generation of cytostatic drugs to combat a malignant melanoma.

4. Tables of activities

Compound	B16	B16-F1	MEL-1	MEL-2
1	53.5	16.1	6.3	1.3
2	83.0	—	—	—
3	—	13.8	—	—
5	—	—	31.8	0.1
13	—	210.1	—	244.1
14	—	—	—	160.0
15	—	26.0	—	—
16	—	—	—	217.9
17	—	—	—	NA
24	80.5	—	—	—
25	72.9	—	—	—
26	80.9	—	—	—
27	66.0	—	—	—
28	41.1	—	—	—
29	63.2	—	—	—
30	—	—	—	—
31	—	—	NA	NA
33	—	—	—	2.9
34	—	—	—	4.1
35	—	—	—	6.8
36	—	—	—	8.0
37	—	—	—	18.1
38	-	-	-	12.7
42	—	>248	—	—
43	—	>228	—	—
44	—	>175	—	—
55	—	—	—	247.0
56	—	—	—	223.7
58	—	7.1	—	—
59	—	3.9	—	—
60	—	11.0	—	—
68	—	—	—	3.4
69	—	—	—	3.4
70	—	—	—	4.2
71	—	—	—	3.7
72	—	—	—	13.4
73	—	—	—	24.9
74	—	—	—	90.0
75	-	-	-	1.5
77	—	—	—	2.4
78	—	—	—	8.3
79	—	—	—	1.3
80	—	—	—	2.2
81	—	—	—	NA

Table 1.

Compound	B16-F1	MEL-1	MEL-2	M14-MEL
91	-	-	18.0	-
92	-	-	20.0	-
93	-	-	3.0	-
96	-	NA	NA	-
97	-	14.1	14.1	-
98	-	21.5	33.3	-
99	-	NA	0.5	-
100	-	NA	0.4	-
102	-	NA	NA	-
103	-	-	23.0	-
109	2.7	-	1.5	-
110	3.0	-	0.21	-
111	0.55	-	0.50	-
112	6.1	-	31.2	-
114	3.1	-	2.2	-
116	-	-	98.0	-
118	67.9	-	65.3	-
119	NA	-	NA	-
123	11.5	-	19.9	-
124	0.4	-	1.2	4.3
125	0.7	-	2.1	-
126	34.3	-	223.7	-

Table 2.

Compound	518A2	Colo38	Bro	WM3211	WM793
15	28.8	—	—	—	—
30	2.6	—	—	—	—
46	—	—	—	0.6	2.5
47	—	—	—	0.7	1.3
48	—	—	—	0.3	1.9
49	—	—	—	0.6	0.8
50	—	—	—	0.3	0.5
51	—	—	—	0.5	0.5
52	—	—	—	0.4	0.4
53	—	—	—	0.5	0.5
54	8.18	—	—	—	—
82	17.8	—	—	—	—
83	51.5	—	—	—	—
84	16.7	—	—	—	—
85	—	—	—	2.6	5.7
87	—	10	10	—	—
88	—	> 10	> 10	—	—

Table 3.

Compound	M14	UACC 62	SK MEL 5
1	5.4	5.0	4.5
38	16.7	22.3	4.7
41	14.6	1.6	2.2
64	21.1	19.0	18.4
75	12.7	12.6	9.3
90	9.2	5.8	6.9
104	54.0	29.8	27.6
116	41.2	14.7	6.3
118	15.8	14.7	11.3
121	6.2	4.6	3.5
122	45.8	13.9	6.4
124	4.9	3.5	3.8
125	4.3	3.5	3.4

Table 4.

Table 1-4: Table 1-3 - cytotoxicity (IC_{50} in μM) of published compounds against melanoma cell lines; Table 4 - cytotoxicity (IC_{50} in μM) selected semisynthetic lupane derivatives against melanoma cell lines which are part of the NCI60 cell lines panel and are therefore well characterized and further studied, e.g. cell line M14 is bearing the mutated p53 protein. NA is not active.

5. Acknowledgment

This study was supported partially by the Czech Science Foundation (305/09/1216 and 301/09/P433), by grant from EHP/Norway (CZ0099) and by the Ministry of Education of the Czech Republic (MSM 6840770040). Special thanks to Dr. Carlos Gerardo Velazco Macías from Áreas Naturales Protegidas, Parques y Vida Silvestre de Nuevo León for providing photos of plants and Andrew Olson for text corrections. The infrastructural part of this project (IMTM) was supported from the Operational Program Research and Development for Innovations (project CZ.1.05/2.1.00/01.0030).

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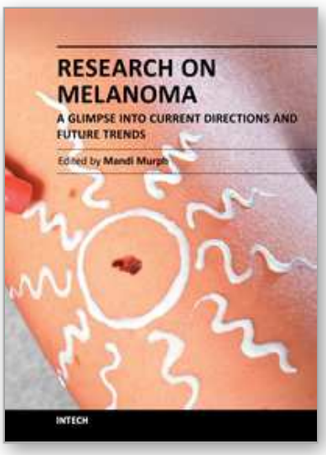

Research on Melanoma - A Glimpse into Current Directions and Future Trends

Edited by Prof. Mandi Murph

ISBN 978-953-307-293-7
Hard cover, 414 pages
Publisher InTech
Published online 12, September, 2011
Published in print edition September, 2011

The book Research on Melanoma: A Glimpse into Current Directions and Future Trends, is divided into sections to represent the most cutting-edge topics in melanoma from around the world. The emerging epigenetics of disease, novel therapeutics under development and the molecular signaling aberrations are explained in detail. Since there are a number of areas in which unknowns exist surrounding the complex development of melanoma and its response to therapy, this book illuminates and comprehensively discusses such aspects. It is relevant for teaching the novice researcher who wants to initiate projects in melanoma and the more senior researcher seeking to polish their existing knowledge in this area. Many chapters include visuals and illustrations designed to easily guide the reader through the ideas presented.

How to reference

In order to correctly reference this scholarly work, feel free to copy and paste the following:

J. Sarek, M. Kvasnica, M. Vlk, M. Urban, P. Dzubak and M. Hajduch (2011). The Potential of Triterpenoids in the Treatment of Melanoma, Research on Melanoma - A Glimpse into Current Directions and Future Trends, Prof. Mandi Murph (Ed.), ISBN: 978-953-307-293-7, InTech, Available from: http://www.intechopen.com/books/research-on-melanoma-a-glimpse-into-current-directions-and-future-trends/the-potential-of-triterpenoids-in-the-treatment-of-melanoma

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