Synthesis and *in vitro* Antitumor activity of Novel Lupane Type Pentacyclic Triterpenoids

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Numbering

1 betulin

1a 3,28-bis(acetoxy)betulin

1b 3-*O*-acetylbetulin

1c 28-*O*-acetylbetulin

1d 28-*O*-chloroacetylbetulin

1e 28-*O*-laurylbetulin

1f 3,28-bis(pivaloxy)betulin

1g 28-*O*-acetylbetulin-3-yl-α-D-tetra-*O*-acetyl glucopyranoside

1h 28-*O*-acetylbetulin-3-yl-β-D-tetra-*O*-acetyl glucopyranoside

1i 28-*O*-acetylbetulin-3-yl-α-D-glucopyranoside

1j 28-*O*-acetylbetulin-3-yl-β-D-glucopyranoside

1k 3,28-bis(ethylcarbamate)betulin

1l 3,28-bis(phenylcarbamate)betulin

1m 3-ethylcarbamate-28-*O*-acetylbetulin

1n 3-phenylcarbamate-28-*O*-acetylbetulin

2 betulinic acid

2a 3-*O*-acetylbetulinic acid

2b 3-*O*-chloroacetylbetulinic acid

2c 3-*O*-hexanoylbetulinic acid

2d 3-*O*-laurylbetulinic acid

2e 28-methylbetulinate

2f methyl(3-*O*-acetyl)betulinate

2g 6-chlorohexyl(3-*O*-acetyl)betulinate

2h 1-hydroxydecyl(3-*O*-acetyl)betulinate

2i 3-*O*-acetylbetulinic anhydride

2j 3-hydroxy-2-(hydroxymethyl)propylbetulinate

2k 2-amino-3-hydroxy-2-(hydroxymethyl)propyl(3-*O*-acetyl)betulinate

2l 2-amino-3-hydroxy-2-(hydroxymethyl)propylbetulinate

2m *N*-(2,3-hydroxy-2-(hydroxymethyl)propyl(3-*O*-acetyl)betulinamide

2n *N*-(2,3-hydroxy-2-(hydroxymethyl)propylbetulinamide

2o 2-amino-3-hydroxy-2-(hydroxymethyl)propylbetulonate

2p 3-ethylcarbamatebetulinic acid

2q 3-phenylcarbamatebetulinic acid

2r 3-ethylcarbamate(methyl)betulinate

2s 3-phenylcarbamate(methyl)betulinate

Abbreviations

HIV: Human Immuno Deficiency Virus

TPA: tumor promoter 12-O-tetradecanoylphorbol-13-acetate

CDDO: 2-cyano-3, 12-dioxoolean-1,9-dien-28-oic acid

NO: nitric oxide

NFκB: nuclear factor kappa-light-chain-enhancer of activated B cells

TNF: tumor necrosis factor

FADD; Fas-Associated protein with death domain

CARD: caspase activating recruitment domain

AIF: apoptosis-inducing factor

FLIP: FLICE-inhibitory proteins

IAP: inhibitor of apoptosis proteins

HtrA2: heat-inducible serine protease A2

SMAC: second mitochondria-derived activator of caspase

DIABLO: direct inhibitor-of-apoptosis protein binding protein with low Pi

Apaf: apoptotic protease activating factor

ROS: reactive oxygen species

DNA: deoxyribonucleic acid

VEGF; vascular endothelial growth factor

Sp: specificity protein

EBV EA: Epstein Barr Virus Early Antigen

SRB: sulforhodamine B

MTT: 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

Tris: 2-amino-2-hydroxymethyl-propane-1,3-diol

TEMPO: 2,2,6,6-tetramethylpiperidine-1-oxyl

Published or accepted papers, enlisted below are included in the present PhD work as appendices

Appendix 1:

H. Kommera, G. N. Kaluđerović, J. Kalbitz, R. Paschke, Microwave mediated synthesis and structure-activity relationships of betulin and betulinic acid derivatives, Archiv Pharm. **2010**, in press.

Appendix 2:

H. Kommera, G. N. Kaluđerović, J. Kalbitz, R. Paschke, Lupane Triterpenoids - Betulin and Betulinic acid derivatives induce apoptosis in tumor cells, *Invest. New Drugs.* **2010**, in press, doi: 10.1007/s10637-009-9358-x.

Appendix 3:

H. Kommera, G.N. Kaluđerović, M. Bette, J. Kalbitz, P. Fuchs, S. Fulda, W. Mier, R. Paschke In vitro anticancer studies of α- and β-D-glucopyranose betulin anomers, *Chem-Biol Interact.* **2010**, *185*, 128–36.

Appendix 4:

H. Kommera, G. N. Kaluđerović, J. Kalbitz, B. Dräger, R. Paschke, Small structural changes of pentacyclic lupane type triterpenoid derivatives lead to significant differences in their anticancer properties, *Europ. J. Med. Chem.* **2010**, in press, doi:10.1016/j.ejmech.2010.04.018.

Appendix 5:

H. Kommera, G. N. Kaluđerović, J. Kalbitz, B. Dräger, R. Paschke, Novel carbamate derivatives of betulinic acid and betulin with selective cytotoxic activity, *Bioorg. Med. Chem Lett.* **2010**, in press doi:10.1016/j.bmcl.2010.04.004.

Summary

Synthesis and *in vitro* Antitumor activity of Novel Lupane Type Pentacyclic Triterpenoids

This work describes synthesis and *in vitro* antitumor activity of new C-3 and C-28 derivatives of betulin and betulinic acid. The derivatives were characterized by ^{1}H and ^{13}C NMR and LC/MS spectroscopies and tested for cytotoxicity evaluation on 15 different tumor cell lines. The effect of their structural variations on anticancer activity as well as their tumor selectivity in comparison with normal cells has been studied. The mode of cell death along with alterations in caspase activity and cell cycle perturbations caused by the derivatives has been investigated.

A lab scale single mode microwave was used to synthesize six novel esters of varying carbon chain length were synthesized. The primary hydroxyl group at C-28 or secondary hydroxyl group at C-3 or both of betulin and the β-hydroxyl group at C-3 position in betulinic acid were reacted with chloroacetic anhydride, hexanoic anhydride, lauric anhydride andpivaloyl anhydride to obtain esters of betulin (**1c**–**1f**) and betulinic acid (**2b**–**2d**). 3-O-acetyl betulinic acid (**2a**) was converted into its acid chloride and reacted with methanol, 6-chloro-1-hexanol and 1,10-decanediol in excess to obtain the four new esters **2f**, **2g, 2h and 2i** respectively.

Four glucopyranose derivatives of 28-*O*-acetoxy betulin (**1c**), **1g**, **1h**, **1i** and **1j** have been synthesized for determination of the differences in the biological properties of α and β

anomers. Crystals of the 28-*O*-acetylbetulin-3-yl-*β*-D-(2',3',4',6'-tetra-*O*-acetyl)glucopyranoside (**1h**) suitable for X-ray diffraction measurements were obtained by the slow evaporation of petrol ether from the solution. **1h** was crystallized in triclinic crystal system and *P*1 space group.

C-28-esters and amides from 2-(hydroxymethyl)propane-1,3-diol and 2-amino-2-(hydroxymethyl)propane-1,3-diol of 3-O-acetyl betulinic acid (**2a**) namely, **2j**–**2l** and C-28 ester of 2-amino-2-(hydroxymethyl)propane-1,3-diol of betulonic acid, **2o**, were synthesized from the respective acid chlorides. The importance of acetyl group at C-3 for cytotoxicity has also been investigated.

A series of new ethyl and phenylcarbamates of betulin (**1k**–**1n**) and betulinic acid (**2p**–**2s**) have been synthesized with an intention to investigate the effects of aromatic and aliphatic substitutions. The primary and secondary hydroxyl groups of betulin (**1**) and 28-*O*-acetyl betulin (**1c**) and only secondary hydroxyl group in betulinic acid (**2**) and methyl betulinate (**2e**) are modified into ethyl and phenylcarbamates.

The cytotoxicity of the most active compounds from the synthesized derivatives is discussed as follows. The chloroacetyl ester derivatives among the short and long chain esters of betulin and betulinic acid at C-28 or C-3 (**1d** and **2b**) were found to be cytotoxic on a broad spectrum of cancers. The acetyl protected glucose anomers (**1g** and **1h**) did not show any activity even at high concentrations whereas their deacetylated counterparts (**1i** and **1j**) were cytotoxic. Among the C-28-esters and amides 2-amino-2-(hydroxymethyl)propane-1,3-diol of 3-O-acetyl betulinic acid and betulonic acid, **2k** and **2o** were highly cytotoxic even at low concentrations. Short chain aliphatic carbamates of betulin **1k** and **1m** showed high antiproliferative effect at lower concentrations on the other hand the phenyl carbamates were inactive. All the active compounds induce cell death by apoptosis in cancer cells which was

envisaged by trypan blue dye exclusion test and confirmed by the induction of DNA fragmentation on treated cells.

Among the most active derivatives the carbamates, **1k** and **1m** were highly selective to cancer cells and well tolerated by normal human fibroblasts (WWO70327). The glucose derivatives of betulin (**1i** and **1j**) showed a similar magnitude of selectivity as betulinic acid, whereas the 2-amino-3-hydroxy-2-(hydroxymethyl)propyl(3-*O*-acetyl)betulinate, **2k** was not selective towards the tumor cells.

The compounds **2b** and **1d** seem to upregulate upstream caspase-9 significantly and downstream caspase 3 on HT-29 cell line, which infers that the mitochondrial pathway in apoptosis induction is triggered by them. The small structural variations in α and β glucose anomers of betulin, **1i** and **1j**, might effect to activate different caspase cascade pathways to induce apoptosis even though they show a same degree of cytotoxicity on HCT-116 colon cancer cells. The new carbamate derivatives, **1k** and **1m** were found to activate all the caspases used for investigations even for short exposure times and regulate the sequential events of initiator caspase 2 and caspase 8 followed by caspase 9 and caspase 3 due to stress induced apoptosis involving mitochondrial damage in lung cancer cell line (A549).

1. Introduction

Plant products have been used extensively in the treatment of malignant disease for thousands of years. A large number of chemical constituents isolated from naturally occurring plant products have proved to be quite efficacious as antitumor agents. Some of the plant products which have been used in treatment of cancers include alkaloids like cochicine and narciclasine, dimeric indole alkaloids like vinblastin and vincristin, hetercyclic amines like campothecin and hydroxy camptothecin, lactones like podophyllotoxin and deoxypodophyllotoxin, glycosides like mithramycin and several other cancer therapeutic agents like etoposide, paclitaxel.

Natural products obtained from plant sources played an important role in cancer treatment. Plant products derivatives comprised 14 of the top 35 drugs in 2000 based on worldwide sales [1]. Two plant derived natural products, paclitaxel and camptothecin were estimated to account for nearly one-third of the global anticancer agents [2]. Only a small portion of two hundred thousand higher plants existing have been explored phytochemically. So, it is anticipated that plants can provide potential bioactive compounds for the development of new lead substances to combat cancer diseases since they are easily available, cost effective, less toxic and show good efficacy with minimum ancillary effects. Among the classes of identified natural products triterpenes are one of the largest families which have been studied vastly for their diverse structures and a variety of biological activities, particularly anticancer activity.

1.1. Triterpenes

Terpenes are a part of our everyday life, such as mono- and sesqui-terpene components of essential oils, which contribute to the aroma of plants, terpenes of different types are present in all higher plants *viz.* tetraterpene carotenoids are abundant in our daily food, or polyterpenes, latex, used in the manufacture of rubbers and other rubber components. Terpenes are vested with biological activities which include bactericidal, fungicidal, antiviral, cytotoxic, analgesic, anticancer, spermicidal, cardiovascular, antiallergic properties. Triterpenoids are metabolites of isopentenyl pyrophosphate oligomers and comprise the largest group of plant natural products. This class of compounds may be synthesized from isopentenyl pyrophosphate through the 30-carbon intermediate squalene [3]. Triterpenes are produced by arrangement of squalene epoxide in a chair-chair-chair-boat arrangement followed by condensation.

Triterpenes comprise a large number of different types of compounds which may be subdivided into more important chemical structure families. The main groups of triterpenoids and their glycosides are represented by tetracyclic derivatives of protostane, cycloartane**,** dammarane, euphane, and pentacyclic derivatives of ursane, gammacerane, lupane and hopane. Pentacyclic triterpenes are all based on a 30-carbon skeleton comprising five six-membered rings (ursanes and lanostanes) or four six-membered rings and one five-membered ring (lupanes and hopanes) [4].

The therapeutically important classes of triterpenoids showing anticancer activity include pentacyclic terpenoids containing:

1. ursane and oleanane groups
2. lanostane group

3. dammarane group
4. lupane group
5. other triterpenoids

1.1.1. The ursane and oleanane groups

The ursane group containing terpenes showing anticancer property include boswellic acid, ursolic acid and glycyrrhetinic acid. Boswellic acids are pentacyclic triterpenes acids isolated from the gum resins of *Boswellia* species (Burseraceae), *viz. B. carterii, B. frereana, B. papyrifera, B. sacra, and B. serrata* [5, 6]. Chemically, boswellic acids can differ in the positions of the two methyl groups on ring-E; β-boswellic acids have 19, 20-dimethyl substitution, while α-boswellic acids have 20, 20-dimethyl substitution, classified as the ursane and oleanane groups of triterpenes, respectively. A boswellic acid derivative, 3-O-acetyl-11-keto-β-boswellic acid was found to possess anticancer activity and induce apoptosis in PC-3 and LNCaP (prostate adenocarcinoma) cell lines through a death receptor (DR-5)-mediated pathway, which is a signal transduction cascade involving the activation of capase-8 and capase-3 in apoptosis [7]. Ursolic acid (3-β-hydroxyurs-12-en-28-oic acid) occurs more prevalently in the plant kingdom. Inhibitory effect of ursolic acid on cancer cells may be due to suppression of inhibitors of the NF-*κ*B pathway and p65 phosphorylation, thereby causing down-regulation of the expression of downstream oncogenes [8]. Glycyrrhetinic acid (3β-hydroxy-11-oxoolean-12-en-30-oic acid) is an abundant constituent of licorice (*Glycyrrhiza glabra*) and inhibits the mutagenicity induced by various mutagens [9]. Glycyrrhetic acid was found to show a spectrum of biological activities including antiinflammatory, antiviral, hepatoprotective, antitumor and immunomodulatory effects [10]. The antitumoral activity of

the glycyrrhetinic acid is regulated by triggering the proapoptotic pathway and by inducing mitochondrial permeability transition [11, 12].

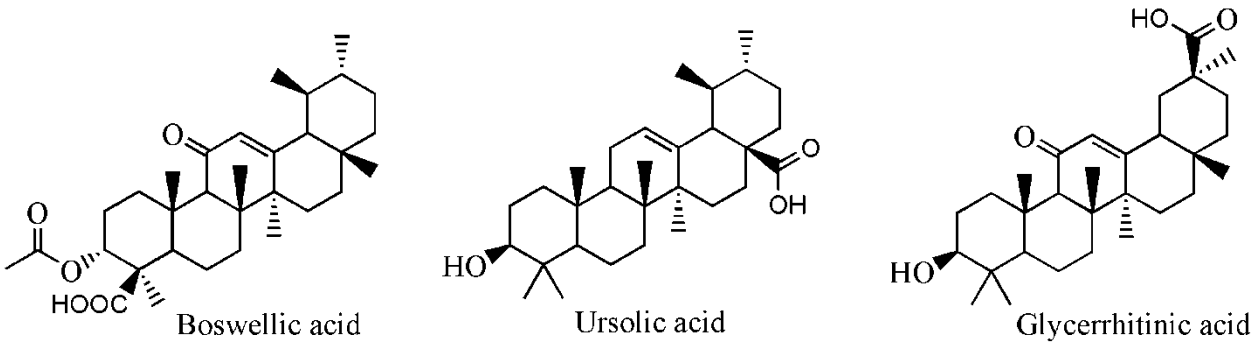


Figure 1. Structures of boswellic, ursolic and glycyrrhetinic acid

The oleanane group of terpenes that are showing anticancer property include oleanolic and masilinic acid. Oleanolic acid (3β-hydroxyolean-12-en-28oic acid) a well known triterpene occurring in numerous varieties of plants, possesses many biological properties, including antiinflammatory, trypanocidal, anti-HIV and cytotoxic activities [13]. Furthermore, it was reported to suppress TPA-induced tumor promotion [14]. Modification of A-ring from oleanolic acid yielded analogues with improved cytotoxicity: 2-cyano-3,12-dioxoolean-1,9-dien-28-oic acid (CDDO) and its C-28 methyl ester (CDDO-Me) which are currently in phase I clinical trials for the treatment of metastatic or unresectable solid tumors or lymphoma. Both of these synthetic derivatives strongly inhibited the production of NO induced by IFN-g in mouse macrophages (IC_{50}: 0.4 nM) [15]. Maslinic acid (2-α,3-β-dihydroxyolean-12-en-28-oic acid) an oleanane triterpene identified from *Olea europaea* or *Crataegus oxyacantha* was found to have potent differentiating and antiproliferative properties [16, 17].

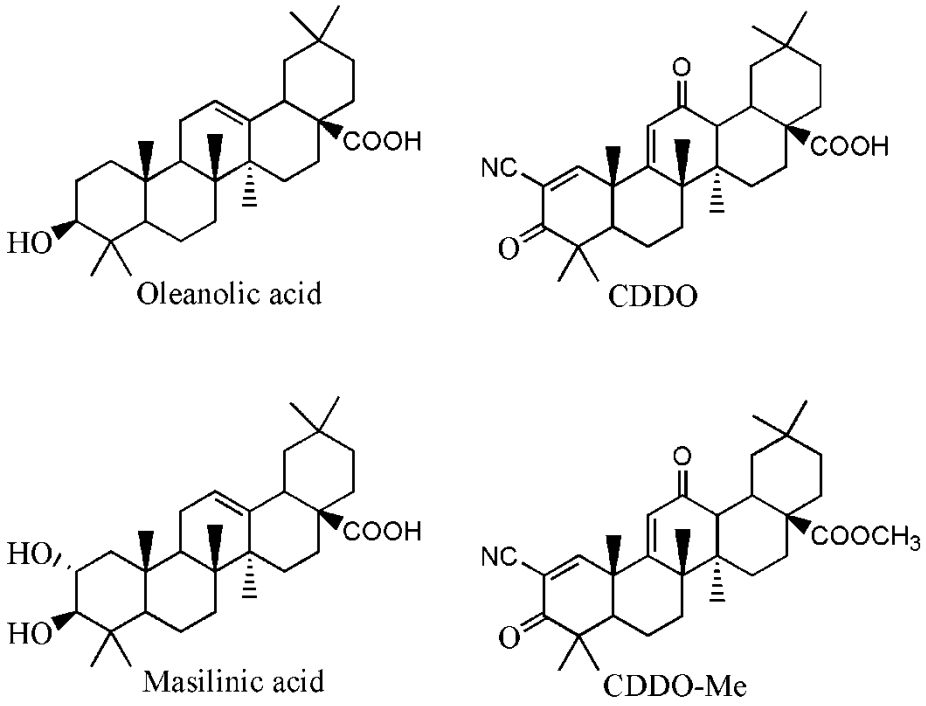


Figure 2. Structures of oleanolic, masilinic, CDDO acids and CDDO-Me

1.1.2. The lanostane group

The lanostane group containing terpenes include ganoderic acid D, impatienside A and bivittoside D. Ganoderic acid D isolated from *Ganoderma lucidum* was assessed found to express antiproliferative effect against human cervical carcinoma cells (HeLa) and arrested cell cycle at the G2/M phase with induction of apoptosis [18]. Impatienside A and bivittoside D were isolated from the sea cucumber *Holothuria impatiens*. The two terpenes exhibited *in vitro* cytotoxicity equal or slightly better than those of the clinical antitumor drug etoposide against different human tumor cell lines [19].

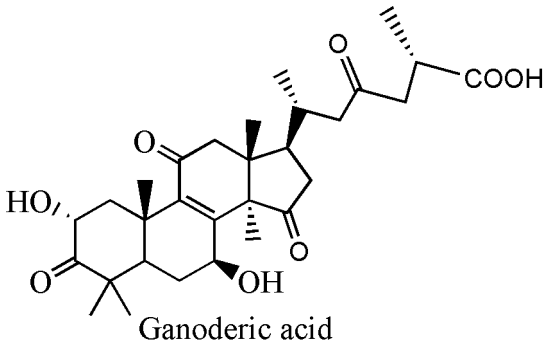


Figure 3. Structure of ganoderic acid

1.1.3. The dammarane group

25-methoxyhispidol is a tirucallane triterpene isolated from the fruit of *Poncirus trifoliata*. The compound demonstrated a slight antiproliferative effect (IC_{50}: 21.0 mM) against the SKHEP-1 cell line, which was postulated to occur through arrest in the G0/G1 phase of cell cycle with induction of apoptosis [20].

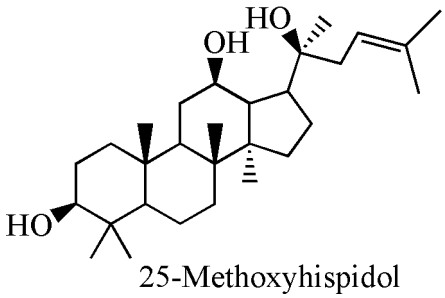


Figure 4. Structure of 25-Methoxyhispidol

1.1.4. The lupane group

Lupeol, betulin (**1**) and betulinic acid (**2**) are natural pentacyclic triterpenes of the lupane-type. Lupeol (3β-lup-20(29)-en-ol) is found in several medicinal plants and possesses strong antioxidant, antiinflammatory, antiarthritic, antimutagenic and antimalarial activity. It also acts as a potent inhibitor of protein kinases and serine proteases. Furthermore, it inhibits the activity of DNA topoisomerase II, a target for anticancer chemotherapy [21]. Lupeol inhibits

nuclear factor (NF)-κB signaling including phosphorylation of IκBa protein, DNA binding of NF-κB complex and NF-κB-dependent reporter gene activity [22]. It has also been shown that lupeol induces differentiation and inhibits the cell growth of mouse melanoma and human leukemia cells. Recently, it was shown in a two-stage model of mouse skin carcinogenesis that lupeol exhibits significant antitumor-promoting activity [22]. A detailed description of betulin and betulinic acid is given in the next section.

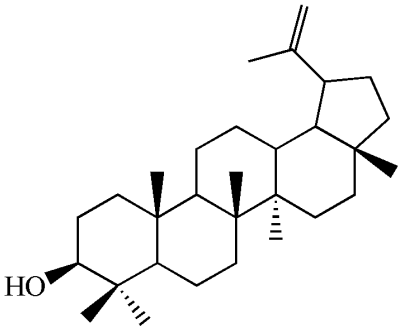


Figure 5. Structure of lupeol

1.1.5. Other Triterpenoids

Celastrol also known as tripterine a quinone methide triterpenoids, isolated from the Chinese Thunder of God Vine [23], was shown to inhibit the proliferation of a variety of tumor cells including those from leukemia [24], gliomas [25] and prostate cancer [26]. Celastrol potentiated the apoptosis induced by TNF and chemotherapeutic agents and inhibited invasion, both regulated by NF-κB activation [27].

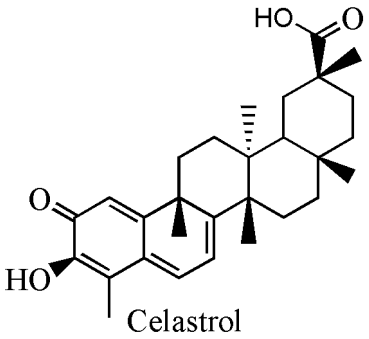


Figure 6. Structure of celastrol

1.2. Betulin and betulinic acid

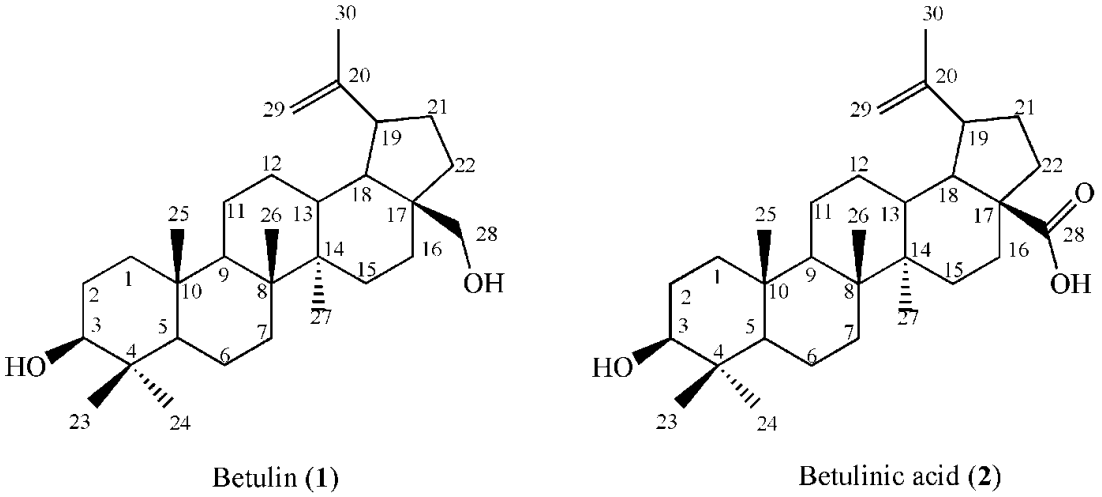


Figure 7. Structures of betulin and betulinic acid

1.2.1 Betulin and its derivatives

Betulin (**1**), lup-20(29)-ene-3β,28-diol, also known as betulinol, betuline and betulinic alcohol is a pentacyclic triterpene alcohol with a lupane skeleton. The oldest reference on betulin dates back to1788 [28] and this substance certainly constitutes one of the first natural products isolated from plants and detailed investigations including elemental analysis were described in 1876 by Hausmann [29]. Betulin can also be isolated from many plant sources in small amounts. But the extractive isolation of betulin on an industrial scale, from birch bark, is done by using high boiling hydrocarbon solvents or with water/alcohol azeotropes [30]. The healing properties of birch bark and birch bark extracts have been known for a long time in folk medicine. Betulin is rather inactive against several tested cancer cell lines such as melanoma (MEL-2), epidermoid carcinoma [31], leukemia (HL60, U937, K562) and neuroblastoma [32]. Betulin induces apoptosis in a similar way like betulnic acid in jurkat cells and was found to be more toxic than betulinic acid on cholesterol sensitized cells [33].

On simple chemical modifications betulin yields many useful compounds, which possess anticancer and other therapeutic properties. Betulin has three positions where chemical modifications can be easily performed: a secondary hydroxy group at position C-3, alkene moiety at position C-20 and primary hydroxy group at position C-28. Modification of these positions may yield derivatives for useful structure–activity relationship (SAR) studies. Betulin can be converted in one [34] or two [35] steps into a very important derivative betulinic acid (**2**). Conversion of secondary and primary hydroxyl groups of betulin into amino acid esters has lead to a structure (*viz.***3**) with improved water solubility and cytotoxicity against human gastric carcinoma cell line (EPG85-257P) [36]. Introduction of an imidazole scaffold at secondary hydroxyl group of betulin at C-3 position yielded a potentially important derivative (**4**) with improved cytotoxic activity on HepG2 (hepatocellular carcinoma) and HeLa (cervical adenocarcinoma) cell lines [37]. A series of monodesmic saponins of betulin have been synthesized in order to enhance the hydrophilicity and cytotoxicity of the lead molecule. Introducing 3β-O-D-mannopyranoside (**5**) on the C-3 position of betulin yielded a strongly potent cytotoxic agent against lung cancer (A549) and colorectal cancer (DLD-1) cell lines [38]. The primary hydroxyl group of betulin at C-28 position can be easily converted to aldehyde (betulin aldehye). Betulin aldehydes are interesting lead substances for the preparation of potent cytotoxic compounds. One such example is the conversion of betulin aldehydes into epoxides which were found to be many folds more toxic than betulinic acid on a broad spectrum of cancer cell lines [39]. Hence, betulin is widely considered by the scientific community that it is an important lead structure from which potential antitumor drugs can be developed.

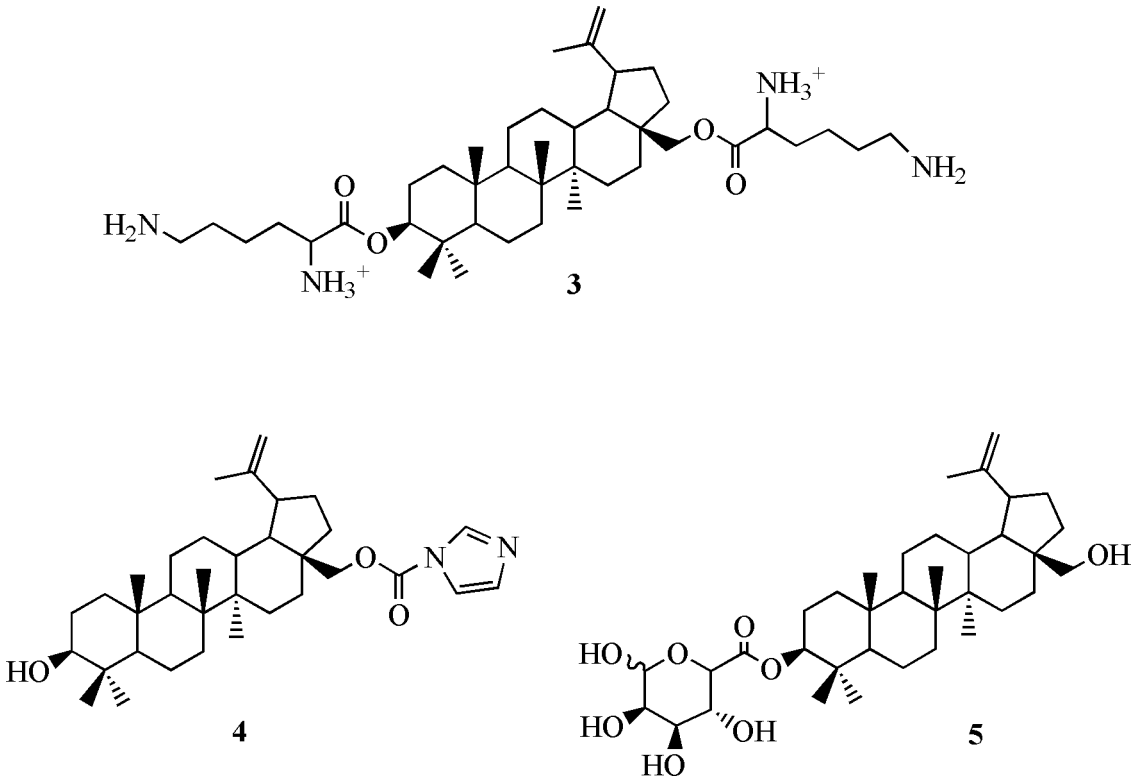
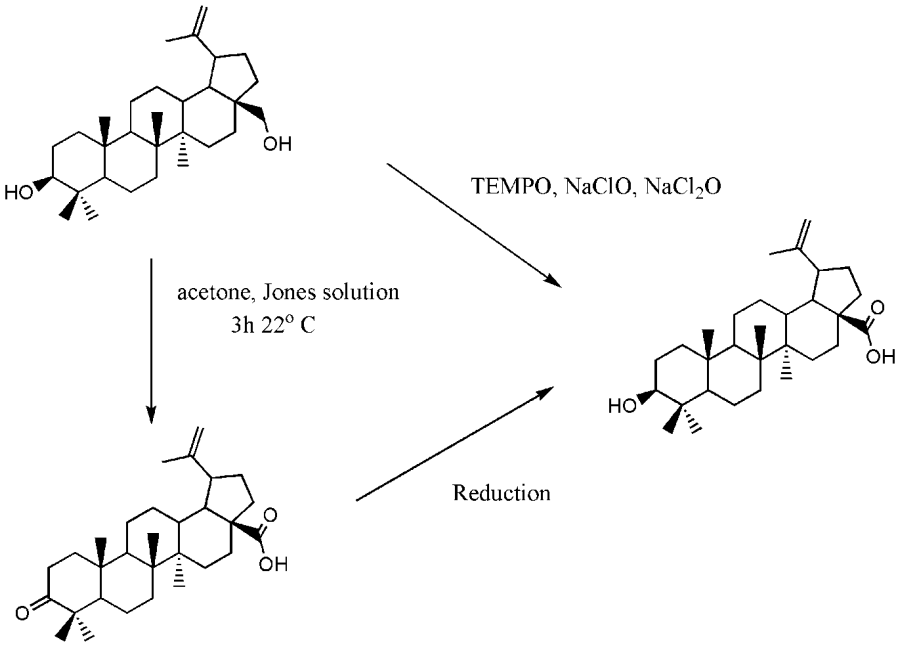


Figure 8. Structures of betulin derivatives

1.2.2. Betulinic acid

Betulinic acid (**2**), 3β-Hydroxy-lup-20(29)-en-28-oic acid, is a C-28 carboxylic acid derivative of the ubiquitous triterpene betulin and is a member of the class of lupane type triterpenes. However, unlike betulin, the oxidized derivative betulinic acid possesses a number of intriguing pharmacological effects including antiinflammatory, anticancer, and anti-HIV activities. The lupane-type triterpene, betulinic acid, is found widely throughout the plant kingdom. One of the most widely reported sources of betulinic acid is the birch tree (*Betula* spp., Betulaceae) where betulin can be obtained in substantial quantities which can be further modified to obtain betulinic acid [40–42]. Plane tree (*Platanus* spp.) bark comprises of around 2.4 % of betulinic acid [43]. Other known sources of betulinic acid include *Ziziphus* spp. (Rhamnaceae), [44–46] *Syzygium* spp. (Myrtaceae), [47–48] *Diospyros* spp. (Ebenaceae), [49–51] and *Paeonia* spp. (Paeoniaceae) [52–54]. A multitude of extraction and

isolation schemes have been used for the procurement of betulinic acid and other related triterpenoids. Typically, dry plant material is extracted with chloroform (for aglycons), [55, 18] methanol (for both aglycons and glycosylated derivatives), [56–57] or even H_2O [58]. The plant may be defatted with hexane prior to extraction to remove nonpolar materials [55, 58]. The resultant extracts can be dried and further extracted with other solvents or directly subjected to column chromatography [53, 60–64]. Betulinic acid can be produced from plane tree by fractional distillation with a solvent of medium polarity and subsequent recrystallization out of methanol [43]. Betulinic acid is a white crystalline solid that exhibits limited solubility in organic solvents such as methanol, ethanol, chloroform and ether. Betulinic acid has low solubility in water, petroleum ether, dimethylformamide, dimethylsufoxide and benzene. However, betulinic acid is highly soluble in pyridine and acetic acid. Betulinic acid can be obtained from betulin according to Scheme 1.



Scheme 1. Synthesis of betulinic acid from betulin

Betulinic acid shows numerous biological activities like antiinflammatory, antimalarial, anti-HIV, antimalarial, antimicrobial, antihelmintic and is widely considered for its anticancer activity. Initially betulinic acid was found to be melanoma specific [44] but later it was discovered that it shows anticancer activity against a broad spectrum of cancers [65]. Moreover, betulinic acid was found to be selective to tumor cells and non toxic to normal non cancerous cells [66] and due to this it is well tolerated in mice even at higher concentrations. Betulinic acid was found to be cytotoxic in different models of drug resistant cell lines, for example in primary pediatric acute leukemia samples that were refractory to standard chemotherapeutic agents [67]. Furthermore, there is evidence that betulinic acid exerts preferential cytotoxicity against metastatic over nonmetastatic melanoma cell lines [68]. Betulinic acid showed a synergistic cytotoxic effect in combination with vincristine on murine melanoma B16F10 cells both *in vitro* and *in vivo* [69]. Betulinic acid may be used to sensitize the cancers which are non responsive for irradiation and also can be used as a complimentary agent in combination with ionizing radiation for resistant melanomas [70]. It has also been reported that betulinic acid acts as a chemopreventive agent and was shown to inhibit tumor formation in two stage skin carcinogenesis mouse model [71].

1.2.3. Anticancer mechanism of action of betulinic acid

Apoptotic pathways are potential targets for therapeutic modulation. The main, best characterized apoptotic pathways are shown in Figure 9. Triggering of a death receptor (left) by an agonistic antibody or natural ligand leads to the recruitment of the adapter protein Fas-Associated protein with death domain (FADD) and pro-caspase-8; this becomes cleaved and activated at the receptor complex, initiating the caspase cascade [72]. The mitochondria–apoptosome intrinsic pathway (right) is triggered by several apoptotic stimuli. An early step, which is currently poorly understood, is the mitochondrial release of apoptosis-inducing

molecules [e.g. cytochrome c, apoptosis-inducing factor (AIF), heat-inducible serine protease A2 (HtrA2) and second mitochondria-derived activator of caspase or direct inhibitor-of-apoptosis protein binding protein with low pI (Smac/DIABLO)] into the cytosol. Initially, cytochrome c and deoxyadenosine triphosphate are associated with apoptotic protease activating factor 1 (Apaf1). This process unmasks the caspase activating recruitment domain (CARD) motif in Apaf1, enabling the binding and activation of pro-caspase-9. Once activated, caspase-9 activates other members of the caspase cascade. A proteolytic cleavage cascade significantly amplifies the initial signal. In addition, positive-feedback loops involving Bid, mitochondria, apoptosome, caspase-9, effectors caspases and caspase-8 are also able to amplify the death signal. The apoptosome pathway is further potentiated by AIF through the increased mitochondrial release of cytochrome c and pro-caspase-9 [72].

Negative modulators of apoptosis, such as FLICE-inhibitory proteins (FLIPs) and inhibitor of apoptosis proteins (IAPs) negatively influence the transmission of the apoptotic signal and are thus important targets for novel anticancer therapies. Smac/DIABLO and HtrA2 halt the apoptosis-inhibitory action of IAPs [72].

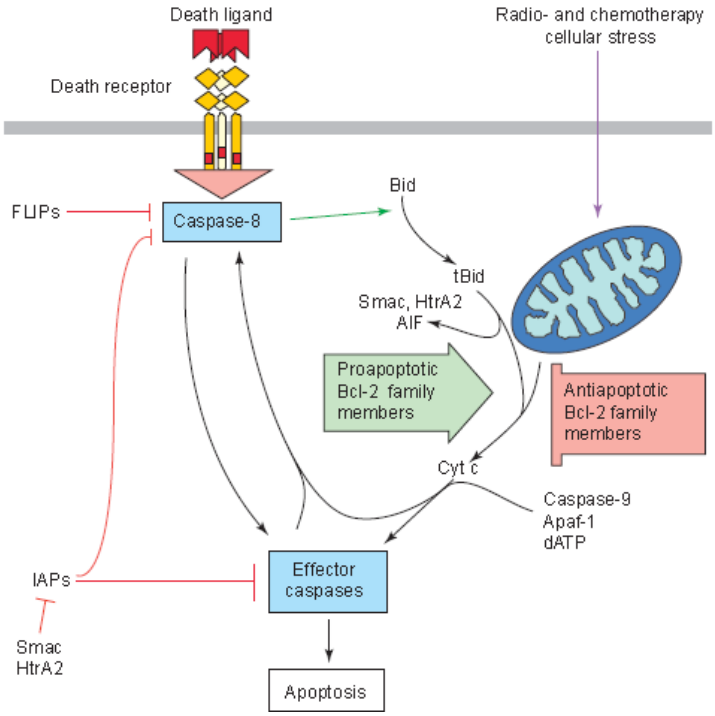


Figure 9. Caspase cascade in apoptosis

A large number of studies in last few years have been aimed at elucidating the molecular mechanisms of betulinic acid - mediated antitumor activity. Eventhough, the anticancer mechanism is not completely clear and well established, betulinic acid was found to cause cancer cell death by induction of apoptosis through changes in the mitochondrial membrane potential, production of reactive oxygen species (ROS), and permeability of transition pore openings. These processes lead to the release of mitochondrial apoptogenic factors, activation of caspases and DNA fragmentation [73–74]. The cytotoxicity of this compound is related to its ability to trigger the mitochondrial or intrinsic pathway of apoptosis in cancer cells. Betulinic acid causes a decrease of outer mitochondrial membrane potential [75]. In a cell-free system comprising mitochondria, cytosol and purified nuclei, betulinic acid induces mitochondria permeability transition mediated cytosolic caspase activation and nuclear fragmentation via the release of soluble factors, such as cytochrome c. Antiapoptotic Bcl-2 family proteins, such as Bcl-2 and Bcl-XL, inhibited all mitochondrial and cellular

manifestations of apoptosis induced by betulinic acid indicating that mitochondrial permeability transition was required for these events. Mitochondria from intact cells treated with betulinic acid, induced cleavage of both caspase-8 and caspase-3 which was preceded by the disturbance of mitochondrial membrane potential and by the generation of reactive ROS in intact cells [70]. Betulinic acid has been reported to induce apoptosis in a p-53 and CD-95 independent manner [76]. Betulinic acid was also identified as a potential activator of NF-κB in a number of cancer cell lines. This activation resulted in an increased IKK activity, phosphorylation of Ikßa at seriene 32/36 followed by degradation of IkBa and nuclear translocation of NF-kB subunit p65 [77]. Betulinic acid was also reported to inhibit aminopeptidase N, an enzyme required for angiogenisis and overexpressed particularly in cancer cells [78]. Betulinic acid also was found to catalytically inhibit topoisomerase I by inhibiting the process of topoisomerase I-DNA complex formation by interacting with the enzyme directly inside the cells [79]. To summarize, betulinic acid causes a fall in membrane potential of outer mitochondrial membrane thereby causing a release of cytochrome c which inturn activates the initiator caspase 9 following the activation of effector caspase 3 leading to DNA fragmentation and apoptotic cell death in cancer cells.

Betulinic acid decreases expression of vascular endothelial growth (VEGF) and the antiapoptotic protein survivin in tumors, due to activation of selective proteasome-dependent degradation of the transcription factors specificity protein 1 (Sp1), Sp3, and Sp4, which regulate VEGF and survivin expression. Thus, betulinic acid acts as a novel anticancer agent through targeted degradation of Sp proteins that are highly over expressed in tumors [80].

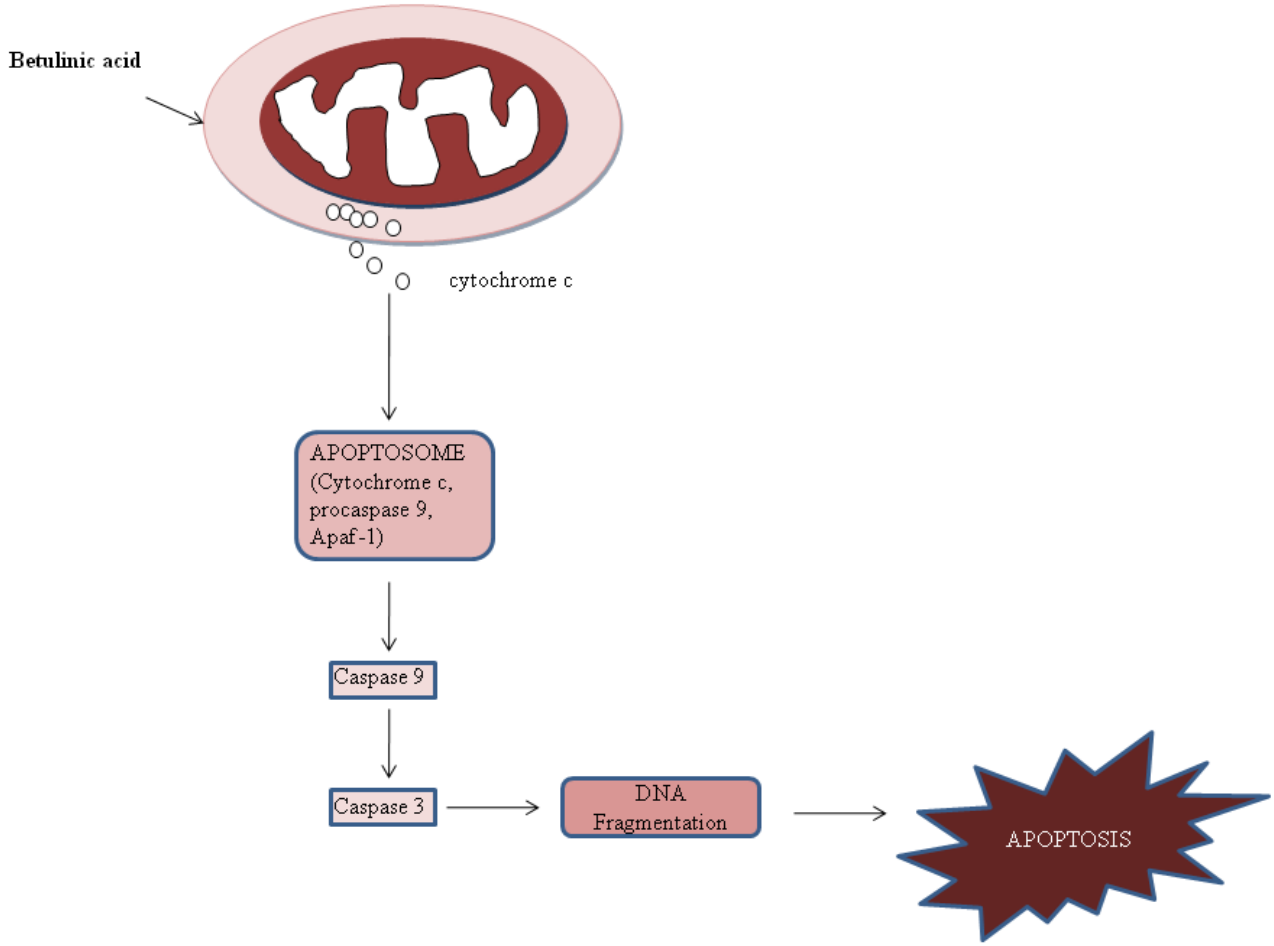


Figure 10. Mechanism of anticancer action of betulinic acid

1.2.4. Betulinic acid derivatives

Remarkably, betulinic acid exhibited no toxic effects in mice even at a concentration of 500 mg/kg, eventhough doses of betulinic acid as low as 5 mg/kg were sufficient to impede tumor development [44]. These encouraging findings have made betulinic acid a very attractive candidate for the clinical treatment of various forms of cancer. As a result, further studies have been performed to derive synthetic betulinic acid analogs in an effort to establish meaningful structure-activity relationships. Three positions in betulinic acid, the C-3

hydroxyl, C-20 alkene, and C-28 carboxylic acid moieties, have served as the target for most derivatization studies.

The β-hydroxyl moiety at C-3 position can be chemically modified readily. A number of synthetic C-3 betulinic acid derivatives have been reported and tested for cytotoxicity and some of these modifications include oxidation to a ketone, acetylation and formation of various nitrogen-containing analogs (amine, oxime). Oxidation of the 3-β-hydroxyl group in betulinic acid to a ketone yielded a highly cytotoxic derivative betulonic acid (**6**) but a loss of specificity against melanoma cells [31]. Introduction of an oxime moiety (**7**) at C-3 did not show any considerable impact on cytotoxicity whereas substitution into a benzyl ester resulted in a loss of cytotoxicity against melanoma and non melanoma cells [31]. Derivatization of β-hydroxyl group by pthalic anhydride into methyl (**8**) and ethylpthalates increased cytotoxic activity [81]. The imidazole carboxylic acid ester at C-3 position has also yielded a derivative (**9**) with good cytotoxicity on HepG2 (hepatocellular carcinoma), HeLa (cervical adenocarcinoma) and Jurkat (leukemia) cells [37]. Despite these efforts, very little can be deduced regarding the role that derivatization of C-3 may play in controlling anticancer activity. Esterifications of betulinic acid at C-3 position with fatty acids have yielded many useful derivatives, *viz.***10**, which can be further developed into cancer preventive agents [82].

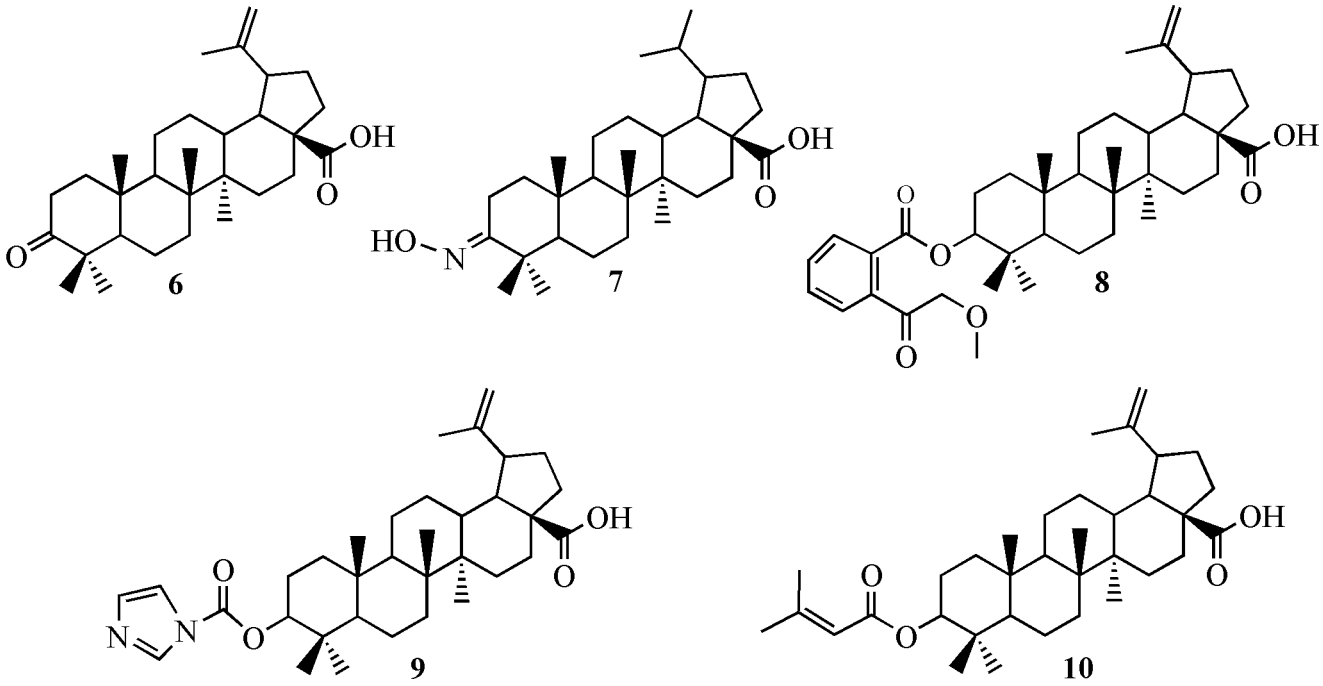


Figure 11. C-3 betulinic acid derivatives

Furthermore, a series of C-28 betulinic acid derivatives have been synthesized and tested against a variety of tumors. A set of C-28 amino acid conjugates, *e.g.* glycine conjugate of betulinic acid (**11**) was synthesized and reported to retain cytotoxicity and specificity towards melanoma cells and also found to have improved water solubility [31, 83–84]. On the other hand, the 28-O-β-D-glucoside of betulinic acid (**12**) did not exhibit any significant cytotoxicity [85]. It is considered that C-28 carbonyl group was necessary for preservation of cytotoxic property of betulinic acid.

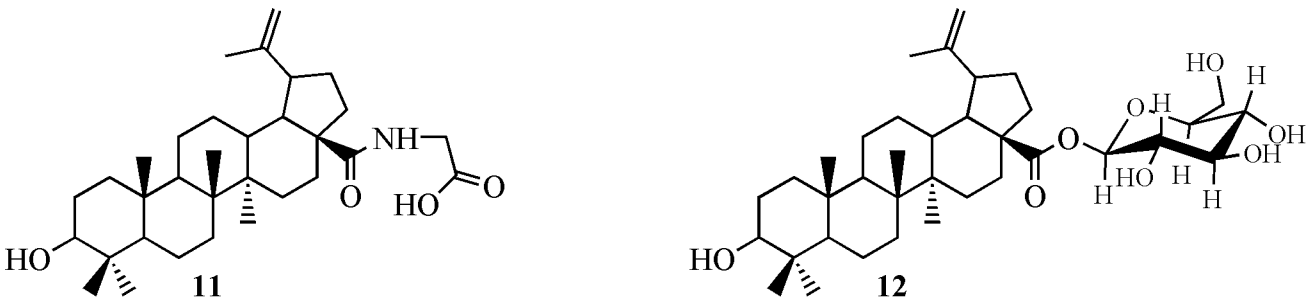


Figure 12. C-28 betulinic acid derivatives

A limited number of studies have examined changes in the cytotoxicity profile of betulinic acid when the C-20 side chain has been modified. Minor changes, such as the introduction of a ketone (**13**) or oxime moiety at C-29, resulted in a loss of activity [86]. However, hydrogenation of the C-20 double bond (**14**) does not adversely affect the cytotoxicity of betulinic acid [31]. Introduction of halogenated cyclopropane group at C-20 yielded *viz*. 20,29-dihydro-20,29-dichloromethylenebetulinic acid (**15**) which also have resulted in a less toxic derivatives than betulinic acid [87]. These results suggest that the cytotoxicity profile of betulinic acid derivatives may be sensitive to the size of the substituents at the C-20 position in addition to the electrostatic sensitivity.

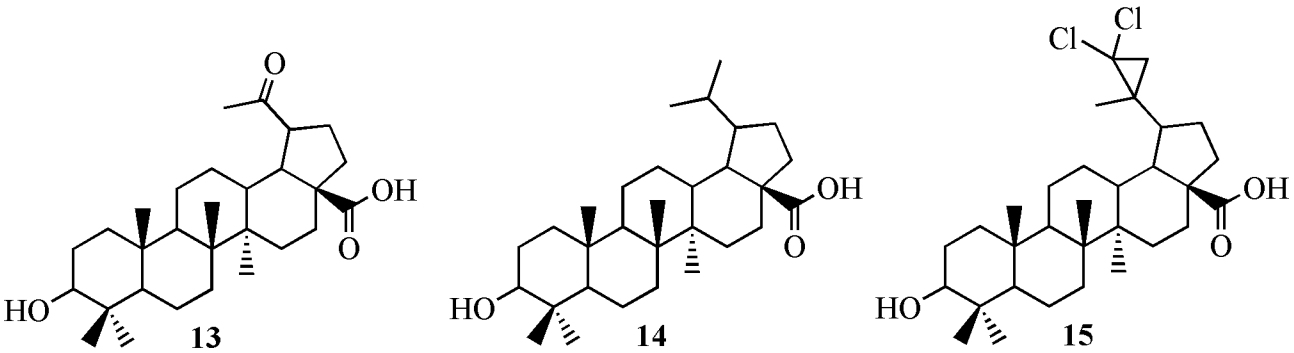


Figure 13. C-20 betulinic acid derivatives

Several other betulinic acid derivatives, modified at various other positions were found to show better anticancer activity than betulinic acid. For example, the compounds bearing 1-ene-3-oxo with electron-withdrawing groups at C-2 *viz.* 3-oxo-1-ene-20, 29-dihydrobetulinic acid (**16**) and 2-cyano-3-oxo-1-en–methyl betulinate (**17**) showed strong cytotoxicity on melanoma (M2) and lung cancer (A549) cell lines [88]. A substitution of bromo at C-2 in 20,29-dihydrobeltulonic acid also yielded a highly cytotoxic derivative (**18**) with activity on a

number of different cancer cell lines. In addition, *seco* anhydrides (**19**) as well as triol (**20**) showed a greatly improved cytotoxicity [89].

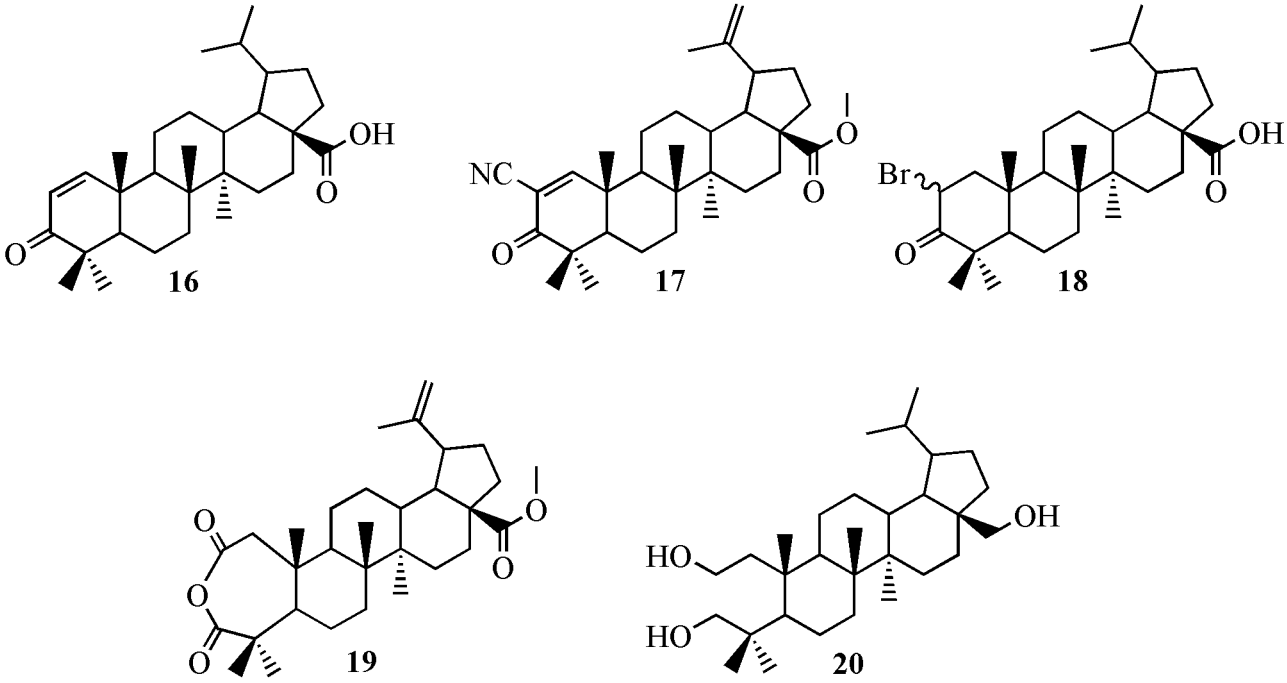


Figure 14. Structures of betulinic acid derivatives with multiple modifications

2. Aim

Betulinic acid is readily available in the local geographical area and has shown great promise as an agent for the treatment of multiple forms of cancer, hence its derivatives, which could easily synthesized have received a significant attention in recent years. A vast majority of derivatives reported possess moderate to good antitumor properties. However, due to various reasons e.g. poor solubility and low selectivity, they are not particularly good candidates for clinical use. A need therefore exists for novel betulinic acid derivatives, which are not only potent, but also clinically safe.

The aim of this work was to synthesize safe, soluble antitumor derivatives of betulinic acid and betulin, perform mechanistic studies on mode of cell death and gain insight into molecular changes leading to apoptosis caused by the novel derivatives. Furthermore, an attempt to relate the structural changes to activity has been investigated to identify a sub lead structure or a new molecular skeleton derived from betulin or betulinic acid to design new anticancer drugs. The C-3 and C-28 positions of betulin and betulinic acid were modified to obtain new promising derivatives against different types of cancers and efforts were made to contribute in understanding the mechanism of action of the potent new derivatives.

3. Materials and Methods

This section defines the terms and explains briefly the methods and techniques used to elucidate the bioactivity of new betulin and betulinic acid derivatives.

3.1. Cancer Cell Lines

The cancer cell lines 8505C and SW1736 (anaplastic thyroid tumor), A253 and FaDu (head and neck tumor), A431 (cervical), A2780 (ovarian), DLD-1, HCT-8, HCT-116, HT-29 SW480 (colon), MCF-7 (breast), 518A2 (melanoma), A549 (lung) and liposarcoma (connective tissue) were included in this study. The cell lines were used at optimal density and cytotoxicity screening studies were performed using SRB assay. All these cell lines were kindly provided by Dr. Thomas Müller, Department of Hematology/Oncology. Cultures were maintained as monolayers in RPMI 1640 supplemented with 10 % heat inactivated fetal bovine serum and 1 % penicillin/streptomycin at 37°C in a humidified atmosphere with 5 % CO_2.

3.2. Cell Viability

Cell viability is a determination of living or dead cells, based on a total cell sample. Cell viability measurements may be used to evaluate the dead or alive cancerous cells. Testing for cell viability usually involves looking at a sample cell population and staining the cells or applying chemicals to show which are living and which are dead.

3.3. Cell Proliferation

Cell proliferation can be defined as an increase in the number of cells as a result of cell growth and cell division.

3.4. Cytotoxicity

Cytotoxicity can be defined as the degree to which an agent possesses a specific destructive action on certain cells or the possession of such action. The ability to measure early indicators of toxicity is an essential part of drug discovery. Most of the commonly used cytotoxic anticancer drugs were discovered through random high-throughput screening of synthetic compounds and natural products in cell-based cytotoxicity assays. Commonly used cytotoxicity assays for primary screening of new anticancer agents are SRB (sulforhodamine B), MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), crystal violet assay etc. SRB assay has been used in the present work for screening betulinic acid and betulin derivatives.

3.5. Sulforhodamine B Colorimetric Assay Method

The SRB assay method is used for determination of cell density based on the measurement of cellular protein content. The sulforhodamine B (SRB) assay was developed by Skehan and colleagues to measure drug-induced cytotoxicity and cell proliferation for large-scale drug-screening applications [90]. Its principle is based on the ability of the dye sulforhodamine B to bind electrostatically in a pH dependent way onto basic amino acid residues of trichloroacetic acid-fixed cells. Under mild acidic conditions it binds to cellular proteins and can be extracted using mild basic conditions which can further be solubilized for colorimetric measurement. After 96 h treatment of exponentially growing cells with the substance, the cells are fixed with 10 % TCA and stained for 30 min. The excess of dye is washed with 1 % glacial acetic acid and the protein bound dye is dissolved in 10 mM Tris base solution for optical density (OD) determination at 570 nm. The IC_{50} and IC_{90} values are defined as the concentrations of the compound at which 50 and 90% cell inhibition relative to untreated control cells and can be estimated from the semi-logarithmic dose-response curves based on

OD values (Figure 15). Results of the SRB assay were reported to be linear with cell number and cellular protein measured at cellular densities ranging from 1 to 200 % of confluence [91]. The SRB assay possesses a colorimetric end point and is nondestructive and indefinitely stable. These practical advances make the SRB assay an appropriate and sensitive assay to measure drug-induced cytotoxicity even at large-scale application [91].

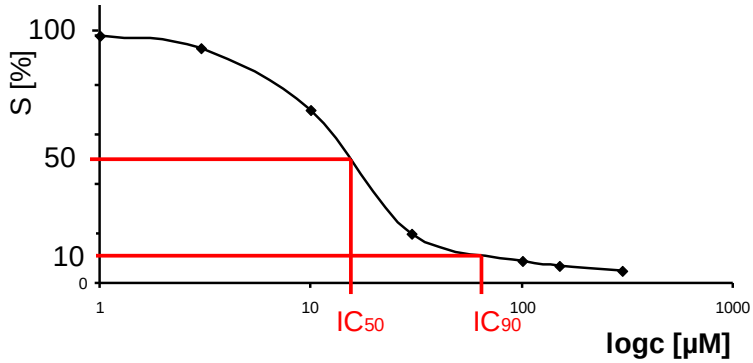


Figure 15. Graphical representation of IC_{50} and IC_{90} values on a dose inhibition curve

3.6. Selectivity and Selectivity index

Selectivity is the degree to which a dose of a drug produces the desired effect in relation to adverse effects. Selectivity index of a substance towards a tumour cell line can be defined as the ratio of concentrations at which same number of non cancerous cells and cancer cells is inhibited (for ex. IC_{50} concentration). It can be represented as:

$$\text{Selectivity index} = \frac{IC_{50}(\text{normal cells})}{IC_{50}(\text{tumor cells})}$$

Higher value → better selectivity

3.7. Dye Exclusion Test (Trypan Blue)

Trypan blue is a diazo dye used to selectively stain dead tissues or cells. The reactivity of trypan blue is based on the fact that the chromophore is negatively charged and does not interact with the cell unless the membrane is damaged. Cell membrane is intact in living cells and the cells undergoing early apoptosis. Therefore, all the cells which exclude the dye are viable or in the early apoptotic state and the cells are blue colored in necrotic cell death. This test, even though not reliable can be used to determine the type of cell death (apoptotic or necrotic) caused by the substance in attachment cultures.

3.8. DNA Fragmenatation Assay - Agarose Gel Electrophoresis

DNA fragmentation is a consequence of apoptosis. Under stress, apoptotic signal endogenous endonucleases are activated with subsequent cleavage of chromatin DNA into internucleosomal fragments of 180 base pairs or its multiples. It can be analyzed using agarose gel electrophoresis where a laddering pattern is observed for apoptotic cell death and a smear is seen in case of necrotic death.

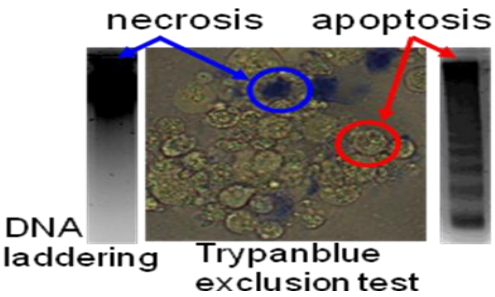


Figure 16. Apoptosis and necrosis determination by Trypan blue and DNA laddering methods

3.9. Caspase activity

Most of the biochemical and morphological features of apoptotic cells result from the selective proteolytic cleavage of a subset of cellular polypeptides [92–93]. These cleavages are mediated by caspases, cysteine proteases that cleave next to aspartate residues. The 12 known human caspases differ in primary structure and substrate specificity but share several common features [92].

1. Each active caspase is a tetramer composed of two identical large subunits and two identical small subunits.
2. Each caspase is synthesized as a zymogen containing a prodomain, a large subunit and a small subunit.
3. Caspase activation involves proteolytic cleavage at multiple aspartate residues, including one between the large and small subunits and another between the prodomain and the large subunit.

The fact that these cuts occur at potential caspase cleavage sites suggests that activation involves a proteolytic cascade [94]. Caspases can be divided into upstream or initiator caspases which mainly include caspase 2, caspase 8 and caspase 9 and downstream or effector caspases which are caspase 3 and 7.

Caspase 2 is one of the earliest identified caspases, but the role caspase 2 plays in the induction of apoptosis is still unclear. Under cytotoxic stress caspase 2 is activated and causes mitochondrial membrane permabilization [95]. Caspase 2 is engaged in intrinsic or mitochondria dependent apoptotic pathway inducing the release of cytochrome c and other apoptogenic factors into cytoplasm [96].

Caspase 8 is an upstream caspase involved in extrinsic or death receptor apoptotic pathway. The ligand binding-induced trimerization of death receptors results in recruitment of the

receptor-specific adapter protein Fas-associated death domain (FADD) which then recruits caspase-8. Activated caspase-8 is known to propagate the apoptotic signal either by directly cleaving and activating downstream caspases or by cleaving the BH3 Bcl2-interacting protein, which leads to the release of cytochrome c from mitochondria, triggering activation of caspase-9 in a complex with dATP and Apaf-1. Activated caspase-9 then activates further downstream caspases, including caspase-8 [97].

Due to apoptotic stimulus when mitochondria released cytochrome c is bound to Apaf-1 together with dATP to form a complex which recruits the initiator caspase-9 leading to its activation. Activated caspase-9 cleaves downstream caspases such as caspase-3 initiating the caspase cascade [98].

Caspase-3, downstream or effector caspase is a frequently activated death protease, catalyzing the specific cleavage of many key cellular proteins. Caspase-3 is also required for some typical hallmarks of apoptosis, and is indispensable for apoptotic chromatin condensation and DNA fragmentation in all cell types. Thus, caspase-3 is essential for certain processes associated with the dismantling of the cell and the formation of apoptotic bodies, but it may also function before or at the stage when commitment to loss of cell viability is made [99].

3.10. Cell cycle analysis

The cell cycle is required for cell growth and cell division into two daughter cells. A eukaryotic cell cannot divide unless it replicates its genome (DNA) and then separates the duplicated genome. To achieve these tasks cells must perform DNA synthesis and mitosis. The cell cycle is an ordered set of events. The G1 phase is required for cell growth and preparation of DNA synthesis. In the S-phase the cell replicates the genome. The G2 is needed for cell growth and preparation for mitosis. The last phase is M is mitotic phase in

which cells segregate duplicated chromosomes. The cells are equipped with the checkpoints that are set at various stages of the cell cycle. When cells have DNA damages that have to be repaired, cells activate DNA damage checkpoint that arrests cell cycle. According to the cell cycle stages, DNA damage checkpoints are classified into at least 3 checkpoints: G1/S (G1) checkpoint, intra-S phase checkpoint, and G2/M checkpoint. Upon perturbation of DNA replication by drugs that interfere with DNA synthesis, DNA lesions or obstacles on DNA, cells activate DNA replication checkpoint that arrests cell cycle at G2/M transition until DNA replication is complete. There are more checkpoints such as Spindle checkpoint and Morphogenesis checkpoint. The spindle checkpoint arrests cell cycle at M phase until all chromosomes are aligned on spindle. This checkpoint is very important for equal distribution of chromosomes. Morphogenesis checkpoint detects abnormality in cytoskeleton and arrests cell cycle at G2/M transition [100]. Anticancer compounds can cause damage to the intracellular proteins and nucleic acids of the cell and may lead to arrest of cell cycle progress in that particular phase under the tight supervision with the aid of check points.

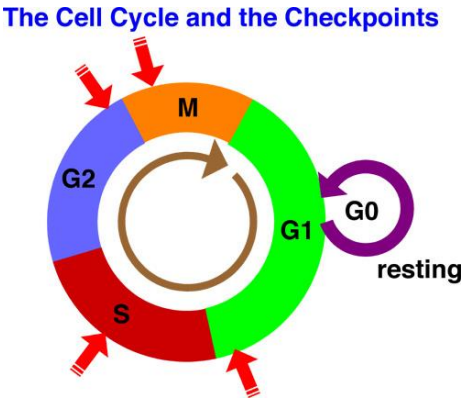


Figure 15. Cell cycle and the cell cycle check points

4. Results and Discussion

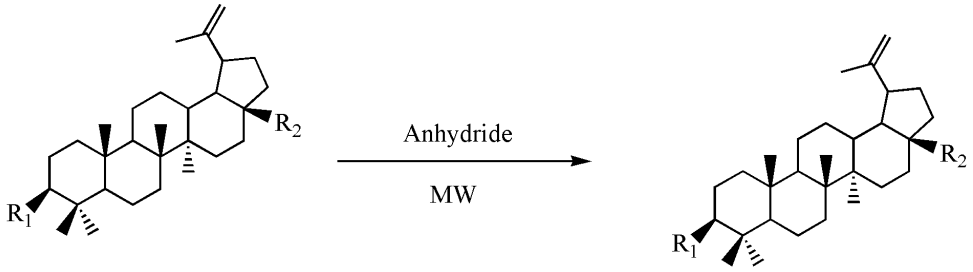
There exists a great deal of interest in probing the structural features responsible for the pharmacological effects of betulin (**1**) and betulinic acid (**2**) and to further optimize its activity profile. As a result, numerous derivatization studies have been performed leading to the production of an array of their analogs. The synthesis and *in vitro* antitumor activity of thirty four derivatives of betulin and betulinic acid are described and discussed. The derivatives are named in the following way: the number signifies the parent compound, *viz.* betulin (**1**) and betulinic acid (**2**) and the alphabetic character denotes its respective derivatives. All the compounds were characterized by ^{1}H and ^{13}C NMR and LC/MS spectroscopy. The derivatives of betulin and betulinic acid were tested for cytotoxicity evaluation against 15 different tumor cell lines and the effect of their structural variations on anticancer activity as well as their tumor selectivity in comparison with normal cells has been studied. A broad panel of tumor cell lines was necessary to find specific toxicity towards particular tumors. In the discussion part only the most active compounds are given (Table 1), the extensive and detailed findings of investigation are given in the publications (appendices 1-5). The mode of cell death along with alterations in caspase activity and cell cycle perturbations caused by the derivatives have been investigated.

4.1. Esters of betulin and betulinic acid *(Appendix 1 and 2)*

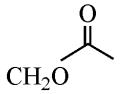
Esters of betulinic acid can be easily prepared due to the presence of hydroxyl and carboxylic acid groups in the main skeleton and were found to be highly active against various types of cancer cell lines [81–82]. Some esters of betulin and betulinic acid were prepared using a single mode lab scale microwave in a closed vessel under pressure (Scheme 2). It is more advantageous to perform a synthesis under pressure in a microwave than in an open vessel

because an increase in boiling points of solvents is more significant with a modest raise of pressure. The temperature versus pressure curves have points of inflection beyond which considerable increase in pressure afford relatively modest elevations. These properties indicate that reaction pressures of 2-3 MPa would facilitate temperatures in the order of 200°C for common solvents such as methanol, methylene chloride or ethanol all of which boil below 85 °C. These benefits could also be obtained from conventional heating procedures but there are heat losses with relative increase of temperature. This could not be minimized even when a number of heating and cooling steps are included in the process. With the appropriate vessels, microwaves would be absorbed predominantly throughout the sample, in addition, the energy could be applied or withdrawn instantaneously and the input could be adjusted readily to match the requirements. Thus it appeared likely that direct bulk heating combined with efficient stirring of the sample would diminish temperature gradients thereby resulting in higher yields in lesser reaction times. The primary hydroxyl group at C-28 or secondary hydroxyl group at C-3 or both of betulin and the β-hydroxyl group at C-3 position in betulinic acid were reacted with corresponding anhydrides to obtain esters of betulin (**1a** – **1f**) and betulinic acid (**2a** – **2d**). Most of the reactions were performed under full power of the microwave except the acetylation of β-hydroxyl group at C-3 which was done at 200 W. The commonly used solvent in all the reactions was dichloromethane as it has a very low dielectric loss or the loss factor which is of the order of 1/1000, therefore it has very less resistance to the movement of either polar molecules or ionic species. Hexane was used in the synthesis of compound **1d** and **2b** *i.e.* 28-*O*-chloracetyl betulin and 3-*O*-chloracetyl betulinic acid in order to avoid differential heating inside the microwave as it was speculated that the chloro acetic anhydride used in the reaction has a high microwave absorption. The ramp time and hold time were estimated depending upon the volume of the contents of the reaction vessel. The temperature chosen for reactions was 25 degrees higher boiling point of the

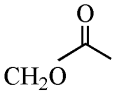
solvent and the reaction hold time in the cavity of microwave was between 20 and 45 min in all pressurized reactions. *(Appendix 1 and 2)*

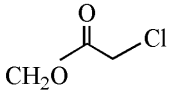


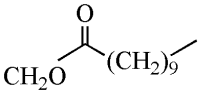
R_1 = OH R_2 = CH_2OH (**1**) or COOH (**2**)

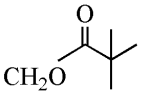
1a R_1 =  R_2 = 

1b R_1 =  R_2 = CH_2OH

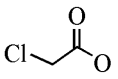
1c R_1 = OH R_2 = 

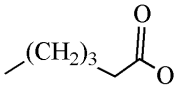
1d R_1 =  R_2 = 

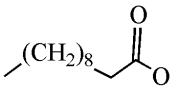
1e R_1 = OH R_2 = 

1f R_1 =  R_2 = 

2a R_1 =  R_2 = COOH

2b R_1 =  R_2 = COOH

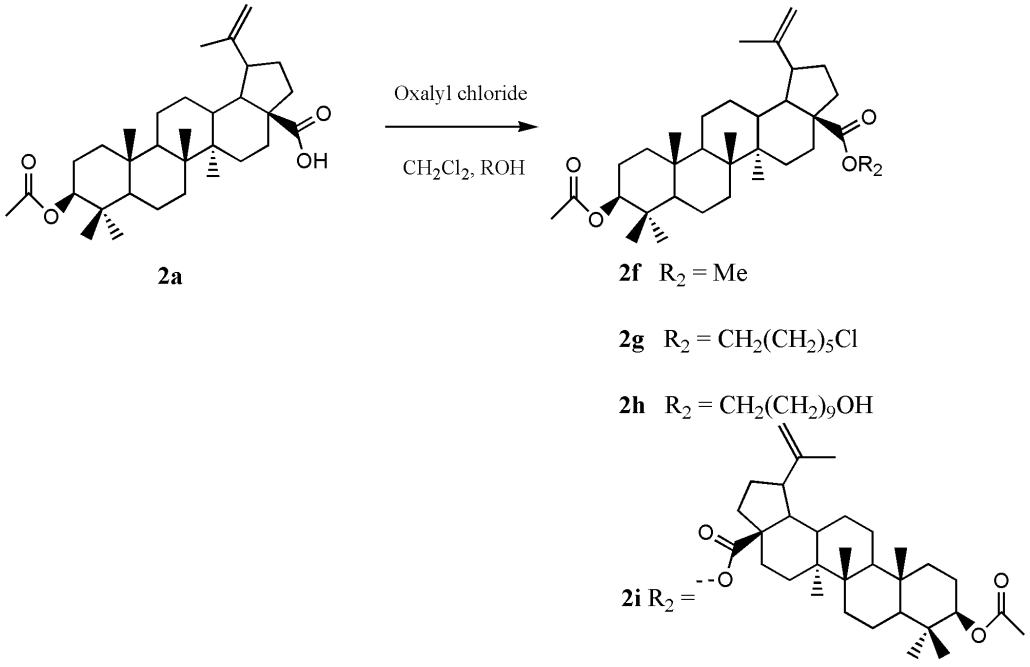
2c R_1 =  R_2 = COOH

2d R_1 =  R_2 = COOH

Scheme 2. Esters of betulin and betulinic acid

In attempts to derivatize C-28 position (carboxylic acid) of betulinic acid long and short chain esters were synthesized (Scheme 3). Methyl betulinate (**2e**) was prepared using diazomethane in ether. Similarly, (3-*O*-acetyl)betulinic acid (**2a**) was converted into its acid chloride by oxalyl chloride and reacted with methanol, 6-chloro-1-hexanol and 1,10-decanediol in excess to obtain the esters **2f**, **2g and 2h** respectively. The anhydride of 3-*O*-acetyl betulinic acid

(**2i**) is obtained as a side product during the synthesis of **2h** and also by refluxing with acetic anhydride and pyridine.



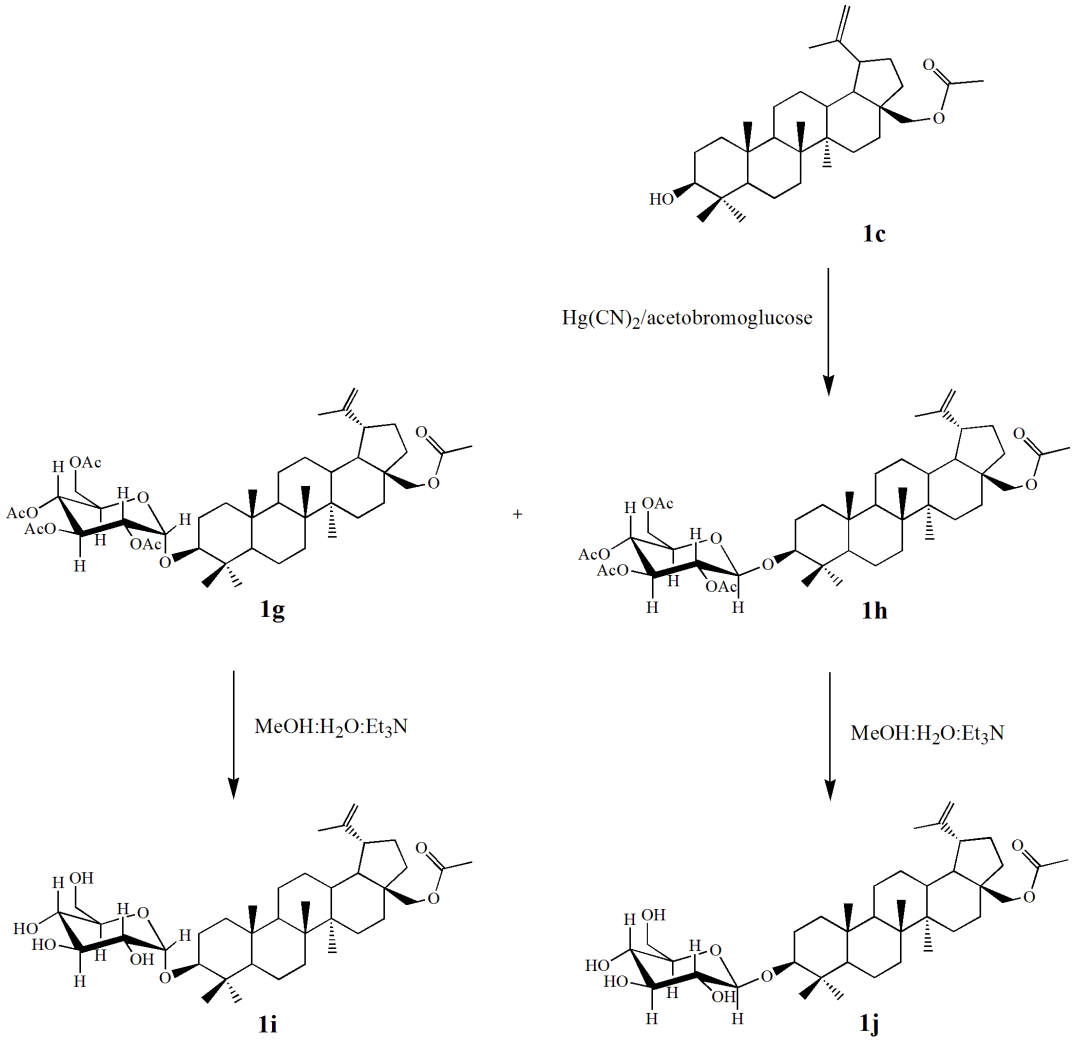
Scheme 3. Esters of 3-*O*-acetyl betulinic acid

Derivatization of C-3 and C-28 positions in betulin and betulinic acid into short and long chain alkyl esters has yielded some interesting compounds. In betulin, esterification of both primary and secondary hydroxyl group into short or branched chain alkyl groups resulted in less toxic derivatives. Selective modification of C-3 group into acetyl group lead to activity similar to betulin on the other hand 28-acetoxy betulin showed activity similar to betulinic acid. Furthermore, derivatization of C-28 with long and medium chain esters was resulted in moderately active compounds. Substitution of chloroacetyl group (**1d**) improved cytotoxicity. Esters of betulinic acid (C-28 derivatives) with short or long chain alkyl groups rendered to be less or non toxic. Esterification of C-3-hydroxy group with acids containing medium and long chain alkyl groups showed moderate or less activity whereas ester with chloroacetyl side chain yielded a potentially toxic derivative (**2b**). **1d** was found to be more active than

betulinic acid on almost all the cell lines used for cytotoxicity evaluation studies. Moreover it was 6 fold more toxic than **2** on cervical cancer cell line (A431). Similarly **2b** was up to 10 times more cytotoxic than betulinic acid and the best activity was observed on anaplastic thyroid cancer cell line (SW1736; IC_{50} = 1.1 μM) (Table 1). Betulin, **1** itself is considered inactive against several cancer cell lines [31]. This may be because of the absence of carbonyl group at, or in the vicinity of C-28 as was observed in case of conversion of the primary alcohol to acid in betulinic acid and also esterification of this position has resulted in derivatives with moderate to good activity depending on the substituent. Conversely, the modification of C-3 (β-hydroxyl) and C-28 (carboxylic groups) positions of betulinic acid into medium or long chain alkyl esters has lead to a fall in activity. The decrease of activity may be due to electron donating property and also makes the derivative more non polar because of long or medium chain bulky group substitutions. The high toxicity of 28-*O*-chloroacetyl betulin (**1d**) and 3-*O*-chloroacetyl betulinic acid (**2b**) could be due to electro negativity of chlorine atom in the molecule which also contributes to an increase in polarity of the derivative. The hypothesis that short chain esters of betulin and betulinic acid result in more polar and cytotoxic derivatives are also supported from the instances in the literature [31, 36] where short chain amino acid esters of betulin and betulinic acid were found to be more cytotoxic than their longer chain counterparts. In a similar way, short chain fatty acid esters at C-3 of betulinic acid were found to show significant potency as antitumor-promoters than long chain fatty acid substituents in EBV-EA activation assay using Raji cells [82]. The 3-*O*-acyl betulinic acid derivative having an electron withdrawing group in aromatic ring at C-3 side chain is a more active anti-angiogenic agent on endothelial (ECV304) cell line than the compounds with bulky pentyl or heptyl group or electron donating group in the aromatic ring [101]. *(Appendix 1 and 2)*

4.2. α - and β-D-glucopyranose betulin anomers *(Appendix 3)*

Cancer cells consume glucose at a much higher rate than normal cells, but use very little glucose to produce energy, spending the rest instead on cell-building material. Moreover glucose is readily passes through the blood brain barrier, hence a betulinic acid derivative containing glucose can be used to target the nervous tissue tumors in brain. Since the cancer cells can be targeted with glucose adjuvants, betulin derivatives substituted with D-glucopyranose on secondary hydroxyl group at C-3 position were synthesized. A slightly modified method already reported has been used [102]. **1c** was reacted with tetra-*O*-acetyl-α-D-glucopyranosylbromide and mercury cyanide in dry toluene for 48 h to obtain 28-*O*-acetylbetulin-3-yl-α-D-tetra-*O*-acetyl glucopyranoside (**1g**) and 28-*O*-acetylbetulin-3-yl-β-D-tetra-*O*-acetyl glucopyranoside (**1h**). A mixture of methanol, water and triethylamine for 4 h at 50 °C was used for deacetylation of these compounds to obtain 28-*O*-acetylbetulin-3-yl-α-D-glucopyranoside (**1i**) and 28-*O*-acetylbetulin-3-yl-β-D-glucopyranoside (**1j**) (Scheme 4).



Scheme 4. Synthesis of α - and β-D-glucopyranose betulin anomers

Crystals of the 28-*O*-acetylbetulin-3-yl-β-D-(2',3',4',6'-tetra-*O*-acetyl)glucopyranoside (**1h**) suitable for X-ray diffraction measurements were obtained by the slow evaporation of petrol ether from the solution (Figure 16). **1h** was crystallized in triclinic crystal system and *P*1 space group. The asymmetric unit of **1h** consists of two crystallographically independent molecules. The bond lengths in **1h** are in expected range for analogous structures [103–104]. All the ring junctions in the triterpenoid are *trans*-fused. In both molecules, the cyclohexane rings adopt chair conformations. In one of the molecules cyclopentane ring has an envelope conformation with atom C17 displaced from the C18/C19/C21/C22 plane by 0.277 (2) Å,

while in the other cyclopentane ring is twisted on C17a–C18a bond. The molecular structure is stabilized by both intermolecular and intramolecular C–H···O hydrogen bonds. *(Appendix 3)*

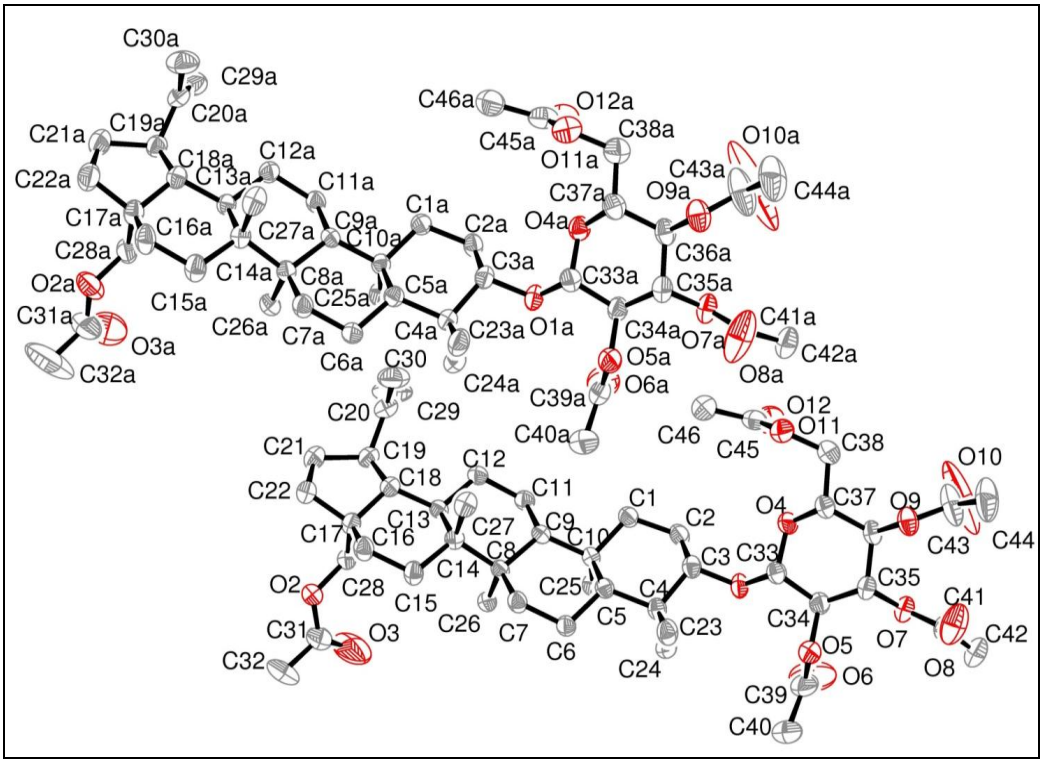


Figure 16. Crystal structure of 28-*O*-acetylbetulin-3-yl-β-D-(2',3',4',6'-tetra-*O*-acetyl)glucopyranoside

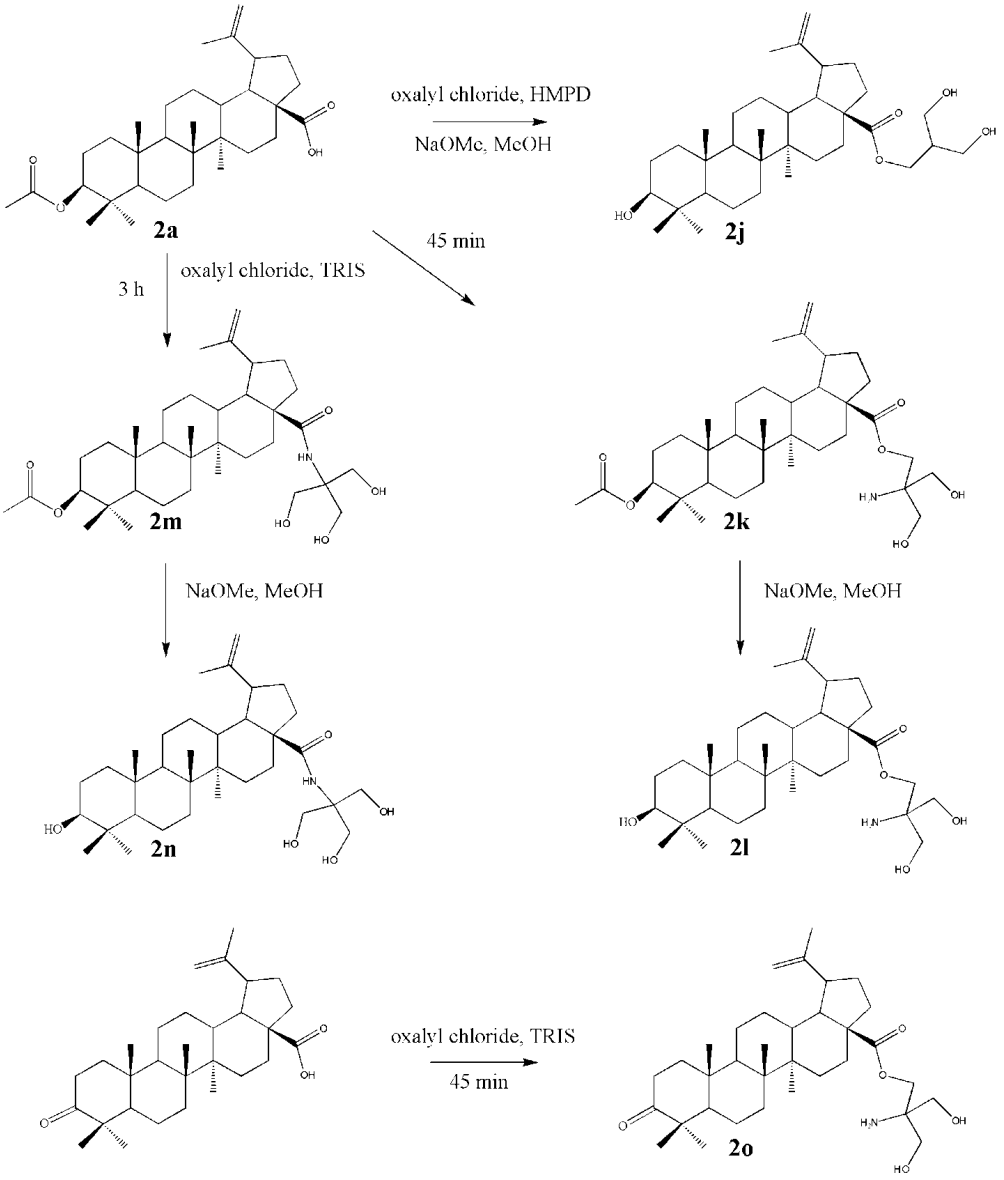
The synthesis of glycosides is an interesting approach to enhance the hydrosolubility and consequently the pharmacological and pharmacokinetic properties of lead compounds [105]. The acetylated D-glucopyranose derivatives of 28-*O*-acetyl betulin did not show any cytotoxicity within the concentration range used for cytotoxicity evaluation studies. The acetyl groups of the glycoside moiety are later converted into free hydroxyl group to obtain **1i** and **1j** which showed slightly better activity than betulinic acid (**2**) on most of the cell lines used and **1i** was found to be most active on DLD-1 (colon cancer) cell line and the compound **1j** on HCT-116 (colon cancer) cell line. Interestingly, both α and β anomers of 28-*O*-

acetylbetulin-3-yl-D-glucopyranoside were equally toxic to the panel of cancer cell lines used, which infers that this small structural variation does not induce significant differences in their toxicities (Table 1). 28-*O*-β-D-glycoside of betulinic acid did not exhibit any significant cytotoxicity [85], hence attempts were made to synthesize C-3 derivatives. The compounds **1i** and **1j** exhibited better cytotoxic activity than C-3 sugar derivatives of betulin and betulinic acid reported in literature [38]. A high rate glycolysis that occurs in solid tumors cells is explained by Warburg effect [106]. Cancer cells often switch glucose metabolism from tricaboxylic acid (TCA) cycle to anaerobic glycolysis for ATP production due to which there exists a 19-fold increase in glucose consumption in cancer cell for the energy needs [107–108]. Thus a greater affinity of cancer cells for glucose aids the accumulation of D-glucopyranoside derivatives inside the cells. A limited number of studies have examined changes in the cytotoxicity profile of betulin and betulinic acid derivatives which have been substituted with sugars. It was found that substitution of rhamnose and mannose at C-3 on betulin has improved the cytotoxicity and rhamnose and arabinose derivatives substituted at C-3 position on betulinic acid [38] whereas a C-28 substitution in betulinic acid with glucopyranose group has resulted in a fall of cytotoxicity [85].

4. 3. C-28-esters and -amides from 2-(hydroxymethyl)propane-1,3-diol and 2-amino-2-(hydroxymethyl) propane-1,3-diol *(Appendix 4)*

Further efforts have been made to synthesize C-28 derivatives of 3-*O*-acetyl betulinic acid (**2a**) and betulonic aid. Unlike the derivatives prepared in section 4.1 where short and long alkyl chain esters which increase the lipophilicity of the molecule, esters and amides with groups that increase polarity were synthesized. The acid chloride of 3-*O*-acetyl betulinic acid (**2a**) was reacted with 2-(hydroxymethyl)propane-1,3-diol for 45 min and then refluxed with

sodium methoxide overnight. The reaction was neutralized by H^+ ion exchange resin in order to obtain **2j**. Similarly 3-O-acetyl betulinic acid chloride when reacted with 2-amino-2-(hydroxymethyl)propane-1,3-diol for 45 min yielded **2k** and when the reaction time was prolonged to 3 h **2m** was obtained. The compounds **2k** and **2m** were stirred under reflux with sodium methoxide overnight and neutralized with H^+ ion exchange resin yielded **2l** and **2n** respectively. Betulonic acid was converted to its acid chloride and treated with 2-amino-2-(hydroxymethyl)pro-pane-1,3-diol for 45 min under dry conditions to obtain **2o** (Scheme 5). Previous investigations suggested that presence of carbonyl group at C-28 was necessary for preserving cytotoxicity [31, 83–84]. Various methyl esters and free acids of amide derivatives of amino acids were prepared and tested for cytotoxicity against the melanoma (MEL-2) cell line, which was comparable to that of betulinic acid [83]. Our investigations (discussed earlier in this chapter) showed that esterification of C-28 carboxylic acid group of betulinic acid with short and long chain esters resulted in a loss or decrease of cytotoxic activity compared to betulinic acid. In contrast, preparation of esters which increase the polarity of the molecule have been performed. Esters of **2a** and betulonic acid with 2-amino-2-(hydroxymethyl)propane-1,3-diol were found to be highly cytotoxic and the corresponding amide **2m**, **2n** showed moderate toxicity on the cancer cell lines (Table 1). Therefore, the presence on an acetyl or ketone group at C-3 and a free amine containing ester at C-28 on betulinic acid skeleton seems to be necessary to render good antiproliferative activity. (*Appendix 4)*

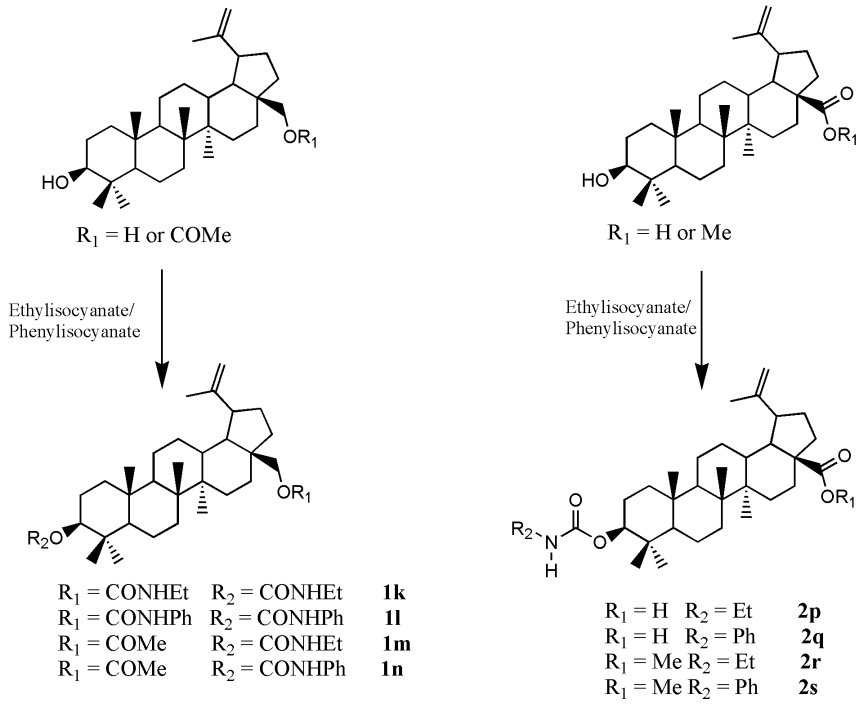


Scheme 5. Synthesis route to esters and amides of Betulinic acid and Betulonic acid with 2-(hydroxymethyl)propane-1,3-diol and 2-amino-2-(hydroxymethyl) propane-1,3-diol

4. 4. Carbamate derivatives of betulin and betulinic acid *(Appendix 5)*

It was reported that novel thiocarbamate derivatives of betulinic acid were highly cytotoxic against cancer cell lines and seem to elucidate anticancer activity by inhibition of topoisomerses [109]. A series of new ethyl and phenyl carbamates of betulin (**1k**–**1n**) and

betulinic acid (**2p**–**2s**) have been synthesized with an intention to investigate the effects of aromatic and aliphatic substitutions of the lead molecules on antitumor activity. The primary and secondary hydroxyl groups of betulin (**1**) and 28-*O*-acetyl betulin (**1c**) and only secondary hydroxyl group in betulinic acid (**2**) and methyl betulinate (**2e**) are modified into ethyl and phenyl carbamates (Scheme 6). **1**/**1c**/**2**/**2e** is reacted with two fold excess of ethyl or phenyl isocyanate in dry chloroform. The reaction mixture was refluxed for 48 h at 60–65 **°**C and later quenched with water for 15 min. The crude mixture is extracted and separated using flash chromatography to obtain ethyl and phenyl carbamates **1k**–**1n** and **2p**–**2s**. (*Appendix 5)*



Scheme 6. Synthesis of carbamate derivatives of betulin and betulinic acid

In a quest to find new lead structures for the synthesis of potential antitumor agents, a series of ethyl and phenylcarbamate derivatives of betulin and betulinic acid have been prepared. The phenylcarbamates **1l**, **1n**, **2q**, **2s** were less or non-toxic within the concentration range used, whereas ethylcarbamates were show moderate to good activity. The compounds **1k** and

1m were 2–8 fold more cytotoxic than betulinic acid on the investigated cell lines with lung cancer cell line (A549) being most sensitive to both these compounds (Table 1). Introduction of an imidalzole scaffold with a carboxylic ester bond into the lupane skeleton [37] yielded similar toxicities as **1k** and **1m**. The reason for low activity of the phenylcarbamates can be due the straight and rigid phenyl groups. Possible interactions with the π-system of the phenyl ring lead to a much more bulky molecule compared to the ethylcarbamates with a lower ability to penetrate the cell membrane which is prerequisite for the biological activity for betulin and betulinic acid derivatives.

4.5. Most active compounds among the synthesized derivatives

Table 1. IC_{50} values of the most active derivatives from each class of compounds

Compound	**2**	**1d**	**2b**	**1i**	**1j**	**2k**	**2m**	**1k**	**1m**
518A2	8.1±0.1	9.9±0.2	8.4±0.4	9.0±0.1	9.6± 0.1	2.9±0.1	29.6±0.1	5.1±0.5	8.2±0.8
8505C	7.3±1.0	5.0±0.7	9.0±2.3	8.2±0.5	8.1±0.6	-	-	5.0±0.5	9.4±0.9
A253	9.2±0.1	4.7±0.5	7.3±1.0	8.4±0.3	9.0±0.2	2.0±0.2	29.5±0.1	4.9±0.5	9.7±1.0
A431	12.6±0.3	2.2±0.4	2.8±0.7	9.4±0.8	9.4±0.1	2.5±0.1	10.3±0.1	5.0±0.5	6.1±0.6
A549	11.1±1.8	11.0±0.7	3.0±0.5	9.9±0.1	9.7±0.1	3.4±0.1	30.5±0.7	1.7±0.7	2.9±0.3
A2780	11.1±0.4	4.6±0.8	4.5±1.9	9.1±0.3	9.7±0.1	3.6±0.6	10.1±0.1	4.3±0.4	4.4±0.4
DLD-1	11.9±0.3	4.6±0.8	3.7±0.4	4.4±0.2	5.4±1.6	-	-	6.9±0.7	8.3±0.8
FaDu	10.2±0.1	19.5±1.6	5.2±0.7	7.4±0.4	7.6±1.8	-	-	4.6±0.4	7.9±0.8
HCT-8	13.1±0.5	12.6±1.9	2.0±0.9	10.2±0.1	9.9±0.1	-	-	4.0±0.4	3.9±0.4
HCT-116	10.8±0.2	4.1±0.7	1.9±0.5	7.5±1.4	5.3±1.6	-	-	4.3±0.4	3.8±0.4
HT-29	13.9±0.5	27.6±1.9	3.6±0.3	9.8±0.1	10.1±0.1	2.4±0.1	10.0±0.1	5.1±0.5	6.2±0.1
Liposarcoma	12.1±0.6	8.5±0.3	9.9±1.1	9.1±0.1	9.6±0.1	-	-	5.3±0.5	12.0±1.0
MCF-7	12.3±0.2	8.1±5.4	7.9±1.4	7.9±0.7	8.6±0.1	2.8±0.1	29.4±0.8	6.0±0.6	17.7±1.6
SW480	6.5±0.1	7.4±0.7	4.1±0.3	9.5±0.1	9.6±0.2	-	-	1.8±0.2	2.8±0.2
SW1736	13.1±0.5	3.5±0.7	1.1±0.3	9.6±0.1	9.4±0.1	3.7±0.1	10.2±0.9	3.1±0.3	3.6±0.3
Ref	*App.1& 2*	*App.1& 2*	*App.1& 2*	*App. 3*	*App. 3*	*App. 4*	*App. 4*	*App. 5*	*App. 5*

The most active compounds from the synthesized derivatives include 28-*O*-chloroacetyl betulin **1d**, 3-*O*-chloroacetyl betulin **2b**, α- and β-D-glucopyranoside anomers of 28-*O*-acetyl

betulin **1i** and **1j**, 2-amino-3-hydroxy-2-(hydroxymethyl) propyl (3-*O*-acetyl)betulinate **2k**, bisethyl carbamate of betulin **1k**, 3-*O*-ethyl carbamate of 28-*O*-acetyl betulin, **1m**. The presence of a carbonyl group at C-28 position in betulin derivatives seems to be necessary for good cytotoxicity. The order of average cytotoxicity of active derivatives synthesized from highest to lowest can be given as

2k > **1m** > **2b** > **1k** > **1m** > **1i** > **1j** > **1d** > **2**

Even though all the active derivatives showed broad spectrum activity, the antiproliferative activity was highly pronounced in certain types of cancer. **2k** was able to inhibit cell proliferation by half at concentrations as low as 2 μM on head and neck cancer cells (A253). Similarly, **1d** showed high cytotoxic activity on cervical cancer cell line (A431) meanwhile interestingly, **2b** showed specific toxicity towards all types of colon cancer cell lines used in the study as well as anaplastic thyroid cancer (SW1736). α- and β-D-glucopyranoside anomers of 28-*O*-acetyl betulin **1i** and **1j** were the most active on DLD-1 whereas **1k** and **1m** with ethylcarbamate substituent were the most suitable against lung cancer.

4.6. Selectivity

It has been a primary motive of cancer chemotherapy to achieve the selective inhibition of tumor cells while minimizing toxicity to normal tissues. Lack of toxicity with significant antitumor activity leads to a favorable therapeutic index. A favorable therapeutic index is a definitive requirement for a new drug to be considered for development. Betulinic acid is selective towards tumor cells and was found to be non toxic to normal cells *in vitro* and well tolerated *in vivo* [50, 74]. It was important to ensure that derivatives of betulin and betulinic acid prepared show high degree of selective cytotoxicity towards tumor cells and are well tolerated by normal cells. The active derivatives were screened for toxicity on normal human fibroblasts (WWO70327) and were less toxic and more selective to tumor cell lines than

betulinic acid. The selectivity index is calculated according to the formula given in chapter 3 (Materials and methods). Glycoconjugates may be promising in cancer chemotherapy as they can selectively target the tumor cells by binding to specific transmembrane glucose transporters. Following preferential uptake of sugar conjugates into cancer cells, they are presumably subject to enzymatic cleavage by β-glycosidases to liberate the free active cytotoxic aglycones that act selectively on cancer cells and spare other noncancerous ones. The glucose derivatives of betulin (**1i** and **1j**) showed a similar magnitude of selectivity as **2**, whereas the tris ester of 3-*O*-acetyl betulinic acid, **2k** was not selective when compared to the tumor cell lines. Interestingly, the carbamate derivatives, **1k** and **1m** elicited 18 and 8 fold more specific cytotoxicity on lung cancer cells (A549), respectively, and chloroacetyl betulinic acid derivative **2b** was 6 fold more specific to anaplastic thyroid cancer cell line (SW1736) than betulinic acid (Table 2). Furthermore **1k** was not cytotoxic on normal cells even at a high concentration (100 µM). The underlying mechanism of selective antitumor activity of betulinic derivatives and betulinic acid is not clear. Transcription factor specificity proteins (Sp) regulate the expression of survivin which is an antiapototic protein. Downregulated survivin causes cytochrome c dependent PARP cleavage and promotes the caspase cascade. The Sp proteins are manifold upregulated in cancer cells compared to noncancerous cells [110]. Therefore, Sp proteins could be potential targets for betulin and betulinic acid derivatives (*Viz.* **1m**). A possible reason for selectivity could be a decrease in expression of these transcription factors in tumor cells which may ultimately cause the cell to commit suicide. (*Appendix 1–5*)

Table 2. IC_{50} values of betulinic acid and the most active derivatives from each class of compounds on human fibroblasts (WWO70327)

Compound	Fibroblasts IC_{50} [µM]
2	20.81
2b	10.31
1k	>100
1m	24.35

4.7. Apoptosis and Caspase activity

Apoptosis also known as programmed cell death occurs in multicellular organisms and involves a series of biochemical events that lead to a variety of morphological changes, including blebbing, changes to the cell membrane such as loss of membrane asymmetry and attachment, cell shrinkage, nuclear fragmentation, chromatin condensation, and chromosomal DNA fragmentation. Most drugs, regardless of mechanistic class and proven effectiveness in the clinical treatment of cancer, will induce apoptosis. Since clinically used compounds have some degree of selectivity for cancer cells versus normal cells, screening for apoptosis is a compelling alternative to target-based screening [111]. The apoptotic mode of cell death induced by the active derivatives from each series on the most sensitive cell lines was investigated and proved by dye exclusion test (trypan blue) and DNA fragmentation assay. The active derivatives **1d**, **2b**, **2k** and **2o** were found to induce programmed cell death on HT-29, colon cancer cell line. The floating cells from HCT-116 colon cancer cells after 24 h treatment with the IC_{90} concentrations of 3-*O*-glucopyranose anomers of 28-*O*-acetyl betulin, **1i** and **1j**, were able to exclude trypan blue and occurrences of DNA ladders confirmed apoptotic cell death. Similarly **1k** and **1m** were able to induce apoptosis in lung cancer cells (A549) (Figure 17). (*Appendix 1–5*)

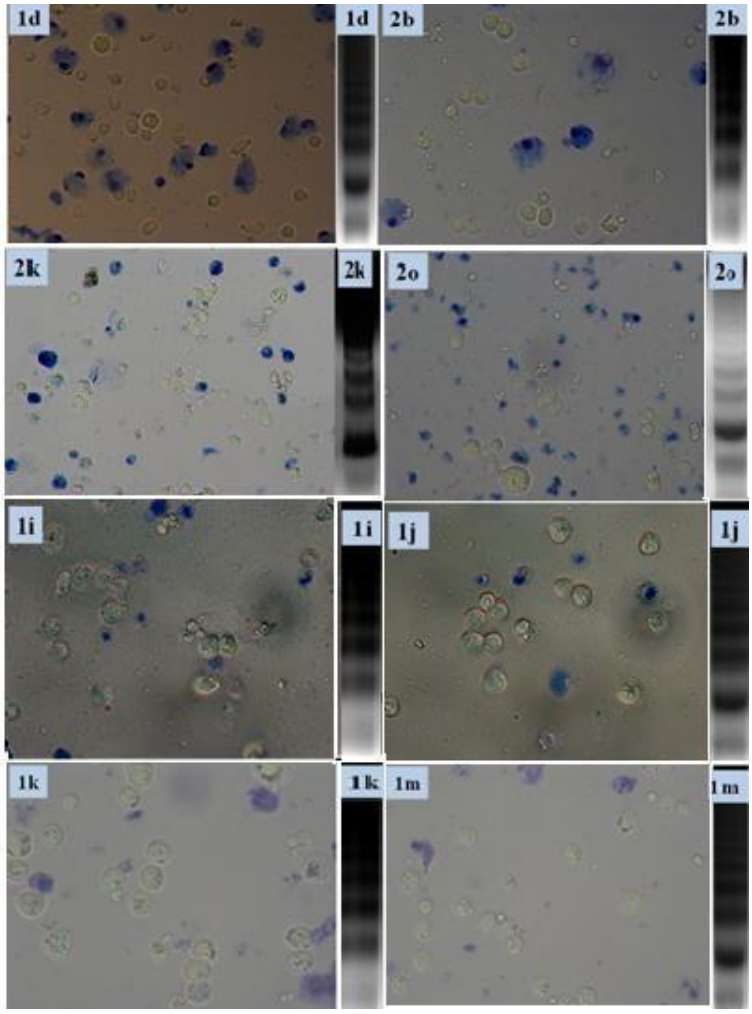


Figure 17. Trypan blue dye exclusion test and DNA fragmentation caused by **1d**, **2b**, **2k** and **2o** on HT-29 cells, **1i** and **1j** on HCT-116 cells and **1k** and **1m** on A549 cells

Apoptosis is an essential physiological process that plays a critical role in development and tissue homeostasis. The progress of apoptosis is regulated in an orderly way by a series of signal cascades under certain circumstances. The caspase-cascade system plays vital roles in the induction, transduction and amplification of intracellular apoptotic signals. Caspases, closely associated with apoptosis are aspartate-specific cysteine proteases and members of the interleukin-1β-converting enzyme family. Apoptosis initiator caspases, caspase 2, caspase 8 and caspase 9 are activated due to cellular stress, death receptor activation and cytochome c release respectively. Caspase 8 can also be activated by the release of cytochrome c for a

further amplification of apoptotic signal. Caspase-3 is an effector caspase and is a key factor in apoptosis execution, can be activated by caspase-8 and caspase-9 [95–99]. In order to understand the molecular changes of the active derivatives-induced apoptosis, we analyzed whether caspases are involved as initiators and effectors in the induced cell death on most sensitive cell lines for 2, 6 and 24 h treatment regimes. The experiments were conducted in triplicate and the standard deviation in all cases did not exceed 10% (data not presented in the figures).

The compounds **2b** and **1d** seem to upregulate caspase-9 significantly on HT-29 cell line, even though a small activation of caspase-8 was observed which may be due to cytochrome C. These results may indicate that compounds **2b** and **1d** traverse the mitochondrial pathway in apoptosis induction (Figure 18). On the other hand so significant caspase activity was observed on treatment with **2k** and **2o** on HT-29 cell line. Besides the classical caspase-dependent apoptosis, also caspase-independent programmed cell death may occur, maintaining key characteristics of apoptosis. Notably, **2k** and **2o** bypassed the caspase-dependent mechanism evinced by occurrence of floating cells showing typical DNA fragmentation despite no caspase activity after 24 h. Cellular responses to **2k** and **2o** indicate that caspases are nonessential effectors of its apoptotic program but facilitate its execution. They seem to be activated as downstream events and more as a sign rather than mechanistic feature of **2k**-triggered on colon cancer HT-29 cell death (Figure 18).

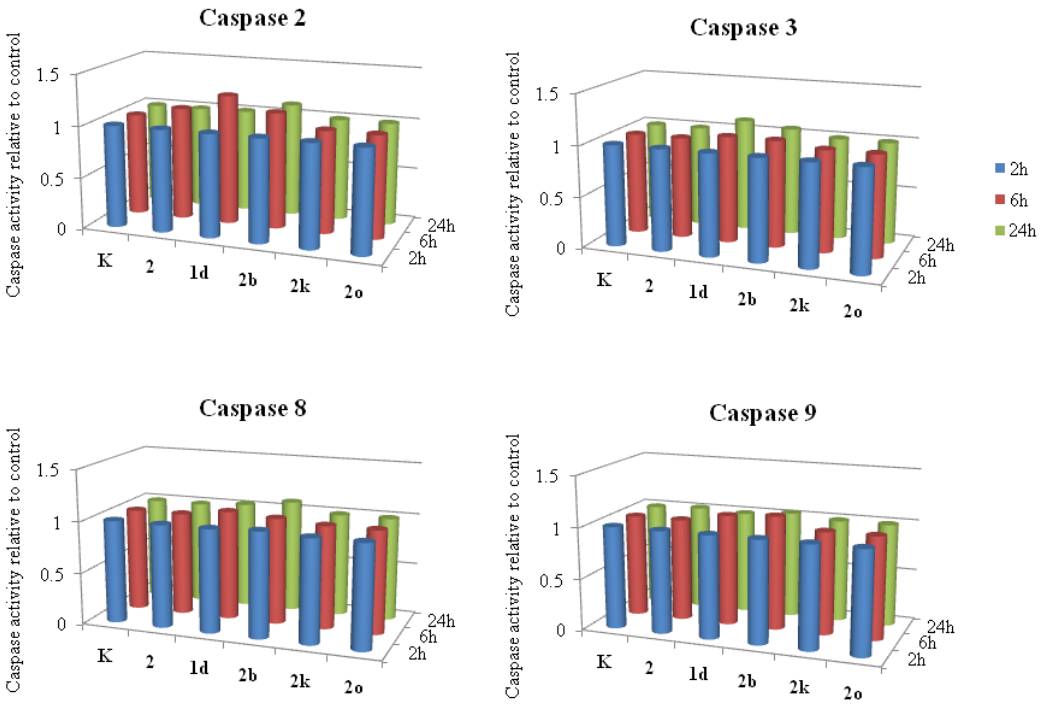


Figure 18. Caspase activity at 2, 6 and 24 h induced by **2**, **1d**, **2b**, **2k** and **2o** on HT-29 cell line (*Appendix 1, 2 and 4*)

Interestingly, 2 h treatment of HCT-116 cells with **1j** has activated only caspases-3 and -9, whereas a 6 h treatment activated caspases-2, -3 and -9. After 24 h treatment all the caspases were upregulated. This gives hint that apoptosis induced by **1j** may be through the mitochondrial or intrinsic pathway in this cell line. In case of compound **1i** caspase 8 has been activated at the end of 2 h exposure. Additionally, caspase-2 along with caspase-8 accumulation was observed for a 6 h treatment, while the caspase-9 seems to be upregulated on 24 h exposure (Figure 19). It can be inferred from these observations that small structural variations in α and β glucose anomers of betulin might effect to activate different caspase cascade pathways to induce apoptosis even though they show a same degree of cytotoxicity on HCT-116 colon cancer cells.

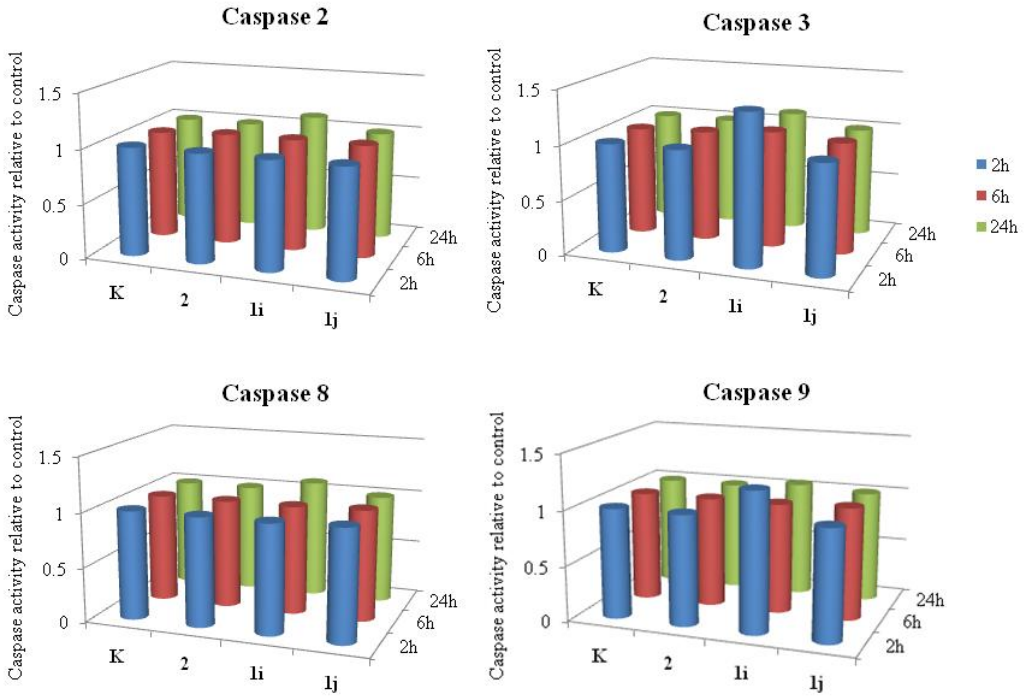


Figure 19. Caspase activity at 2, 6 and 24 h induced by **2**, **1i** and **1j** on HCT-116 cell line (*Appendix 3*)

The new carbamate derivatives, **1k** and **1m** were found to activate all the caspases used for investigations even for short exposure times (Figure 20) and seem to regulate the sequential events of initiator caspase 2 and caspase 8 followed by caspase 9 and caspase 3 due to stress induced apoptosis involving mitochondrial damage in lung cancer cell line (A549) similar to ceramide or etoposide induced apoptosis [112]. The anticancer activity of carbamate derivatives may be mediated by apoptotic cell death after cellular damage passes a critical threshold level. Both the intrinsic mitochondrial-interceded pathway and the extrinsic death receptor-induced pathway culminate in initiation of caspase cascade and in an irreversible cellular commitment to and execution of apoptosis. (*Appendix 5*)

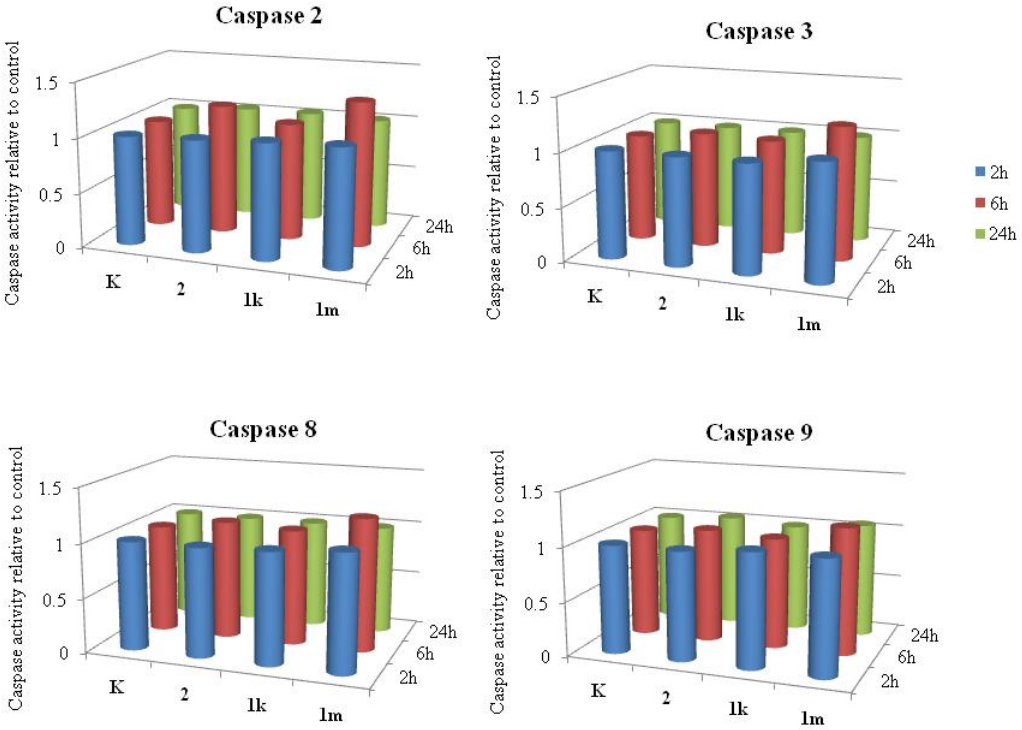


Figure 20. Caspase activity at 2, 6 and 24 h induced by **2**, **1k** and **1m** on A549 cell line (*Appendix 5*)

4.8. Cell Cycle Analysis

Cell cycle perturbations induced by the active compounds **2k**, **1i**, **1j** and **1k** on HT-29, HCT-116 and A549 cell lines respectively were analyzed. Exponentially growing HT-29 cells were treated with IC_{90} concentration of **2k** for 24 and 48 h and when compared to control, the compound (IC_{90}; 24 h) caused no changes in G1 and S phases but a decrease in the number of cells in G2/M-phase with an increase in number of apoptotic cells (SubG1-peak) whereas 48 h did not induce significant cell cycle arrest in any cell cycle phase but rather directly provoked an increase of number of apoptotic cells with concomitant decline of all other cell cycle phases indicating that apoptosis caused by **2k** on HT-29 cells may be due to disturbances caused in G2/M phase in the cell cycle. Similarly, HCT-116 cells were treated

with equitoxic IC_{50} and IC_{90} concentrations of **1i** and **1j** for 24 and 48 h. Interestingly, **1i** was found to show cytostatic effect at lower concentrations (IC_{50}) even after 48 h treatment as the number of cells in sub G1 phase were comparable to control whereas a cytotoxic effect with higher concentrations (IC_{90}) which could be envisaged by an increase in number of apoptotic cells. On the other hand **1j** showed cytotoxic effect at low and high concentrations without arrest in any specific phase of cell cycle. Likewise, A549 cells when treated with IC_{50} concentration for 24 and 96 h with bisethylcarbamate of betulin, **1k** no significant changes were observed at the end of 24 h but directly provoked a small increase of number of apoptotic cells with a decline in the number of cells in G1 phase when compared to control (Table 3). (*Appendix 1–5*)

Table 3. Impact of treatment with **1i, 1j, 1k** and **2b** on cell cycle progression in human tumor cell lines HCT-116, A549 and HT-29 cells

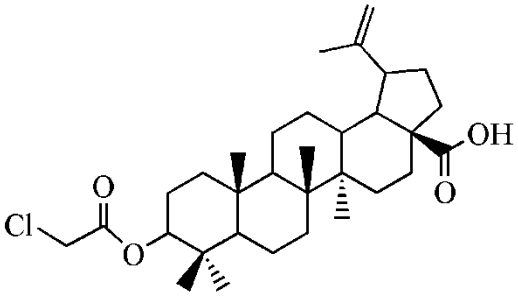
	Sub G1	G1	S	G2/M
HCT-116				
Control 24h	0.53 ± 0.03	19.80 ± 2.03	46.53 ± 6.70	33.55 ± 7.90
Control 48h	2.47 ± 0.71	32.58 ± 4.45	45.58 ± 7.21	19.37 ± 5.82
1i IC_{50} 24h	1.97 ± 0.65	26.58 ± 4.28	49.96 ± 5.45	21.90 ± 3.63
1j IC_{50} 24h	2.15 ± 0.29	25.23 ± 3.15	49.25 ± 9.22	23.84 ± 4.80
1i IC_{50} 48h	7.34 ± 0.96	39.71 ± 2.11	23.23 ± 5.40	29.83 ± 3.66
1j IC_{50} 48h	38.62 ± 5.30	19.75 ± 3.42	27.55 ± 6.18	14.58 ± 2.80
1i IC_{90} 24h	72.30 ± 8.42	9.19 ± 1.91	13.22 ± 2.22	5.54 ± 1.68
1j IC_{90} 24h	67.59 ± 6.86	7.21 ± 0.88	15.78 ± 3.50	9.64 ± 2.72
1i IC_{90} 48h	83.89 ± 11.20	8.44 ± 1.09	7.38 ± 1.46	0.50 ± 0.11
1j IC_{90} 48h	90.39 ± 14.53	4.72 ± 0.68	4.35 ± 0.78	0.70 ± 0.23
HT-29				
Control 24h	2.95 ± 1.54	45.40 ± 3.58	21.62 ± 2.93	30.12 ± 4.88
Control 48h	2.33 ± 1.90	46.22 ± 5.40	37.24 ± 3.86	14.62 ± 3.44
2b IC_{50} 24h	25.11 ± 2.10	38.41 ± 3.90	19.83 ± 2.28	16.39 ± 3.60
2b IC_{50} 48h	60.66 ± 9.00	18.07 ± 4.24	13.85 ± 2.12	7.60 ± 2.38
A549				
Control 24h	10.99 ± 2.80	19.55 ± 4.22	38.08 ± 7.18	19.95 ± 1.88
Control 96h	18.20 ± 4.64	18.44 ± 4.80	18.80 ± 1.90	45.03 ± 6.24
1k IC_{50} 24h	16.66 ± 3.80	14.05 ± 2.14	30.39 ± 2.48	39.50 ± 4.72
1k IC_{50} 96h	40.27 ± 7.30	9.20 ± 1.33	19.75 ± 5.44	31.25 ± 5.83

5. Conclusion

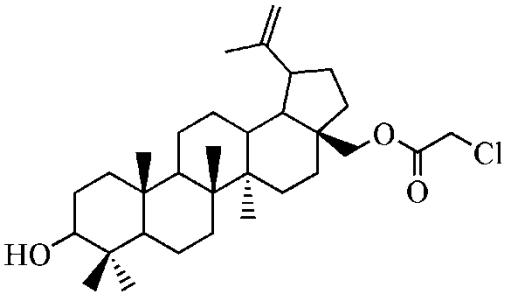
The preclinical development of bioactive natural products and their analogs as chemotherapeutic agents is a major objective of anticancer research programs. Betulinic acid and betulin are abundantly available in nature and are ascribed with many biological properties, but they are highly regarded for their anticancer activity. Various modifications of substituents at positions C-3, C-20 and C-28 of betulinic acid and betulin have been the subject matter of all research efforts to obtain potent lead compounds. Modifications have been performed on C-3 and C-28 positions of betulinic acid as it was observed and reported that any modifications at C-20 would either cause no change in the cytotoxic effect or lead to a fall in anticancer activity. Novel betulin and betulinic acid derivatives have been synthesized and characterized (NMR and LC/MS spectroscopy). Furthermore, all new compounds, along with parent compound betulinic acid have been tested on different tumor cells lines. From each group of derivatives selectivity index of the most active compounds have been determined. In addition, mode of cell death along with involvement of caspases has been studied. The cell cycle perturbation caused by potential sublead compounds has also been investigated. The results can be summarized as follow:

1. The compounds **1d**, **2b**, **2k**, **2o, 1i**, **1j**, **1m** and **1k** display a cytotoxicity superior to that of natural triterpenes betulin and betulinic acid.
2. The presence of carbonyl group at C-28 seem to be necessary to preserve the cytotoxic activity and esterification of carboxylic acid on 28 position with short or long chain alkyl esters results in less toxic derivatives. In the same way esterification of 3-β-hydroxy group with medium and long alkyl chains causes a lowering of toxicity. The compounds with chloroacetyl side chain at C-3 in betulinic acid (**2b**) and at C-28 in betulin (**1d**) were

found to be selectively cytotoxic on tumor cells and traverse intrinsic mitochondrial pathway in apopto**si**s induction.

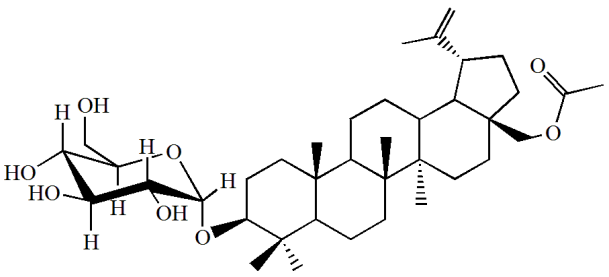


2b

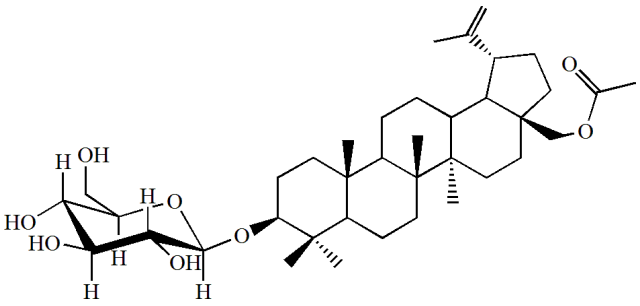


1d

3. The anticancer activity was slightly improved upon derivatization of C-3 of 28-*O*-acetyl betulin into corresponding α and β anomers of D-glucopyranose (**1i** and **1j**) when compared to betulinic acid and they seem to induce apoptosis by activation of different caspase cascade pathways. Moreover at lower concentrations *α* anomer seem to show a cytostatic effect unlike the β anomer which was cytotoxic. The crystal structure of acetylated α anomer of D-glucopyranose derivative of betulin has been described.



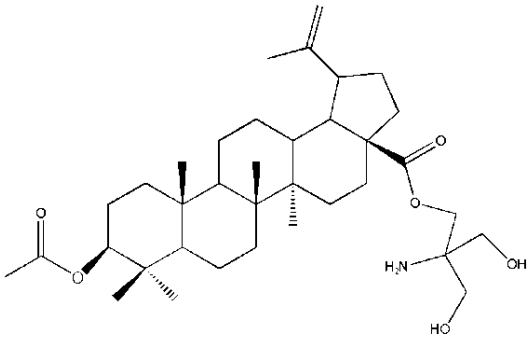
1i



1j

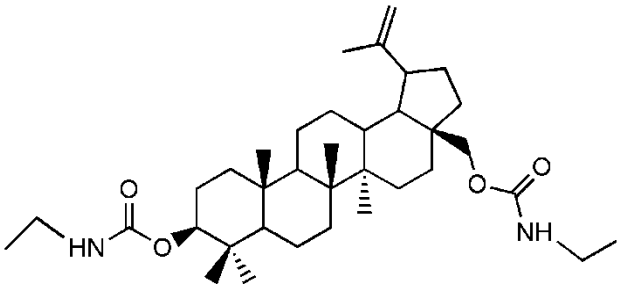
4. The modification with acetyl or keto group at C-3 and C-28 carboxylic acid group into ester of tris(hydroxymethyl)amino methane, (**2k** and **2o**) yielded a highly cytotoxic derivative whereas the conversion into an amide has resulted in a moderately active derivative. **2k** and **2o** seems to show apoptosis without the involvement of caspases

indicating that caspases are nonessential effectors of its apoptotic program and **2k** seem to cause cell death by perturbations in G2M phase of cell cycle on HT-29 cells.

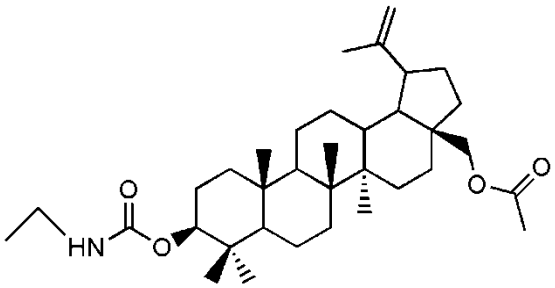


2k

5. The carbamate derivatives of betulin (**1k** and **1m)** were found to show toxicity on a broad panel of cancer cells and furthermore they show a high degree of selectivity to tumor cells than betulinic acid. Apoptosis is induced in a cell cycle independent with an up regulation of caspases involved in both extrinsic and intrinsic pathways of apoptosis which may be due to critical cellular damage caused by these compounds.



1k



1m

6. The aim of the work was to find a molecular modification of betulinic acid and betulin which is selectively cytotoxic to cancer cells and non toxic to normal non cancerous cells. To achieve this many types of modifications on C-3 and C-28 positions of the parent compounds have been performed. After thorough analysis it was resolved that a chloroacetyl group at C-3 secondary hydroxyl group and an untouched C-28 carboxylic acid in betulinic acid and small alkyl chain carboxylic acid esters at C-3 and C-28 in

betulin could be new lead molecules with desired properties to design new anticancer drugs . However, results from a more extensive investigation using a greater number of derivatives are needed for structure activity relationship (SAR) study for the design and ultimate synthesis of a more effective betulinic acid and betulin derived antitumor agent.

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Eidesstattliche Erklärung

Hiermit erkläre in an Eides statt, dass ich die vorliegende Arbeit selbständig verfasst und keine anderen Hilfsmittel als die angegebenen verwendet habe. Ich habe die Arbeit an keiner anderen Hochschule vorgelegt und mich zu keinem früheren Zeitpunkt um den Doktorgrad beworben.

Harish Kommera

List of Publications

H. Kommera, G.N. Kaluderović, M. Bette, J. Kalbitz, P. Fuchs, S. Fulda, W. Mier, R. Paschke In vitro anticancer studies of α- and β-D-glucopyranose betulin anomers, *Chem-Biol Interact.* **2010**, *185*, 128–36.

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