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Investigation of Novel Antitumor agents for New Approaches in Cancer Therapy

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Numbering of Antitumor agents

1,BA Betulinic acid (3β-Hydroxy-lup-20(29)-en-28-oic acid)

2 3-O-Acetyl-28-betulinic acid

3 3-O-Acetyl-28-betulinic acid (2-(2-aminoethyl)aminoethyl)amide

3(PtClS) κN',N''-(3-O-Acetyl-28-betulinic acid (2-(2-aminoethyl) amino ethyl)amide) chlorido κS dimethylsulfoxide platinum(II) chloride

3($PtCl_2$) κN',N''-(3-O-Acetyl-28-betulinic acid (2-(2-aminoethyl)amino ethyl)amide)dichloridoplatinum(II)

4 3-O-Acetyl-28-betulinic acid (1,3-bis(tert-butylcarboxyamino)-2-propyl)ester

5 3-O-Acetyl-28-betulinic acid (1,3-diamino-2-propyl)ester

5(PtClS) κN,N'-(3-O-Acetyl-28-betulinic acid (1,3-diamino-2-propyl)ester)chloride κS-dimethylsulfoxide platinum(II) chloride

5($PtCl_2$) κN,N'-(3-O-Acetyl-28-betulinic acid (1,3-diamino-2-propyl)ester)dichlorido platinum(II).

6($PtCl_2$) Chlorido-κN,N'-(1,3-diamino-2-propanol) platinum(II) dichloride

6(PtClS) Chlorido-κN,N'-(1,3-diamino-2-propanol) κS-dimethylsulfoxide platinum(II) chloride

Pt1,CP Cisplatin (Cis-diamminedichloroplatinum(II))

Pt2 Carboplatin (Cis-Diammine(1,1-cyclobutanedicarboxylato)platinum(II))

Pt3 Hydroxy-$(CH_2)_{11}$-Carboplatin

Pt4 Tetrahydropyran-$(CH_2)_{11}$-Carboplatin

Pt5 Choli acid-$(CH_2)_{11}$-Cisplatin

Pt6 Choli acid-(CH_2)4-Carboplatin

Pt7 Choli acid-(CH_2)6-Carboplatin

Pt8 Choli acid-(CH_2)8-Carboplatin

Pt9	Choli acid-(CH_2)11-Carboplatin
NVX-207	3-acetyl-betulinic acid-2-amino-3-hydroxy-2-hydroxy methyl-propanoate
NVX-207E	Cyclodextrine Encapsulated NVX-207
B10	28-O-acetylbetulin-3-yl-β-D-glucopyranside
2,4DNPH1	3-[(2,4-dinitrophenyl)hydrazono]-(20R)-29-oxolupan-28-oic acid
2,4DNPH2	3-hydroxy-20-[(2,4-dinitrophenyl)hydrazono]lupan-28-oic acid
CAI1	Betulin 3,28-disulfamate
CAI2	28-Acetoxybetulin 3-sulfamate
CAI3	3-Acetoxybetulin 28-sulfamate
CAI4	3-Sulfamoyloxybetulinic acid
CAI5	3-Sulfamoyloxybetulin 28-ethlycarbamate
CAI6	28-Sulfamoyloxybetulin 3-ethylcarbamate

List of Abbreviations

ADP	Adenosine diphosphate
AIF	Apoptosis-inducing factor
AMP	Adenosine mono phosphate
AO	Acridine orange
Approx.	Approximately
ATCC	American Type Culture Collection
Avg.	Average
BA	Betulinic acid
BP	Base pairs
Ca	Calcium
$CaCl_2$	Calcium chloride
CP	*cis*-diamminedichloroplatinum(II)
$CHCl_3$	Chloroform
CO_2	Carbon dioxide
Conc.	Concentration
DIABLO	Direct inhibitor of apoptosis-binding protein with low Pi
Dist. water	Distilled Water
DMF	Dimethyl formamide
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EB	Ethidium bromide
EDTA	Ethylene diamine tetra acetic acid
Eppis	Eppendorf's
EtOH	Ethyl alcohol
FACS	Fluorescence-activated cell sorting
FADD	Fas-Associated protein with death domain
FITC	Fluorescence isothiocyanate
HCl	Hydrochloric acid
HIV	Human Immuno Deficiency Virus
H_2O	Water
HSV	Herpes simplex virus
LDH	Lactate dehydrogenase
MAP kinase	Mitogen activated protein kinase
MeOH	Methyl alcohol
Mg	Magnesium
MOMP	Mitochondrial outer membrane permeabilization
MPT	Mitochondrial permeability transition
MTT	3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NaCl	Sodium chloride
NaN_3	Sodium triazide
NaOH	Sodium hydroxide
NFkB	Nuclear factor kappa-light-chain-enhancer of activated B cells
OD	Optical density
OTC	Over the counter
PARP	Poly(ADP-ribose) polymerase
PBS	Phosphate buffered saline

PCD	Programmed cell death
PI	Propidium iodide
PTPC	Permeability transition pore complex
PTs	Pentacyclic triterpenes
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPM	Rotations per minute
RPMI	Roswell Park Memorial Institute medium
RT	Room temperature
SDS	Sodium dodecyl sulphate
S.D	Standard deviation
S.E	Standard error
SMAC	Second mitochondria-derived activator of caspase
SP	Specificity protein
SRB	Sulforhodamine B
TAE	Tris-Acetate-Ethylene diamine tetra acetic acid
TCA	Trichloroacetic acid
THP	4-(tetrahydro-2*H*-pyran-2-yloxy
TNF	Tumor necrosis factor
Tris	2-amino-2-hydroxymethyl-propane-1,3-diol
UV light	Ultra violet light

List of Publications

Published and submitted research papers, enlisted below are included in the present PhD work as appendices

Appendix 1:

D. Emmerich, **K. Vanchanagiri,** L. C. Baratto, H. Schmidt, R. Paschke.*"Synthesis and studies of anticancer properties of lupane type triterpenoid derivatives containing cisplatin fragment", European Journal of Medicinal Chemistry, 75 (2014), 460-466.*

Appendix 2:

K.Vanchanagiri, T.Mueller, R. Paschke. "*Elucidation of Anticancer Mode of action of Betulinic acid-Cisplatin Conjugates on Lung cancer A549 cells In vitro*". *Valley International Journals.* https://dx.doi.org/10.18535/ijmsci/v3i10.11.

Appendix 3:

Sebastian Paschke, Thomas Mueller, Hans-Joachim Schmoll, **Kranthi Vanchanagiri.** "*More insight into the mode of action of lipophilic antitumor drugs containing a platinum (II) fragment*". Submitted Manuscript.

Appendix 4:

G. Liebscher, **K. Vanchanagiri,** Th. Mueller, K. Feige, J.-M.V. Cavalleri, R. Paschke. *"In vitro anticancer activity of Betulinic acid and derivatives thereof on equine melanoma cell lines from grey horses and in vivo safety assessment of the compound NVX-207 in two horses", Chemico-Biological Interactions, 246 (2016), 20-29.*

Appendix 5:

Leopoldo Clemente Barattoa, Thomas Müller, Brás Heleno de Oliveira, Reinhard Paschke, **Kranthi Vanchanagiri**. "*Betulinic acid 2,4-dinitrophenylhydrazone derivatives induce caspases activation in A549 lung cancer cells*". *Valley International Journals.* https://dx.doi.org/10.18535/ijmsci/v3i11.05.

Appendix 6:

K. Vanchanagiri, Daniel Emmerich, Monique Bruschke, Matthias Bache, Franziska Seifert, Dirk Vormark, Reinhard Paschke. " *Synthesis and Biological Investigation of New Carbonic anhydrase (CA IX) Inhibitors*". Submitted Manuscript.

Summary

Investigation of Novel Antitumor agents for New Approaches in Cancer Therapy

This work describes *in vitro* antitumor activity of Betulinic acid-Platinum and Bile acid-Platinum conjugates, C-3 and C-28 substituted Betulinic acid derivatives (2,4DNPH derivatives, NVX-207 and B10) and Betulinic acid-Sulfamate conjugates. The derivatives were evaluated for their cytotoxicity against 10 different tumor cell lines. The effect of their structural variations on anticancer activity as well as their tumor selectivity in comparison to 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 on specifically targeted cancer cell lines. Preclinical testing is an important part of cancer drug development. The aim of this thesis is to establish and evaluate preclinical *in vitro* assay methods useful in the development of new anticancer drugs. The results from each appendix can be summarized as follows:

Appendix 1 and Appendix 2: *Betulinic acid-Platinum Conjugates*

Both Betulinic acid (**BP**, **1)** and Cisplatin (**CP, Pt1**) are promising antitumor agents, which induce apoptotic cell death of cancer cells. In the present investigation a new series of Betulinic acid-Cisplatin conjugates (**BA-CP**) were synthesized and cytotoxicity and selectivity were assessed against five different tumor cell lines. The *in vitro* anti-tumor activity of C-3 & C-28 substituted Betulinic acid (**1**) and its derivatives with and without platinum ligands was reported. The main objective of this work was to determine the mode of action and to establish structure activity relationships of compounds which contain two cytotoxic groups. In general, when two cytotoxic groups are covalently linked in one molecule the more pronounced cytotoxic activity can be expected. The property of having two cytotoxic groups is called Double loading. The investigation has been successfully carried out to check whether the property of double loading leads to increased toxicity to cancer cells when compared with their precursors.

Appendix 3: *Bile acid-Platinum Conjugates*

Carboplatin (**Pt2**) is a second generation platinum anticancer drug following Cisplatin (**Pt1**). The goal of this investigation is to understand, by using an *in vitro* model (cultured cells), whether and how the modification of the prototypal Cisplatin and Carboplatin molecule with chelating diamines (useful to link bioactive vectors) via spacers (contributing to increase lipo-philicity) is detrimental to the overall cytotoxicity. The Cisplatin and Carboplatin analogues are attached to the acid group of the bile acid via an ester link called as ChAPt(n)Cis and ChAPt(n)Carbo. The compounds exerted a dose dependent antiproliferative action at micro molar concentrations and the effect of these structural variations on anticancer activity was elaborated and discussed more in detail. To summarize, several compounds revealed signif-

icant antitumor activity and surprisingly the ChAPt(11)Cis and ChAPt(11)Carbo induce programmed cell death with molecular features different from each other, suggesting that both drugs induce apoptosis but through different initial pathways.

Appendix 4: *C-28 ester (NVX-207) and C-3 glucopyranoside (B10) derivatives of Betulinic acid*

In this *in vitro* study, Betulinic acid and its two derivatives **B10** and **NVX-207**, both with an improved water solubility compared to Betulinic acid, were tested on two equine melanoma cell lines (MelDuWi and MellJess/HoMelZh) and a human melanoma (A375) cell line. The idea of use of equine melanoma cell lines is introduced here for the first time for the *in vitro* antitumor activity investigation. The aim of this project was to test Betulinic acid and its derivatives **B-10**, the Tris ester **NVX-207** and cyclodextrine encapsulated **NVX-207E** thereof on equine melanoma cell lines and to show that they induce apoptosis comparable to human cancer cell line e.g. A375 (Melanoma) cell line. Additionally, the formulation with the highest prospects of clinical efficacy was evaluated for safety after intratumoral injection in two horses. In a first tolerability evaluation *in vivo* the formulation **NVX-207** was administered every one week for 19 consecutive weeks and well tolerated in two adult melanoma affected horses.

Appendix 5: *Betulinic acid 2,4-dinitrophenylhydrazone derivatives*

2,4-DNPH1 and **2,4-DNPH2** are 2,4-dinitrophenylhydrazone derivatives of Betulinic acid. The influence of the modifications at C-3 hydroxyl (**2,4-DNPH1**) and C-20 aldehyde (**2,4-DNPH2**) with 2,4-dinitrophenylhydrazone reported here. The biological results showed that the 2,4-DNPH moiety plays an important role for the cytotoxicity of these molecules and also for the activation of caspases and apoptosis induction. The introduction of 2,4-DNPH moiety in Betulinic acid altered significantly the kinetics of the molecules, since the mechanism of action of the derivatives was slower than the precursor.

Appendix 6: *Betulinic acid-Sulfamate conjugates*

In this section the *in vitro* anticancer activity and Carbonic anhydrase IX (CA IX) inhibition of new sulfamate conjugates of Betulinic acid was demonstrated. The Betulinyl sulfamates were targeted to inhibit carbonic anhydrases (CA), such as CA IX, an attractive target for tumor-selective therapy strategies in cancer cells. The Betulinyl sulfamates were tested against five tumor cell lines and normal human skin fibroblasts. The mode of cell death on MCF7 breast cancer cells induced by the most active compounds **CAI1**, **CAI3**, **CAI6** was investigated by Fluorescence Activated Cell Sorting (FACS) experiments. The compounds showed inhibitory activity against CA IX which was determined by *in vitro* enzyme assay. Our preliminary investigations revealed that all compounds showed potent anticancer properties with IC_{50} values below 20 µM against all tumor cells. Interestingly, among the panel of sulfamate

conjugates, **CAI3** was found to be highly cytotoxic (average IC_{50}=5-10 µM) and possess high inhibitory activity (Ki=1.25 nM) against CA IX.

In conclusion the work presented here contributes to different levels of the preclinical drug screening and methods may aid in the characterization of anticancer compounds for future development of potent anticancer agents.

1. Introduction

Plant products have been used extensively in the treatment of malignant diseases 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. Natural products obtained from plant sources played an important role in cancer treatment. Plant product derivatives comprised 47% of a total of the 155 anticancer drugs approved up to 2014 worldwide as promising anticancer agents[1]. Among the classes of identified natural products pentacyclic 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. Betulinic acid (3β-Hydroxy-lup-20(29)-en-28-oic acid) (**1, BA**) is a member of pentacyclic triterpenes. **BA** showed numerous biological activities like anti-HIV[2-4] , anti-bacterial[5-8], anti-malarial[9-11] anti-inflammatory[12-17], anti-helminthic[18], antinociceptive[19], and is widely considered for its anticancer activity[20-23]. Initially **BA** was thought to be melanoma specific[23] but later it was discovered that it showed anticancer activity against a broad spectrum of cancers[20, 24]. Moreover, **BA** was found to be selective for tumor cells and nontoxic to normal non-cancerous cells[13]and due to this, it is well tolerated in mice (500 mg/kg) even at higher concentrations. **BA** exerts cytotoxicity against metastatic over non metastatic melanoma cell lines and showed a synergistic cytotoxic effect in combination with vincristine on murine melanoma B16F10 cells both *in vitro* and *in vivo*[25]. Betulinic acid (**1**) and its derivatives are pluripotent compounds with numerous biological activities. Therefore they have been investigated widely over the past few years[15, 25-28], focusing in the field of cancer therapy[29-33]. Currently, the development of new anticancer agents focused on discovering diverse compounds with either novel structures or a new mechanism(s) of action. Discovery of novel plant derived natural products as potential new lead compounds for anticancer agents as well as the modification of these new lead compounds is continuing goals of our laboratory[30-36]. A vast majority of Betulinic acid 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.

Metal complexes are used in many fields of drug discovery. Especially in the field of anticancer chemotherapy a number of complexes have already been tested in clinical trials or at an advanced stage of clinical testing[37-39]. They seem to have crucial advantages for use as drugs in drug-targeting conjugates. Cisplatin (**Pt1**) and Carboplatin (**Pt2**) are the most popular metal complexes used as potent chemotherapy drugs for cancer therapy[40]. They are small, highly cytotoxic molecules, and their mode of action is so far well investigated. Although both of these compounds are now in clinical use because of their drug toxicity profiles, there is always need to look for new approaches to overcome their disadvantages i.e., Chemo resistance,

lipophilicity and toxicity. In order to make platinum antitumor drugs more effective but also to gain drug-targeting properties many research groups attempt an approach either to combine two antitumor drugs or to combine an antitumor drug with tissue specific molecules[39, 41-43].

As a contribution, to solve some of these drawbacks from plant derived cytotoxic agents like Betulinic acid and metal complex based chemotherapeutic agents like Cisplatin, three different approaches practiced in this work mainly at preliminary stage using *in* vitro and some *in vivo* models. Elucidation of antitumor activity of new Betulinic acid derivatives and Platinum conjugates (total 30 anticancer drugs of which 18 are newly synthesized compounds) carried out and classified into the following three categories. The hypothesis is that the results can open new possibilities for potent antitumor agents in preclinical anticancer drug development. The following are the three strategies,

A. *Combinational therapy/Double loading*
B. *Targeted Drug Delivery*
C. *Derivatization of Betulinic acid lead to potent and polar antitumor agents*

A. Combinational therapy/Double loading

Combinational therapy is common in the field of chemotherapy[39, 43-46]. The efficiency of this therapy depends strongly on the nature of the single components: how they can be delivered, how they are metabolized, and how and to which extent they can enter the cell and reach the targets for the action. An insufficient process of apoptosis is not only an important factor in the genesis of tumours, but also the main reason for malignant tumours getting resistant against chemo and radiotherapies. Therefore, it could be advantageous when the components are covalently linked to each other. There are several examples for this approach. As a result of the combination of Wortmannin and Cetuximab in a “double drug” concept, the antiproliferative activity of both compounds could be improved[47-48]. Similarly, the cytotoxic and phototoxic properties of a Ruthenium-Porphyrin [48]conjugate are combined [48].

Compounds that exert a direct action on mitochondria present promising experimental cancer therapeutics, since they may trigger cell death under circumstances in which standard chemotherapeutics fail. Thus, mitochondrion-targeted agents such as Betulinic acid (**1, BA**) and Cisplatin (**Pt1**) hold great promise as a novel therapeutic strategy in the treatment of human cancers. Betulinic acid is a known bioactive pentacyclic triterpene, which has gained a lot of attention in the recent years since it exhibits a variety of biological and medicinal properties. In contrast to the cytotoxicity of Betulinic acid against a variety of cancer types, normal cells and tissue are relatively resistant to Betulinic acid, pointing to a therapeutic window. Platinum complexes are clinically used as adjuvant therapy of cancers aiming to induce tumor cell death. Cisplatin is one of the most potent chemotherapy drugs widely used

for cancer treatment[49]. Betulinic acid and Cisplatin both are promising antitumor agents, and both induce the apoptotic cell death of cancer cells[50-51]. Both have broad spectrum anticancer activity and were shown to be effective against a vast variety of carcinoma cell lines derived from lung, ovarian, cervical, head and neck carcinomas, as well as from lymphoma, neuroblastoma, medulloblastoma, glioblastoma, and other types of tumours[52]. Additionally Betulinic acid has potential clinical value as an anti-HIV, anti-bacterial and anti-malarial agent[53-54]. The aim was to find out if a combination of these two different apoptosis causing structures would lead to a significant influence on the overall cytotoxicity of the conjugates (Figure 5). In this context it is also important to understand the anticancer mechanism of action of Betulinic acid and Cisplatin, which could further help to establish the structure activity relationship.

A.1. Anti-Cancer Mechanism of action of Betulinic acid

Apoptotic pathways are potential targets for therapeutic modulation. Simplified schematic representation of caspase-dependent apoptotic cell death pathway induced by Betulinic acid was shown in Figure 1. Over the past few years', numerous studies have been aimed at elucidating the molecular mechanisms of **BA**-mediated antitumor activity. One characteristic feature of **BA**'s cytotoxicity is its ability to trigger the mitochondrial pathway of apoptosis in cancer cells 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[21, 55]. The cytotoxicity of **BA** is related to its ability to trigger the mitochondrial or intrinsic pathway of apoptosis in cancer cells.

BA causes a fall in membrane potential of outer mitochondrial membrane thereby causing a release of Cytochrome C which in turn activates the initiator caspase-9 following the activation of effector caspase-3 leading to DNA fragmentation and apoptotic cell death. Antiapoptotic Bcl-2 family proteins, such as Bcl-2 and Bcl-XL, inhibited all mitochondrial and cellular manifestations of apoptosis induced by **BA**, indicating that mitochondrial permeability transition was required for these events. Mitochondria from intact cells when treated with **BA** 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 reactive oxygen species (ROS)[56]. **BA** also decreases the expression of vascular endothelial growth factor (VEGF) and the antiapoptotic protein survivins in tumours (prostate (LNCaP) and melanoma (SK-MEL2) cancers), 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, BA may act as a novel anticancer agent through targeted degradation of Sp proteins that are highly overexpressed in tumours[57].

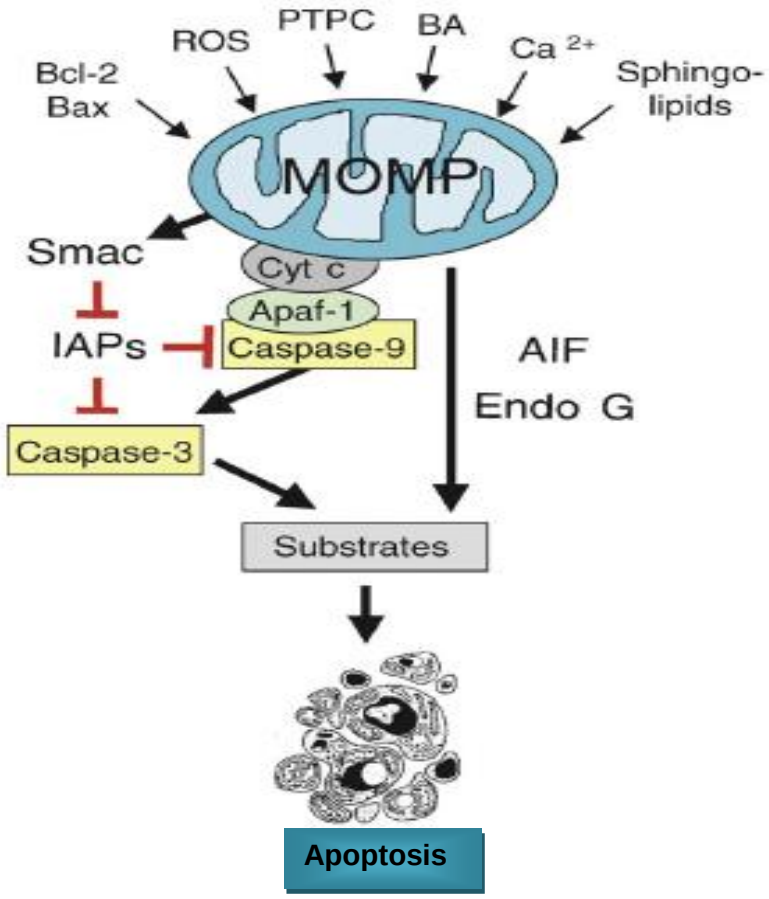


Figure 1. Mitochondrial pathway of apoptosis. The intrinsic (mitochondrial) pathway of apoptosis is linked to mitochondrial outer membrane permeabilization (MOMP), which is regulated by various factors including pro- and antiapoptotic Bcl-2 proteins, reactive oxygen species (ROS), proteins from the mitochondrial permeability transition pore complex (PTPC), ions, sphingolipids and Betulinic acid (**BA**). MOMP in turn results in the release of soluble intermembrane proteins from mitochondria into the cytosol such as Cytochrome C, SMAC/DIABLO, AIF and endonuclease G. Cytochrome C and SMAC/DIABLO promote activation of caspases, where as AIF and endonuclease G contribute to caspase-independent chromatolysis[58].

Recently it was published that **BA** inhibits the steroyl-CoA desaturase and consequently reduces the desaturation levels of cardiolipins in cancer cells. This leads to ultrastructural changes in mitochondrial membranes. As cancer cells depend on *de novo* lipogenesis, this w ould explain the selective effect of **BA** on cancer cells[59]. Direct interaction with cardiolipins wi th subsequent MMP formation seems also to be possible, because it was shown that **BA** inte racts with in the human mitochondria with most abundant Tetralinoleol cardiolipin[60]. Additiona lly, the production of reactive oxygen species (ROS) plays a role in MMP formation since the co-treatment with the antioxidant alpha-DL-Tocopherol inhibited the induction of apoptosis[61-62]. As a consequence of the pore formation apoptogenic factors release from the mitochondri a and caspases are activated[63]. Additionally, the inhibition of other target proteins such as pr- olidase, proteins of collagen biosynthesis, the mammalian DNA topoisomerase I[64] and acyl-

CoA:cholesterol acyl transferase were found[65]. These multiple modes of action show the high anticancer efficacy of **BA**.

A.2. Anti-Cancer mechanism of action of Cisplatin

The best characterized biochemical anticancer mechanism of action of Cisplatin (**CP**) was shown in Figure 2. **CP** is a well-known DNA-damaging agent, and DNA platination is an essential first step in its cytotoxic activity[66]. Only 1% of **CP** is linked to nuclear DNA[67], besides, mitochondrial DNA, RNA and other cellular components, including membrane phospholipids, cytoskeletal microfilaments, and thiol-containing proteins, are potent reactants for the platinum structure[68]. Platinum compounds damage tumours via induction of apoptosis, which is mediated by the activation of various signal transduction pathways. This effect is related to inhibition of DNA synthesis and repair that might result in cell cycle arrest at the G1, S, or G2/M phases, followed by cell death (Apoptosis)[69].

CP-DNA damage induces a fall in the mitochondrial permeability transition (MPT), and this MPT fall releases factors such as reactive oxygen species (ROS), Bax, and Ca^{2+} which facilitates the rupture of mitochondria[70]. Mitochondrial rupture releases Cytochrome C and procaspase-9 that bind to cytosolic Apaf-1 and ATP in an apoptosome complex, leading to the activation of caspase-9. Activated caspase-9 induces other caspase interactions, resulting in activation of caspase-3, caspase-6, and caspase-7 with the subsequent cleavage of key substrates[71]. The final outcome is the dismantling of the cell by formation of apoptotic bodies. An alternative pathway of apoptosis may be initiated by injury of phospholipids of the cell membrane, which may induce the sphingomyelin-ceramide signalling system of cell death[72]. **CP** activate the Fas receptor by Fas ligand (FasL) which induces the formation of an apoptosome complex between Fas-associated death domain (FADD) and procaspase-8 that subsequently activates caspase-8. Then caspase-8 activates the caspase-3-6-7 system that finally cleave key substrates, and the cell is digested through apoptosis. Caspase-8 may also activate the proapoptotic protein Bid that triggers apoptotic cell death through the mitochondrial pathway. The cleavage of PARP by caspase-3, -6, or -7 switch the cell death mechanism from necrosis to apoptosis[73].

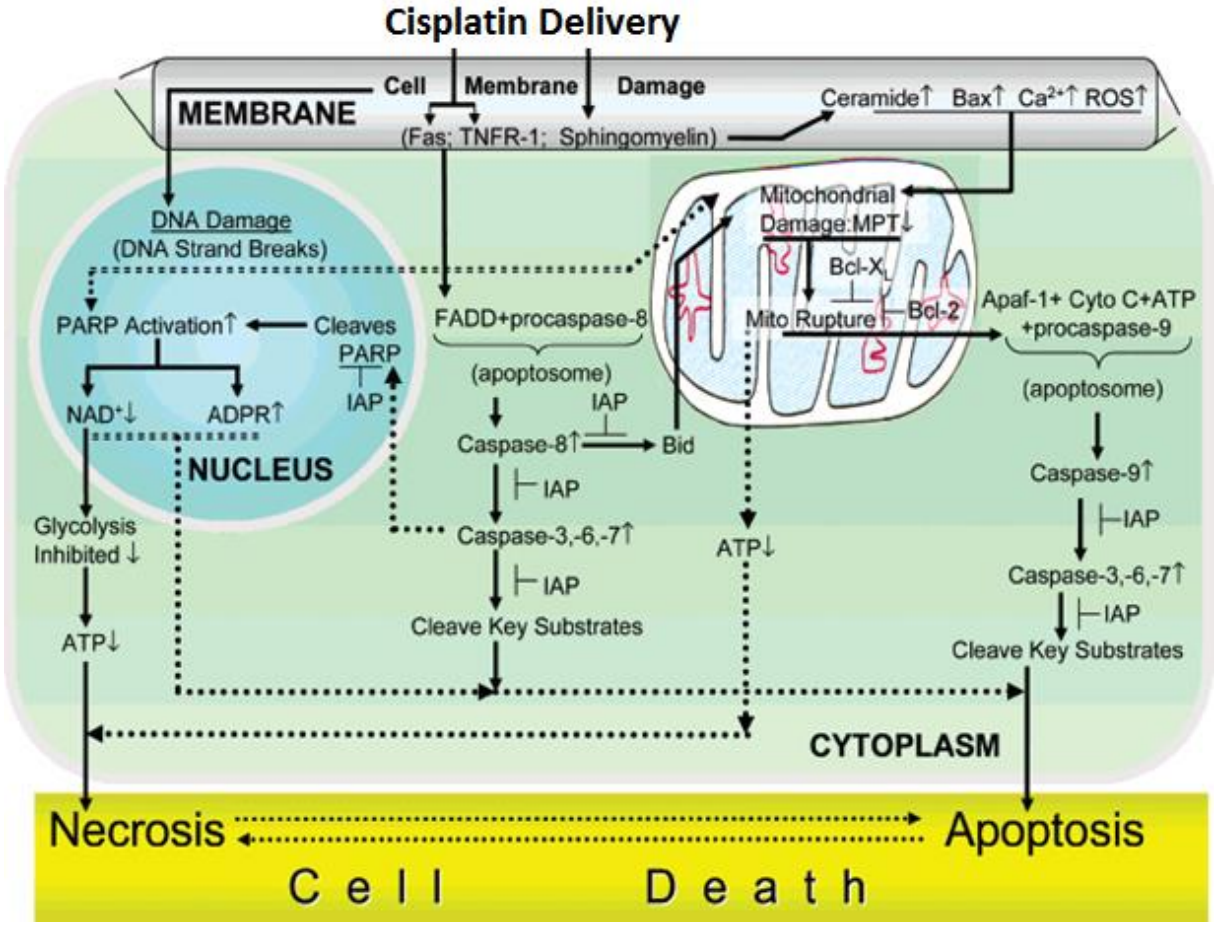


Figure 2. CP-induced DNA damage results in the triggering of apoptosis. Cancer cells when exposed to CP show internucleosomal DNA degradation to approximately 180 base pair fragments, blebbing of the cell surface and cell shrinkage. All these features are consistent with apoptosis as a mode of cell death. CP also produce characteristic features of necrosis, which is considered a mode of cell death due to general cell machinery failure. Necrosis was considered the mode of cell death induced by DNA-damaging anticancer agents because of the activity of poly (ADP-ribose) polymerase (PARP). PARP is activated by the DNA strand breaks caused by some anticancer agents, including CP[68].

B. Targeted drug delivery

B.1. Biomolecules linked to transition metal complexes

One of the most common and interesting design strategies is “Drug targeting and delivery”. This concept is applied in the present work, which involves the development of any drug molecules to target specific tumor cells with the expectation of higher lipophilicity and chemical stability, along with higher antitumor activities and lower systemic toxicity[74-76]. This method of anticancer chemotherapy relies on agents with selective access to tumours to deliver drugs to the target[77]. Two examples shown in Figure 3, a steroidal-platinum(II) complex and modified Bile acid-Cisplatin conjugate. From Figure 3 , compound A was reported to exhibit binding affinity for estrogen receptor and to have similar antitumor acitivity to that of Cisplatin, while compound B named as Bamet-R2 (Ba=Biomolecule, Met=Metal complexes) has lower

cytotoxicity compared to Cisplatin *in vitro,* but similar cytotoxicity *in vivo.* Cisplatin is often used as a fragment because the mode of action is well investigated and it is possible to link this structure to other molecules by a variety of synthetic methods[43, 78]. Approaches to find such agents often depend on novel design strategies employing bioactive vectors/biomolecules, such as intercalators, amino acids, sugars, bile acids, folates, and oestrogen receptors[76, 79]. There are also several examples for this approach.

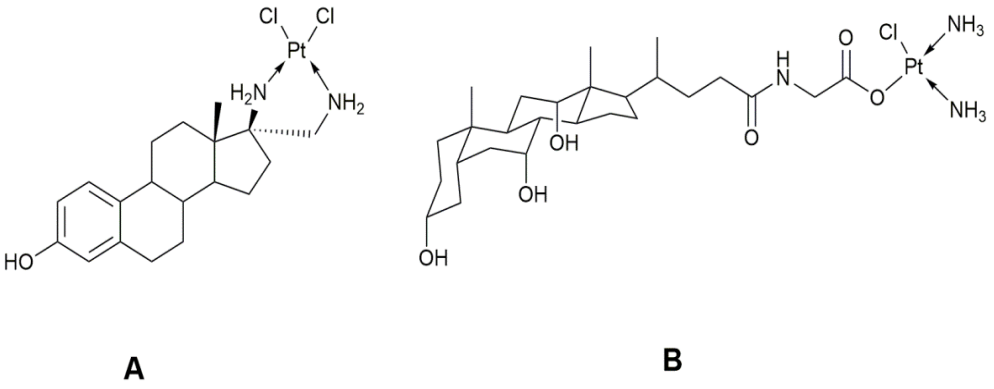


Figure 3. Structures of A. steroidal-platinum(II) complex, B. Bamet-R2

In the last few years' bile acids have become important as drug vehicles in medicinal chemistry. Bile acids have been used in several attempts to shuttle drugs to the liver[80]. Binding biomolecules to drugs always causes a drastic change in the structure of the carrier as well as in the structure of the pharmacologically active compound. The combination of a pharmacologically active compound to bile acid offers the possibility to exploit the natural resorption and could lead to an improved uptake of pharmaceuticals[81-82]. Sometimes such changes in structure can make them both lose the specific and unique moieties that make them less active than their precursors[83]. To avoid or minimize this effect, spacers are often used to separate the biomolecule and the drug. Platinum complexes are clinically used as adjuvant therapy of cancers aiming to induce tumor cell death[75].

Cisplatin (**Pt1**) and Carboplatin (**Pt2**) are well-known DNA-damaging agents, and it is currently thought that DNA-platination is an essential first step in their cytotoxic activity. Depending on cancer cell type and the used concentration they induce cytotoxicity, e.g., by interference with transcription and/or DNA replication mechanisms. It was discussed that this effect is related to inhibition of DNA synthesis and repair that might result in cell cycle arrest at the G1, S, or G2/M phase, therefore apoptosis induced [19-21]. Additionally **Pt1** and **Pt2** damage tumours via induction of apoptosis, mediated by the activation of various signal transduction pathways, including calcium signalling, death receptor signalling, and the activation of caspases through

mitochondrial pathways[84]. Unfortunately, neither cytotoxicity nor apoptosis are exclusively induced in cancer cells, thus, **Pt1** might also lead to diverse side effects such as neuro- and/or renal-toxicity or bone marrow-suppression. Moreover, the binding of **Pt1** to proteins and enzymes may modulate its biochemical mechanism of action. Apoptosis, a type of programmed cell death, controlling the development and homeostasis of multicellular organisms, has been shown to be one of the key cellular event responsible for exhibiting the anticancer activity of Cisplatin. There are two types of apoptotic caspases: initiator (apical) caspases and effector (executioner) caspases. Initiator caspases (e.g. caspase-2, -8, -9, and -10) cleave inactive pro-forms of effector caspases, thereby activating them. Effector caspases (e.g. caspase -3, -6 and -7) in turn cleave other protein substrates within the cell, to trigger the apoptotic process[85].

Previous reports described three types of lipophilic platinum conjugates, a Cisplatin-conjugate (ChAPt-Cis)[86] [24], a Carboplatin conjugate (ChAPt-Carbo)[84] and a series of long Tethrahydropyran-Cispatin conjugates (THP(11)Cis)[35, 49] linked by alkyl chains. The THP-conjugates and the Cisplatin-conjugates were intensively investigated because of their ability to break the Cisplatin resistance. This property is also reported for a similar group of compounds. In the present investigation we report the apoptosis inducing anticancer mode of action of conjugates which consist of three functional parts, where Cisplatin and Carboplatin (biologically active fragments) linked to bile acid (transport fragment) via alkyl spacers (-CH_2-) which are designated as ChAPt(n)Cis and ChAPt(n)Carbo (Figure 6). In case of ChAPt(n)Carbo, the length of the alkyl chain was varied [n=4,6,8,11] in order to investigate the influence of the distance between drug and transport fragment. Bile (Cholic) acid is used as a transport fragment because of its complete reabsorption in the enterohepatic circulation makes this compound attractive for drug targeting[87]. In this context, the question to be answered is whether the similarities in the structure of these Bile acid-Cis/Carboplatin conjugates finally lead to a similar pattern in the biological behaviour, e.g. cell cycle, caspase activity to that of parent compounds. The former investigations revealed that those lipophilic platinum compounds enter the cell very quickly in comparison to Cisplatin and cause cell death in a different manner[35, 49].

B.2. Sulfamate conjugates of Betulinic acid

The aim of this work was to produce safe, soluble antitumor derivatives of Betulinic acid, perform mechanistic studies on the mode of cell death and gain insight into the 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 Betulinic acid to design new anticancer drugs. The C-3 and C-28 positions of Betulinic acid were modified to obtain new promising derivatives against different types of cancers and efforts were made to contribute to the understanding of

the mechanism of action of the potent new derivatives. In the present study, we report about the combination of a Betulinic acid (**BA**) fragment with a sulfamate substituent (Figure 9), which is able to inhibit Carbonic acid anhydrase (CA)[88]. Carbonic anhydrases (CAs), a family of zinc metalloenzymes, play a key role in intracellular and extracellular pH regulation. They catalyse the hydrogenation of CO_2 to HCO_3^- and H^+ and regulates the intra and extracellular pH level of cells[89-90]. The transmembrane protein Carbonic anhydrase IX (CAIX), is a member of CAs and is transcriptional, and regulated by hypoxia induced factor-1α (HIF-1α)[91-92].

In various tumor types, such as lung, cervical, head-and-neck or breast cancer, high CAIX expression levels are closely associated with a poor prognosis[93]. A special criterion for drug targeting is the saving of healthy tissue by treatment with chemotherapeutic agents. CAIX inhibition is therefore an attractive target for tumor-selective treatment strategies[92-95]. Hypoxia and acidosis are associated characteristics of solid tumors with increasing size[96].. Small interfering RNAs, therapeutic antibodies or small molecule inhibitors are strategies to evaluate the tumor therapeutic potential of CAs (e.g. CAIX). Actually, some drugs inhibiting CA function are already under consideration in clinical studies[97].

To date, no extensive research regarding the *in vitro* antitumor activity of sulfamate conjugates of Betulinic acid has been published[98-99]. For many years, we have been searching for molecules combining both antitumor activity and a drug targeting moiety. Betulinyl sulfamates belong to this category of antitumor agents. The compounds tested here were synthesized by derivatization at C-3 (hydroxyl) and C-28 (carboxylic group) positions of **BA** and the structure activity relationship developed in relation to modifications at the corresponding positions. The present investigation demonstrates that simple modifications of the parent structure of **BA** can produce a number of highly potent derivatives, which may improve the selective toxicity profile or to introduce general toxic effects.

C. Derivatization of Betulinic acid lead to potent and polar antitumor agents

Betulinic acid has three sites that are highly amenable to derivatization, including C-3 hydroxyl, C-20 alkene, and C-28 carboxylic acid positions[100]. Here the studies have been performed for the synthetic C-3, C-20 alkene and C-28 substituted Betulinic acid derivatives which is an additional effort to establish meaningful structure activity-relationships in the context of novel Betulinic acid derivatives.

C.1. C-28 ester (NVX-207) and C-3 glucopyranoside (B10) derivatives of Betulinic acid

Cancer is a threat to equine health; Especially grey horses suffer from cutaneous melanocytic tumours, since 80% of grey horses older than 15 years bear melanoma lesions[101]. Equine malignant melanoma (EMM) is reported to progress to malignancy and metastasize in the surrounding tissue, lymph nodes or other internal organs in more than 60% of cases[102]. Like

humans also grey horses suffer from melanoma as the mutation causing their grey hair phenotype likewise increased risk for melanoma formation with a risk of metastasizing [103]. For medical and ethical reasons, a treatment is necessary. Local chemotherapy with Cisplatin or a local excision show only successful results for small tumours. But due to the high number of melanomas and the risks when administering Cisplatin, a new treatment strategy has to be found. Chemotherapy using Cisplatin and surgical excision are still the most commonly used treatment modalities for EMM. Despite promising local antimelanoma effects[104-105], due to its cytotoxic properties to normal cells, the use of Cisplatin carries a high risk for the horse owner and the treating veterinarian[106]. Therefore, a new treatment is needed, and a possible new drug is Betulinic acid or one of its derivatives. Betulinic acid and its derivatives have been known for about 25 years as efficient anticancer drugs. The substances proved their efficacy on several human cancer cell lines and *in vivo* model systems. Due to their poor water solubility they have not been used as drugs for cancer treatment. To solve this problem a plethora of new derivatives were synthesized in the last decade, and two promising derivatives are the glucopyranoside derivative **B-10** and the Tris ester **NVX-207**[31, 33] (Figure 7). The latter agent already showed promising results in treatment of canine cancer patients[33]. Another way to improve the water solubility is with the use of drug delivery system. In the last years different formulations were tested *in vitro* as well as *in vivo* in xenograft models, e.g. liposomes [107]or nanoparticles consisting of cellulose and Poly(L-lactate) and encapsulating Betulinic acid[108].

The encapsulation in β cyclodextrine was already used for encapsulating Betulinic acid[109] and hydroxy propyl-β-cyclodextrine for encapsulating **NVX-207**. Cyclodextrine showed no effects on the activity of the encapsulated compounds[33]and is very likely degraded into non-toxic saccharides[110]. The idea of use of equine melanoma cell lines was introduced here for the *in vitro and in vivo* antitumor activity investigation. The main goal of this part of the project was to test Betulinic acid and its derivatives **B-10**, Tris ester **NVX-207** and cyclodextine encapsulated **NVX-207E** thereof on equine melanoma cell lines and to show that they induce apoptosis comparable to human cancer cell line e.g. A375 (Melanoma) cell line. Additionally, the formulation with the highest prospects of clinical efficacy was evaluated for safety after intratumoral injection in two horses.

C.2. C-3 and C-20 substituted Betulinic acid 2,4-dinitrophenylhydrazone derivatives

The antitumor properties of Betulinic acid motivated further studies on the structure activity relationship for its new derivatives. The structure activity relationships are proposed and could contribute to the understanding of the cytotoxic profile of this class of compounds. The synthesis, characterizations and *in vitro* antitumor activity of two novel Betulinic acid 2,4-dinitrophenylhydrazone (2,4-DNPH) derivatives was reported previously[111] (Figure 8). The results showed that some of these derivatives were more cytotoxic and selective towards different cancer cell lines than Betulinic acid. Since the derivatives showed anticancer potential

at micro molar concentrations and considerably better selectivity, the derivatives were selected for the elucidation of anticancer mechanism of action of apoptosis induction, which was assumed to be a good contribution for understanding their structure-activity relationship (SAR).

Preclinical models for efficacy Assessment-Importance of early characterization of mechanism of action:

To date most of the anticancer drug treatments used were developed prior to 1975, at a time when much of the knowledge of genetic and biochemical mechanisms of cancer pathogenesis was yet to be discovered[112]. The main mechanism of action of the classic cytotoxic drugs is inhibition of the increased rate of DNA synthesis and replication, or to destroy DNA in tumour cells. Cytotoxic drugs can interact with cells via different mechanisms and are divided into groups accordingly; alkylating agents (e.g. melphalan), antimetabolites (e.g. cytarabine, fluorouracil), topoisomerase inhibitors (e.g. etoposide, doxorubicin) and microtubule interacting agents (e.g. vincristine, paclitaxel)[113]. In contrast to the traditional DNA-targeting cytotoxic agents, later some drugs were designed to specifically act on their targets and thereby to be less toxic to normal cells. Efforts have been made to develop *in vitro* assays for predicting toxicity profiles and therapeutic index. Normal cells are often less proliferative and more fragile than tumour cells, making *in vitro* culturing a big challenge.

Screening for cancer drugs and screening for novel compounds with cytotoxicity or affinity for certain targets is still establishing in anticancer drug development and can be applied to different collections of compounds[112]. For example, screening for cytotoxic activity can be performed among natural product extracts or drugs isolated through rational drug design i.e. substances synthesized to act against specific molecular targets[114-116]. The initial stage of drug screening often involves a large number of compounds and is usually carried out using tumour cell lines[117]. Some of the desirable qualities of a drug screening assay therefore include simplicity, low costs and reproducibility, and the assays must allow screening of a large number of compounds as well as provide a reasonably accurate assessment of drug sensitivity[116].The advancement from preclinical models to clinical trials is mainly based on both *in vitro* and *in vivo* investigations. The difficulty of finding the ideal preclinical model for prediction of diagnosis-specific activities is difficult from species differences is ever present[118-119]. The National Cancer Institute has a panel of 60 cell lines (NCI60) that have been used to discover and develop novel anticancer drugs. The NCI60 panel has also been used together with the COMPARE algorithm to reveal the mechanism of action of new drugs primarily at *in vitro* level, since it has been shown that drugs with similar mechanisms of action tend to have similar patterns of growth inhibition in the NCI60 screen[114]. In the present work the newly synthesized Betulinic acid derivatives, Platinum conjugates, Sulfamate conjugates were along with precursors were subjected to *in vitro*, some *in vivo* assays with the goal of establishing

structure activity relationship and elucidating early stage anticancer mechanism of action. The *in vitro* cancer lines, mainly A549 (lung cancer), MCF7 (Breast cancer) and DLD1 (colorectal adenocarcinoma) which belongs to NCI60 screen were used as targets for some of the investigated compounds. The evaluation of the whole set of results from three different strategies practiced here would lead to new insights in the field of drug development.

2. Aim

Betulinic acid (**1, BA**) is a known natural product which has gained a lot of attention in the recent years since it exhibits a variety of biological and medicinal properties. A vast majority of Betulinic acid derivatives have been reported in recent years; they received a significant attention and possess moderate to good antitumor properties. Platinum complexes are clinic ally used as adjuvant therapy of cancers aiming to induce tumor cell death. Cisplatin (**CP**, **Pt1**) is one of the most potent chemotherapy drugs widely used for cancer treatment. While both of these compounds are now in clinical use because of their drug toxicity profiles there is always need to look for new approaches to overcome their disadvantages. A need therefore exists for novel anticancer derivatives, which are not only potent, but also clinically safe.

The main objectives of the current work are:

1. To perform mechanistic studies on mode of cell death caused by the C-3, C-20 and C-28 derivatives of Betulinic acid.
2. To establish and evaluate preclinical methods useful in the development of new anticancer agents with favourable efficacy, selectivity and toxicity profiles.
3. To develop a simple, robust and high-throughput series of assays for preliminary investigation of antitumor activity of new anticancer drugs and its conjugates of Betulinic acid and Platinum derivatives
4. To scrutinize the anticancer mode of cell death induced by the new anticancer agents along with their precursors, and to establish the structure activity relationship, further that could contribute to the understanding of their cytotoxicity profiles
5. To answer whether, the properties of Double loading, Biomolecule linked platinum drug targeting would lead to significant influence on the overall cytotoxicity or not.
6. To understand the mechanism of action of bifunctional molecules like Betulinic acid-Sulfamate conjugates, which further contribute to the development of potent new anticancer derivatives.

3. Materials and Methods

The contents of this section defines the terms and describes the techniques used in this work. All investigated compounds were provided by Prof. Dr. Reinhard Paschke, “Laboratory of Medicinal Pharmaceutical chemistry” at “Martin-Luther University Halle-Wittenberg.

3.1 Preparation of solutions of compounds

The investigated compounds were insoluble in water, They were initially dissolved either in Dimethyl sulfoxide (DMSO) or Dimethyl formamide (DMF) and further diluted with culture medium for analysis. 20 mM stock solutions of test compounds were prepared. The final concentration of DMSO or DMF never exceeded 0.5%, which was found to be non-toxic to the cells.

3.2 Cell line and culture conditions

The cell lines 518A2 (melanoma cancer), A2780 (ovarian cancer), A549 (lung cancer), MCF7 (breast cancer), 8505C (anaplastic thyroid cancer), DLD1 (colorectal adenocarcinoma) and HepG2 (hepatocellular carcinoma), A375 (melanoma cancer) as well as non-tumorous cell line-human skin fibroblasts (WWO70327) were included in this study. All these cell lines were supplied by Bio-Solutions Halle GmbH, Halle, Germany. The equine melanoma cell lines MelDuWi provided by Dr. Saskia Willenbrock, University of Veterinary Medicine, Hannover, Foundation, Germany, MellJess/HoMelZh provided by Dr. Monika Seltenhammer, Veterinary University of Vienna. The cell lines were used at optimal density and cytotoxicity screening studies were performed after evaluation of cell viability by cell counter using Tryphan blue staining. Cultures were maintained as monolayers in RPMI 1640 (PAA Laboratories, Pasching, Austria) supplemented with 10% heat-inactivated fatal bovine serum (Sigma Aldrich, Steinheim, Germany) and 1% penicillin/streptomycin (PAA Laboratories, Pasching, Austria) at 37 °C in a humidified atmosphere with 5% CO_2.

3.3 *In vitro* antitumoral studies

3.3.1 Cytotoxicity

Cytotoxicity can be defined as the degree at 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.

In the present work the cytotoxicity of all compounds was evaluated by sulforhodamine-B (SRB) (Sigma-Aldrich) micro culture colorimetric assay. In short, exponentially growing cells were seeded into 96-well plates on day 0 at the appropriate cell densities to prevent confluence of the cells during the period of experiment. After 24 h, the cells were treated with serial dilutions of the compounds (0–100 µM) for 96 h. The percentages of surviving cells related to untreated controls were determined 96 h after the beginning of drug exposure. After 96 h treatment, the supernatant medium from the 96-well plates were discarded and the cells were fixed with 10% TCA. For a thorough fixation plates were allowed to stand at 4 ºC for at least 2 h. After fixation the cells were washed in a plate washer (Tecan Austria GmbH, Austria). The washing step was done five times with water using alternate dispensing and aspiration procedures. The plates were then stained with 100 µL of 0.4% SRB for about 45 min. After staining, the plates were washed with 1% acetic acid to remove the dye and allowed to air dry overnight. 100 µL of 10 mM Tris base solution was added to each well and the absorbance was measured at 570 nm using the plate reader (TECAN Infinite F200 PRO, Tecan GmbH, Austria).

3.3.2 Apoptosis tests

3.3.2.1 Microscopic Investigation

The microscopic investigation was performed to identify the morphological behaviour of treated cancer cells compare to untreated controls. The aim of microscopic investigation is to identify the morphological behavior of treated cancer cells and to check whether the cells are undergoing Programmed cell death (PCD) or not.

Programmed Cell Death (PCD):

PCD referring to the death of cell in any pathological format when mediated by an intracellular program. There are three types of PCD identified, that are Apoptosis, Necrosis and Autophagy [71, 120]. Apoptosis and Necrosis are two different types of PCD show distinct morphological and biochemical changes they were first described and characterized by Kerr et al.[121]. Apoptotic cells share morphological features of cell shortening, loss of intercellular adhesion, membrane blebbings, apoptotic bodies, and DNA laddering[122]. Recent studies have provided evidence that there is another mechanism of PCD which is associated with the appearance of auto phagosomes and depends on autophagy proteins. Autophagy is an evolutionarily conserved catabolic process beginning with formation of auto phagosomes, double membrane-bound structures surrounding cytoplasmic macromolecules and organelles, destined for recycling[120]. Autophagy plays an important role in cancer – both in protecting against cancer as well as potentially contributing to the growth of cancer. The fluorescent agents used to differentiate apoptotic, necrotic and autophagic cells are given below.

Acridine orange/Ethidium bromide (AO/EB staining):

Acridine orange/Ethidium bromide staining mainly used here to differentiate the live and dead (apoptotic/necrotic) cells. AO is cell permeable, and interacts with DNA and RNA by electrostatic attractions respectively. When bound to DNA, spectrally it is very similar to fluorescein, with an excitation maximum at 502 nm and an emission maximum at 525 nm (green). When it associates with RNA, the excitation maximum shifts to 460 nm (blue) and the emission maximum shifts to 650 nm (red)[123]. AO binds to DNA and form a complex and the emitted radiation is green. When it binds to RNA the emitted light of formed complex is orange. Based on this the dead (Apoptotic) cells are differentiated from live cells[124]. Ethidium bromide (EB) is an intercalating agent commonly used as a fluorescent tag (nucleic acid stain). When exposed to UV light, it fluoresces an orange color intensifying almost 20-fold after binding to DNA. EB is the most commonly used dye for DNA and RNA detection in gels[123]. EB is a DNA intercalator, inserting itself between the base pairs in the double helix. EB is impermeable for live cells. EB has UV absorbance maxima at 300 and 360 nm, and an emission maximum at 590 nm. Although EB is routinely used to stain DNA in gels. Acridine orange and Ethidium bromide mixture has also been used here to differentiate live and dead (Apoptotic/Necrotic) cells[124]. Exponentially growing cancer cells were seeded in µ-Slide (chambered coverslip) with 8 wells (Sigma Aldrich, Germany) with 500 µL nutrient medium and kept at 37 ^{o}C and 5% CO_2. After 24 h the cells were treated with the corresponding compounds. After 48 h drug treatment the medium was discarded and the chamber slide was air dried for 2 min. The micro wells of the chamber slide washed with 500 µL of PBS (with Ca^{2+} and Mg^{2+}) and rinsed thoroughly. Further sample preparation steps differ for different microscopic method which are described below.

Visible Microscopy:

Under visible microscopy the treated cancer cells were visually checked for morphological changes, there was no fluorescent reagent used here. After rinsing with PBS the chamber slide was dried and 20 µM of PBS (with Ca^{2+} and Mg^{2+}) was added to each well, covered with a coverslip and visualized using Bright field microscope (Carl Zeiss, Germany).

Autophagy:

In case of Autophagy the degradation and recycling of cellular components takes place. When these cells were stained with AO, they enters acidic compartments such as lysosomes and become protonated and sequestered. In these low pH conditions, the dye emits orange light when excited by blue light. Thus, AO can be used to identify engulfed apoptotic cells (Auto phagosomes), because it fluoresces upon engulfment[120]. To check Autophagy for the treated cancer cells, after rinsing with PBS the chamber slide was dried and 100 µL of AO was added to each well of chamber slide, allowed to stand for 2-3 min. Then the dye was discarded, the chamber slide was dried and 20 µL of PBS (with Mg^{2+} and Ca^{2+}) was added and covered with a coverslip and analyzed under fluorescent microscope (390-700 nm, Carl Zeiss, Germany).

Additionally, AO/EB staining performed to check apoptotic and necrotic features for the treated cancer cells prepared in flasks. Exponentially growing cancer cells were seeded in 25cm^2 cell culture flasks with 10 mL nutrient medium and kept at 37 ^oC, 5% CO_2. After 24 h the cells were treated with the corresponding compounds. After 48 h drug treatment the adherent cells and the supernatant was harvested, centrifuged (1500 rpm, 5 min, 4 ^oC) and the pellet was washed with PBS. The supernatant was discarded and the pellet was resuspended in 100 µL PBS (with Mg^{2+} and Ca^{2+}). To the 100 µL of resuspended pellet 100 µL of AO/EB mixture (1 mg/mL) was added. 25 µL of an aliquot was transferred onto a slide and covered with a cover slip. The suspension was immediately analyzed under fluorescent microscope.

3.3.2.2 DNA Fragmentation Assay

Apoptotic DNA fragmentation is a key feature of apoptosis, which is a type of programmed cell death. DNA fragmentation is a consequence of apoptosis. Under stress and when the compounds treated with anti-tumor agents 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[124]. The assay performed as follows. Exponentially growing cancer cells were seeded in 25 cm^2 flasks (10 mL RPMI medium), then the flasks were kept in an incubator at 37 ^oC, 5% $_{CO2}$. After 24 h medium replaced with respective concentration of the compounds. After 48 h drug treatment floating cells induced by drug exposure were collected, washed with PBS and lysed with DNA lysis buffer (100 mM Tris HCl pH 8.0; 20 mM EDTA; 0.8% SDS; all from Sigma Aldrich). Then the cells were treated with RNAse A at 37 ^oC for 2 h. 10 µL proteinase K (20 mg/mL) was added to the sample and incubated at 50^oC overnight. 2% agarose gel was prepared (6 g agarose in 300 mL TAE-Buffer + 15 µL Ethidium bromide). 10 µL DNA loading buffer (6X) was added to samples and loaded on to the gel. The DNA samples were electrophoresed on a 2% agarose gel for 2 h at 40 V. The gel was examined and photograph ed by an UV Transilluminator (Biometra GmbH, Germany).

3.3.2.3 Cell cycle analysis:

Cell cycle analysis is a method in cell biology that allows the flow cytometry to distinguish cells in different phases of the cell cycle[125]. Before analysis, the cells were permeabilized and treated with a fluorescent dye that stains DNA quantitatively. The fluorescence intensity of the stained cells at certain wavelengths will therefore correlate with the amount of DNA they contain. During the S phase of the cell cycle the DNA content of cells duplicates, the relative amount of cells in the G0 phase and G1 phase (before S phase), in the S phase, and in the G2 phase and M phase (after S phase) can be determined, as the fluorescence of cells in the G2/M phase will be twice as high as that of cells in the G0/G1 phase[126]. Cell cycle anomalies can be symptoms for various kinds of cell damage, for example DNA damage, which cause

the cell to interrupt the cell cycle at certain checkpoints to prevent transformation into a cancer cell (carcinogenesis)[127]. Other possible reasons for anomalies include lack of nutrients, for example serum deprivation. The cell cycle analysis was done by Florescence Activated Cell sorting (FACS) machine using propidium iodide (PI) as a fluorescent dye. Exponentially growing cancer cells were seeded in 25 cm^2 flasks (10 mL RPMI medium), then the flasks were kept in an incubator at 37 ^oC, 5%CO_2. After 24 h the medium was replaced with respective concentration of the compounds. Following 24 h, 48 h and 72 h of incubation, the adherent c ells and th-e supernatant were harvested, centrifuged (1500 rpm, 5 min, 4 ^oC) and the pellet was washed with PBS. The cells (1×10^6 cells/mL) were fixed with ethanol (70%, -20°C, for 2 h). The fixed cells were centrifuged and the pellet resuspended in 1 mL staining buffer (PBS + 2% FCS + 0.01% NaN_3) and centrifuged. Further the cell pellet was resuspended in 100 µL of RNase A (1 mg/mL) and incubated at 37 °C, for 30 min. 1 mL propidium iodide (PI) (20 mg/mL of staining buffer) was added and the samples were kept in a dark at room temperature for at least 30 min before the analysis. Doublet cells were excluded from the measurements and for each cell cycle distribution 20,000 events were collected. Each sample was measured in triplicate and the results were compared with untreated controls. Cell Cycle distribution was calculated using Attune software (Life technologies, Darmstadt, Germany).

3.3.2.4 AnnexinV assay:

This assay is intended for detection of early apoptotic cells by flow cytometry. AnnexinV is a member of the Annexin family of intracellular proteins that binds to phosphatidylserine (PS) in a calcium-dependent manner. PS is generally found on the intracellular leaflet of the plasma membrane in healthy cells, but during early apoptosis, membrane asymmetry is lost and PS translocate to the external leaflet. During apoptosis, the cell membrane remains intact; whereas, during necrosis, the cell becomes leaky and loses its integrity[128]. Fluorescence iso-thiocyanate AnnexinV (FITC-AnnexinV)/Propidium iodide staining used here to target and identify both apoptotic and necrotic cells. Annexin V binding alone cannot differentiate between apoptotic and necrotic cells. Early apoptotic cells exclude PI, while late stage apoptotic cells and necrotic cells stain positively which is due to the passage of these dyes into the nucleus where they bind to DNA. Exponentially growing cancer cells were seeded in 25 cm^2 flasks (10 mL RPMI medium), then the flasks were kept in an incubator at 37 ^oC, 5% CO_2. After 24 h, the medium replaced with respective concentration of the the compounds. Following 24 h, 48 h and 72 h of incubation, adherent and floating cells were harvested, centrifuged (1500 rpm, 5 min, 4 ^oC) and washed with 1 mL PBS (with Ca^{2+}/Mg^{2+}). The cell pellet was resuspended in AnnexinV binding buffer (BioLegend®, San Diego, US) to a concentration of 1×10^6/ml. 100 µL of each sample was stained with PI solution (3 µL, 1 mg/ml) and FITC Annexin V solution (5 µL, BioLegend®, San Diego, US) for 15 min in the dark at room temperature. After adding Annexin V binding buffer (400 µL) the suspension was analysed using the Attune® FACS

machine (life technologies, Darmstadt, Germany). For each sample 20,000 events were collected. Each sample was measured in triplicates and the results were compared with untreated controls.

3.3.2.5 Caspase activation Assays:

Apoptosis is a type of programmed cell death. A group of intracellular proteases called caspases are responsible for the deliberate disassembly of the cell into apoptotic bodies during apoptosis. Caspases were identified as a family of cysteine dependent aspartate directed proteases, which are synthesized as precursors (procaspases) and are converted into active enzymes by apoptotic stimuli. There are two types of apoptotic caspases: initiator (apical) caspases and effector (executioner) caspases. Initiator caspases (e.g. Caspase 2, 8, 9, and 10) cleave inactive pro-forms of effector caspases, thereby activating them. Effector caspases (e.g. Caspase 3, 6, and 7) in turn cleave other protein substrates within the cell, to trigger the apoptotic process. They differ in primary structure and substrate specificity but share several common features, 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[71, 85, 129]. Apoptotic pathways are potential targets for therapeutic modulation[72]. Simplified schematic representation of essential pathways for caspase-dependent apoptotic cell death are shown in Figure 4.

In general apoptosis is triggered by internal cellular stress (TYPE II-Intrinsic pathway) or extracellular signals (TYPE I-Extrinsic pathway) that mediate effects via the binding of ligands (e.g. Fas,TNFR1, and DR5) to cell surface death receptors. Extrinsic pathways directly activate executioner caspases (caspase 3) through initiator caspases (e.g., caspase 8 and 9) ultimately leading to cell death. In intrinsic pathways, death signals are conducted through mitochondria, increasing permeability that leads to the release of Cytochrome C. Cytosolic Cytochrome C binds Apaf-1 to activate the apoptosome and caspase-9 which ultimately leads to downstream activation of executioner caspase 3[130]. The sample preparation for caspase assays carried out as follows. Exponentially growing cancer cells were seeded in 25 cm^2 flasks (10 mL RPMI medium), then flasks were kept in an incubator at 37 ^oC, 5% CO_2. After 24 h, medium replaced with respective concentration of the compounds. Following 24 h, 48 h and 72 h of incubation, cells were harvested by mild trypsinization and washed twice in PBS buffer (with Mg^{2+} and Ca^{2+}). The cell pellet was resuspended in PBS buffer with (Mg^{2+} and Ca^{2+}) to a concentration of 1×10^6/mL. 300,000 cells were treated with Caspase 3, 8 and 9 staining kit solution (1 µl, PromoKine, Germany) for 1 h at 37 °C and 5 % CO_2. Following the incubation, the cell samples were washed twice in caspase washing buffer (PromoKine, Germany), resuspended in 300 µl

caspase washing solution (PromoKine, Germany) and analysed using an Attune® FACS machine (Life technologies, Darmstadt, Germany). For each sample 20,000 events were collected and duplicates were measured.

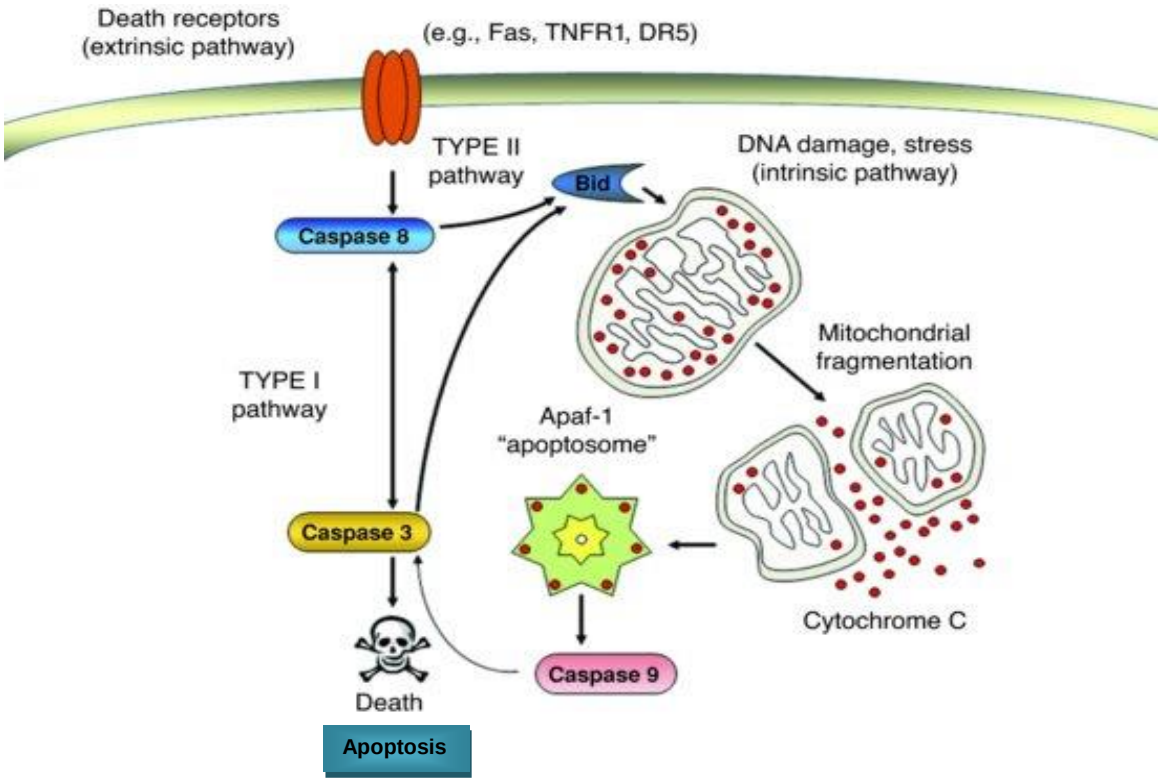


Figure 4. Caspase cascade in apoptosis [131].

Elucidation of antitumor activity of 30 anticancer agents (in which 18 newly synthesized compounds) carried out and classified into five chapters. Under each chapter the methods and preparation procedures employed are described.

Chapter I: Betulinic acid-Platinum Conjugates

The compounds tested here were synthesized by derivatization at the C-3 (hydroxyl) and C-28 (carboxylic group) positions of Betulinic acid. The *in vitro* cytotoxic activity of Betulinic acid (**1, BA**) and its derivatives containing Cisplatin (Figure 5) similar ligands were studied on five different cancer cell lines: 518A2 (melanoma cancer), A2780 (ovarian cancer), A549 (lung carcinoma), MCF7 (breast cancer) and 8505C (anaplastic thyroid cancer) as well as on one non-tumorous cell line (WWO70327) by SRB colorimetric assay method. Stock solutions (20 mM) of Betulinic acid (**1**)**, 2, 3, 4, 5** were prepared in DMSO and **CP, 3($PtCl_2$), 3(PtClS), 5($PtCl_2$), 5(PtClS), 6($PtCl_2$), 6(PtClS)** were prepared in DMF. With the goal of evaluating the

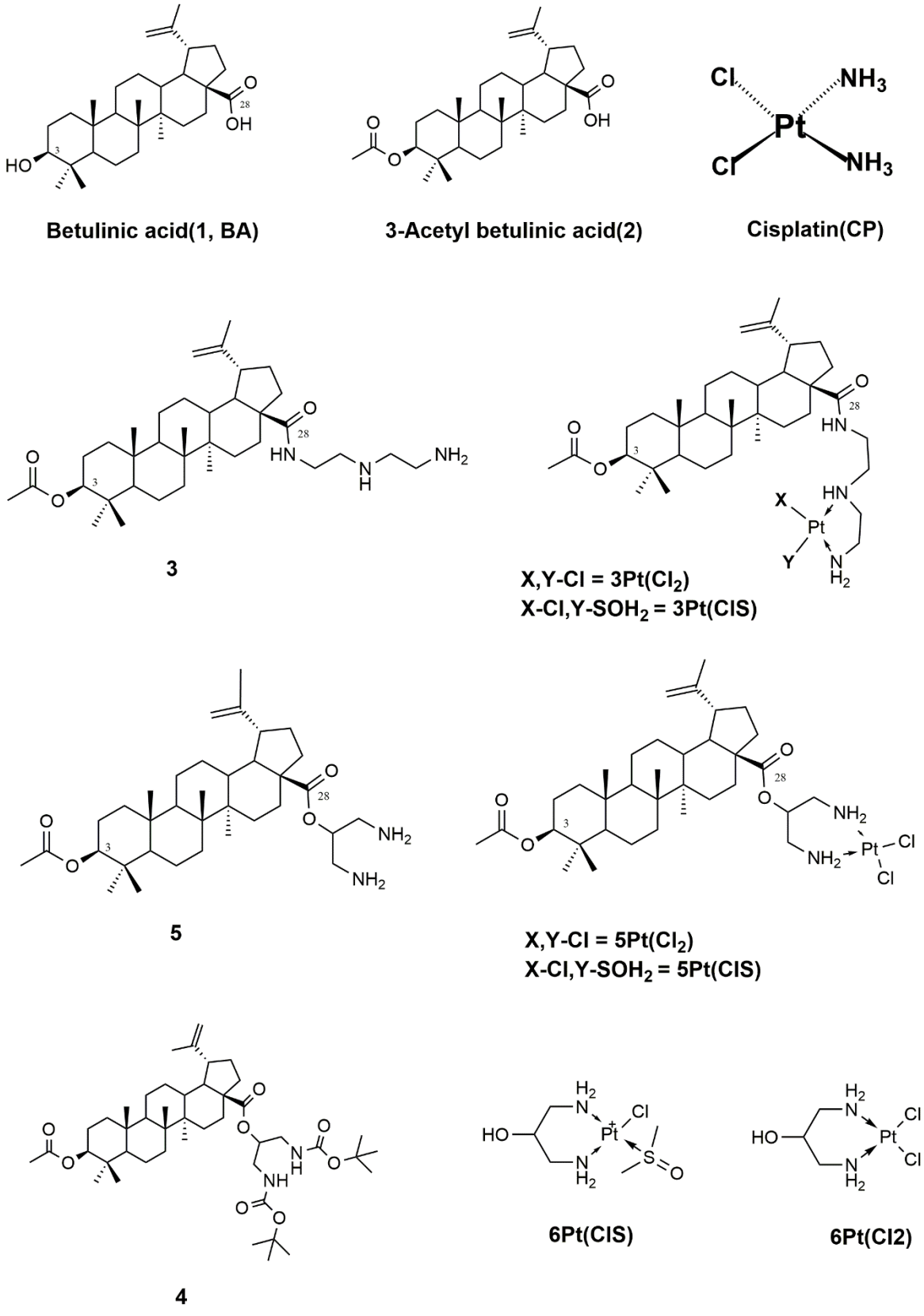


Figure 5. Structures of Betulinic acid-Platinum Conjugates

anticancer mode of cell death, based on the promising cytotoxicity, the compounds **3, 3($PtCl_2$), 5, 5($PtCl_2$)** along with **1** and **CP** were further selected for the more extensive investigations

i.e., cell cycle analysis, AnnexinV and Caspase activation assays were determined along with microscopic investigation and DNA fragmentation assay. The elucidation of compound induced cell death was carried out on A549 (lung cancer) cell line.

Chapter II: Bileacid-Platinum Conjugates

Stock solutions (20 mM) of all tested compounds were prepared in Dimethyl formamide (DMF). The *in vitro* antitumor activity of Cisplatin (**Pt1**) along with the corresponding ChAPt derivatives evaluated against a panel of five tumor cell lines of different histogenic origin (Figure 6).

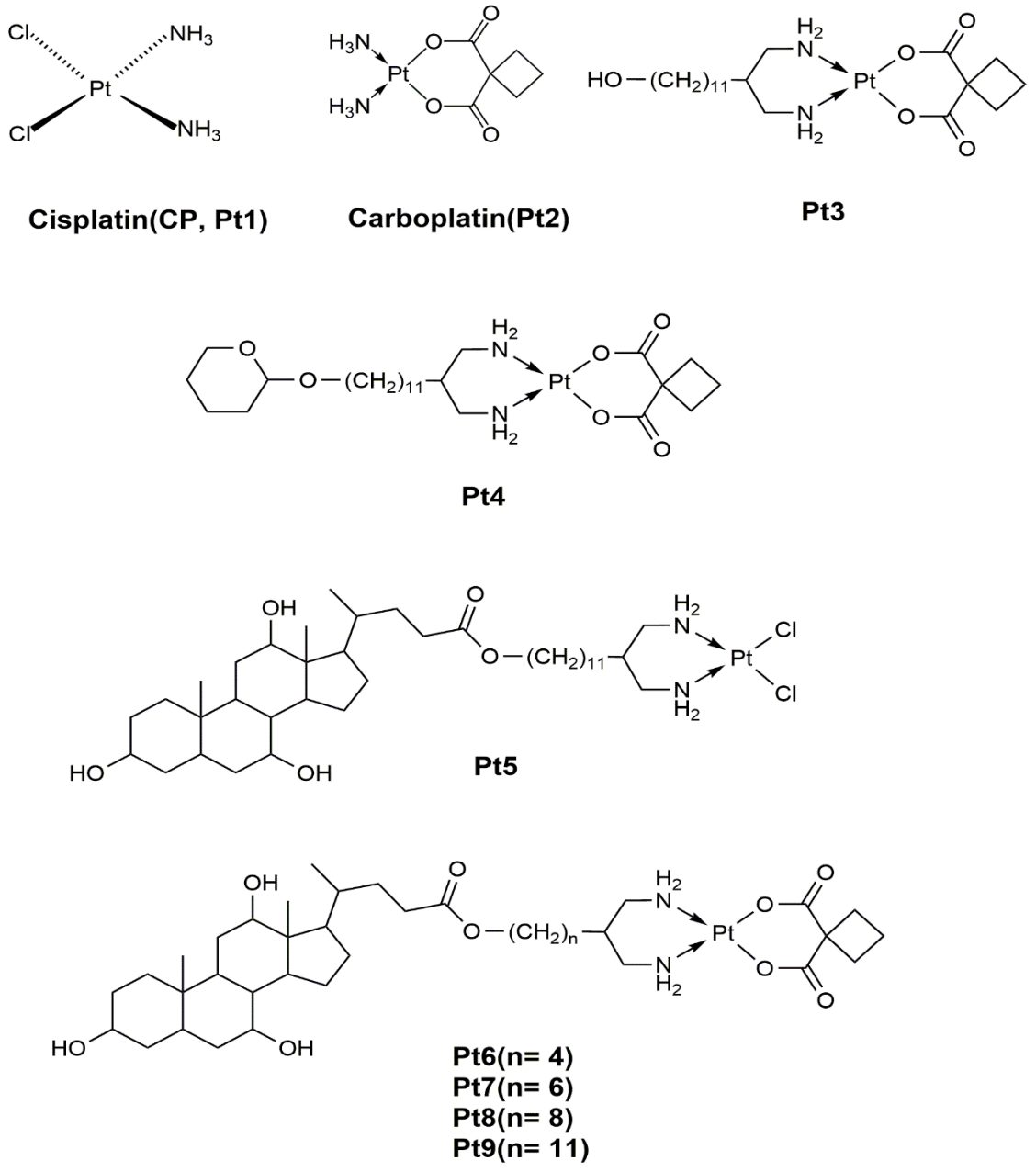


Figure 6. Structures of Bile acid-Platinum Conjugates

The cell lines A2780 (ovarian cancer), DLD1 (colorectal adenocarcinoma), HepG2 (hepatic-ellular carcinoma), 8505C (anaplastic thyroid), MCF-7 (breast cancer) were included in this study. A series of biological methods starting from Sulforhodamine B (SRB) assay to determine IC_{50}, Cell cycle analysis, AnnexinV assay and Caspase assays were performed with the aim of scrutinizing the anticancer mode of action Platinum conjugates against the HepG2 (hepatocellular carcinoma) cell line.

Chapter III: C-28 ester (NVX-207) and C-3 glucopyranoside (B10) derivatives of Betulinic acid

Stock solutions (20 mM) of Betulinic acid (**1, BA**), and its derivatives **B-10, NVX-207** and **NVX-207E** (Cyclodextrine conjugate of NVX-207) (Figure 7) were prepared in DMSO. **NVX-207E** was prepared as follows: 1 mg of **NVX-207** in 1,25 ml of 96% ethanol added to 1,25 ml hydroxyl-ß-cyclodextrine solution (0,05 M). After stirring, evaporation and lyophilisation the conjugate dissolved in saline (0,9% w/v). The cytotoxicity of the compounds was evaluated against two equine melanoma cell lines MelDuWi, MellJess/HoMelZh and A375 (Melanoma from human origin) using SRB micro culture colorimetric assay. The equine cancer cell lines were treated with the test compounds with their double IC_{50} values determined and analysed by flow cytometry.

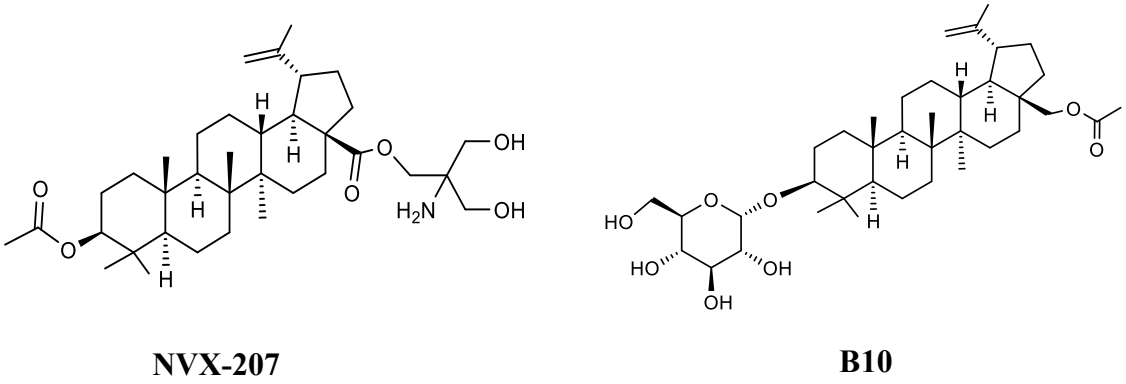


Figure 7. Structures of C-28 ester (NVX-207) and C-3 D-Glucose coupled ester (B10) derivatives of Betulinic acid

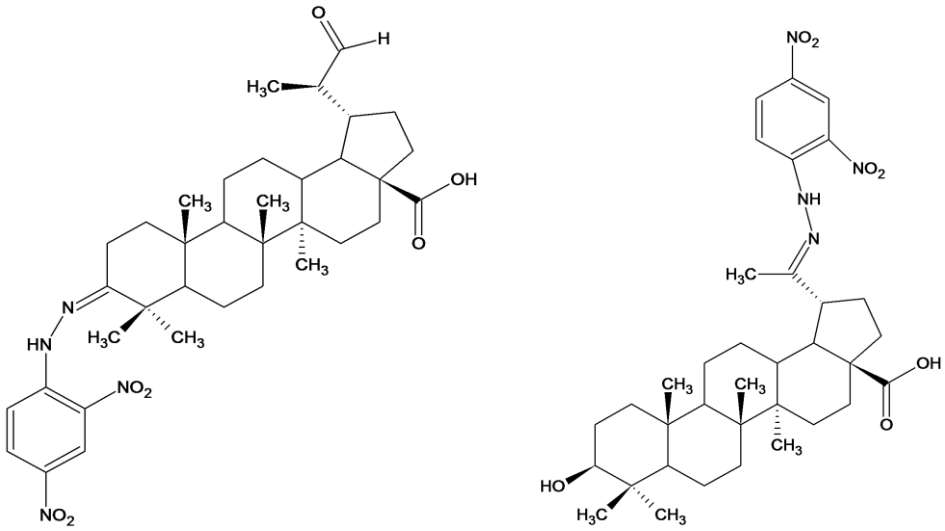
In vivo tolerability study on Horses: The *in vivo* experiments were carried out by J.-M.V. Cavalleri, University of Veterinary Medicine, Hannover, Foundation, Germany. Two adult grey horses bearing at least one melanoma were treated intratumorally with the **BA** derivative **NVX-207**. Horse 1 was a 13-year-old Warm blood mare with a weight of 695 kg and horse 2 was an 18-year-old Warm blood mare weighing 587 kg. Both horses were housed in standard conditions with daily access to pasture and standard feeding with hay ad libitum. The experiment was approved by the German national authorities (LAVES 33.14-42502-04-14/1588). Horse 1 had a solitary melanoma lesion at the root of the tail, horse 2 had multiple

melanoma lesions in predilection areas (underneath the tail, around the anus and vagina and in the head-neck area).

Study design: Each horse was clinically and clinical pathologically examined on each injection day and monitored daily for any clinical reaction. The horses were injected intratumorally in one (horse 1) respectively three dermal lesions (horse 2). **NVX-207** was diluted in sterile saline to a concentration of 0.06 mg/ml. Horses were injected the maximum possible amount per tumor (approximately 1.5 ml in horse 1 and a total of approximately 6 ml in horse 2) every 7 days for 19 consecutive weeks. Monitoring of horses for adverse events included attitude, heart rate, respiratory rate, rectal temperature, mucus membrane colour, capillary refill time and local signs such as swelling, pruritus, pain, reddening, depigmentation and ulceration. Blood was collected for haematology and blood biochemistry at each day of injection and 5 days after injection. Haematological parameters were determined using the ADVIA 120 (Siemens Healthcare Diagnostics, Eschborn, Germany). Blood biochemistry was assayed using the Cobas c311 (Roche Diagnostics GmbH, Mannheim).

Chapter IV: Betulinic acid 2,4-dinitrophenylhydrazone derivatives

Previously, a series of novel Betulinic acid 2,4-dinitrophenylhydrazone (2,4-DNPH) derivatives were designed and synthesized and found to show potent anticancer properties[111]. The two derivatives **2,4-DNPH1** and **2,4-DNPH2** (Figure 8) along with Betulinic acid (**1)** were assayed by cytotoxicity, selectivity, cell cycle arrest, AnnexinV and DNA laddering tests.



2,4DNPH1 **2,4DNPH2**

Figure 8. Structures of Betulinic acid 2,4-dinitrophenylhydrazone derivatives

In order to complement the previous results, the current work carried out for the elucidation of mechanism of action of apoptosis induction by these two derivatives in A549 lung cancer cell line. Caspase activation assays performed to contribute the understanding about the cytotoxic

potential and their structure activity relationship (SAR). Stock solutions of test compounds were prepared in DMSO (20 mm) and then diluted with nutrient RPMI-1640 medium. Staurosporin (0.5 µM) was used as a positive control.

Chapter V: Betulinic acid-Sulfamate conjugates

The *in vitro* anticancer activity and Carbonic anhydrase IX (CA IX) inhibition of new sulfamate conjugates of Betulinic acid (**1**, **BA**) was carried out. Stock solutions (20 mM) of test compounds **BA**, **CAI1-6** (CAI-Carbonic anhydrase inhibitor) (Figure 9) were prepared in DMSO. The cell lines 518A2 (melanoma cancer), A2780 (ovarian cancer), A549 (lung cancer), MCF-7 (breast cancer) and 8505C (anaplastic thyroid cancer) and non tumor human skin fibroblasts (WWO70327) were included in this study. The cytotoxicity of all compounds was evaluated by sulforhodamineB (SRB) (Sigma-Aldrich) micro culture colorimetric assay. The mode of cell death against MCF7 breast cancer cells induced by the most active compounds **CAI1**, **CAI3**, **CAI6** was investigated by the Fluorescence activated cell sorting (FACS) experiments along with microscopic investigations (AO/EB Staining).

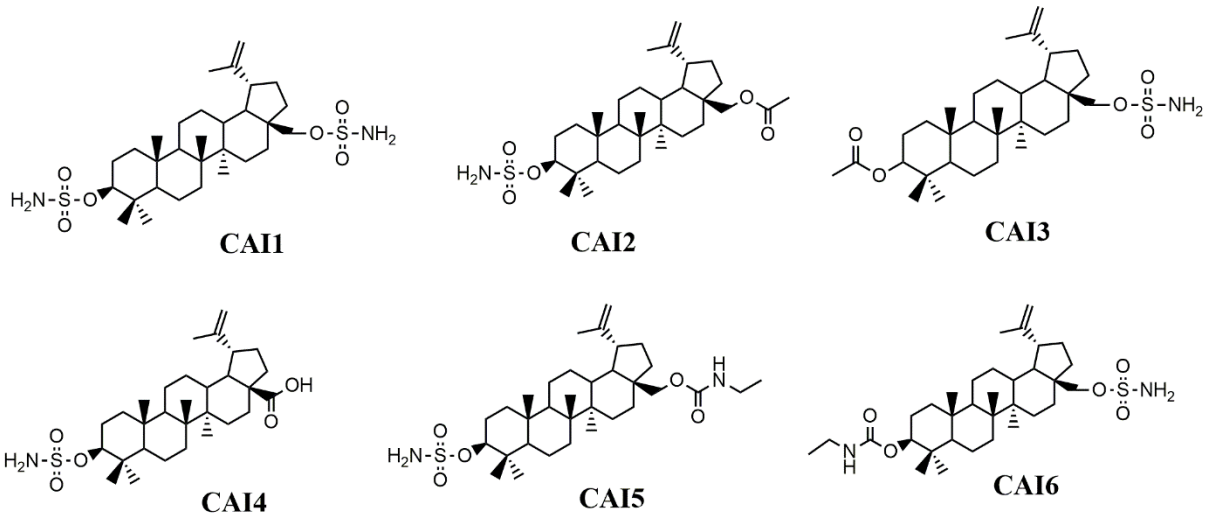


Figure 9. Structures of Betulinyl Sulfamates

Carbonic anhydrase IX (CA IX) inhibition:

The potential of the selected Betulinyl sulfamates to act as an inhibitor of CA IX was determined in a photometric assay employing recombinant CA IX derived from *E.coli* and, 4-nitrophenyl acetate (4-NPA) as a substrate. A mixture of PBS buffer solution pH 7.4 (76 µL), CA IX enzyme (20 µL) and compounds (**CAI1**, **CAI3**, **CAI6)** solutions (2 µL) was prepared in quartz cuvette and incubated at 20 ^{o}C for 3 min. 4-NPA (2 µL) was added to start the enzymatic reaction. The absorbance data (λ = 405 nm) was recorded at 25 ^{o}C for 3 min with 2 sec intervals using UV Spectrophotometer (Ultrospec 2100 pro, Amersham Biosciences, germany). All measure-ements were performed in duplicate. The substrate [4-NPA] concentrations are 10 mM, 8

mM, 5 mM, 4 mM, 2.5 mM, 1.25 mM, 0.625 mM and compound concentrations of 1 nM, 5 nM , 10 nM, 20 nM, 40 nM are used in this assay. The mode of inhibition as well as inhibitory co-nstants (Ki) were determined by Lineweaver Burk plots and Dixon plots using Graphpad Pris m6 respectively.

4. Results and Discussion

The whole set of results and the structure-activity relationship for all the investigated antitumor drugs discussed and described below as five chapters and related publications referred in the text as appendices.

4.1 Chapter I: Influence of double loading on cytotoxicity of novel conjugates *(Appendix 1 and 2)*

The **BA-CP** conjugates exerted a dose-dependent antiproliferative action at micro molar concentrations and the effect of these structural variations on anticancer activity was studied and discussed. The compounds **1, 2, CP, 6($PtCl_2$)**, **6(PtClS)** are the precursors used to synthesize the new derivatives. The lead compound **1** showed high cytotoxicity (avg. IC_{50}= 8-14 µM) against all used cell lines. Compound **2** was slightly more cytotoxic than **1**; the presence of an acetyl group at C-3 seemed to influence the more pronounced activity[131]. Compound **1** more strongly leads to changes in morphological behavior, and apoptotic & necrotic properties were observed for A549 cells by AO/EB staining (see supplementary data Figure S1). Compound **1** more strongly leads to autophagy than other tested compounds (see supplementary data Figure S2). Compound **1** induced apoptotic cell death which was evidenced by the occurrence of typical DNA ladder pattern and which was mediated by cell cycle independent apoptosis (directly provoked apoptosis). **CP** showed superior cytotoxic activity (avg. IC_{50} = 0.33-1.15 µM) against all tumor cell lines. **6($PtCl_2$)** showed 18 times less cytotoxicity than **CP**. **6(PtClS)** was less cytotoxic (IC_{50}=19.05 to >100 µM) than **6($PtCl_2$)**. Probably the presence of chloride groups in **6($PtCl_2$)** contributes more favorably to the activity than the sulfoxide groups in **6(PtClS)**. **CP** produced apoptotic cell death which was evidenced by microscopic investigation (Figure S1 and Figure S2) and fragmented DNA observed for the treated A549 cells. After 24 h **CP** found to show "S" phase arrest and after 48 h it caused "G2/M" phase arrest which was associated with slight increase in the number of cells in SubG1. **BA** and **CP** were sensitive to AnnexinV and up regulates caspase cascade (caspase 3,8,9) involved in both extrinsic and intrinsic pathways of apoptosis which may be due to cellular damage by **BA** and DNA damage by **CP** (Appendix 1 and 2).

Compound **3** is a C-3 acetyl and C-28 alkyl amide derivative of Compound **1**, and **3($PtCl_2$)** and **3(PtClS)** platinum conjugates of Compound **3** in which chloroplatinum and Sulfochloroplatinum ligands covalently linked to C-28 alkyl amide. Compound **3** showed high cytotoxicity (avg. IC_{50}=1.3-2.24 µM) against all types of tumor; the presence of both acetyl and (2-(2-aminoethyl)aminoethyl) amide moieties at C-3 and C-28 positions could be responsible for the high cytotoxicity[132]. However its platinum derivatives **3($PtCl_2$)** and **3(PtClS)** exhibited significantly less cytotoxicity than its precursor. It was found that **3** was 5 to 14 times more cytotoxic than its platinum conjugates. Compounds **3** and **3($PtCl_2$)** induced apoptosis through

the caspase cascade pathway (caspase 3,8,9) without cell cycle arrest and it was further confirmed by microscopic investigation and occurrence of typical DNA ladder during fragmentation assay both compounds induced nearly same amount of apoptosis during AnnexinV assay (Appendix 1 and 2).

Compound **5** is C-3 acetyl and C-28 nonlinear alkyl amine ester derivative of Compound **1**. Compound **5** shared similar range of IC_{50} values with Compound **1.** Even though Compounds **3** and **5** have symmetrical groups, it was found that **3** was 5 to 6 times more cytotoxic than **5**. The reason could be the linear alkyl amide (polyamine) group present at compound **3** that strongly influenced the ability of the substance to enter the cell through the membrane as well as the capability to interact with cell components[133]. It was found that the cytotoxicity of **5** was 1 to 3 times greater than its platinum conjugates **5(PtCl$_2$)** and **5(PtClS).** The compounds **5** and **5(PtCl$_2$)** led to changes in morphological behaviour and autophagy induction (Figure S1 and Figure S2), both compounds directly provoked apoptosis with concomitant decline in cell cycle phases. A weak DNA ladder was observed for compounds **5** and in case of **5(PtCl$_2$)** relatively strong DNA ladder was observed. From the AnnexinV assay results it was observed that the amount of apoptosis was high for **5(PtCl$_2$)** when compared to Compound **5** (Appendix 1 and 2).

The following major outcomes were made based on all experimental results from this chapter. Among the panel of **BA-CP** conjugates Compound **3** was found to be highly cytotoxic and it induced apoptosis mediated by caspase cascade pathway similar to **BA**. The presence of alkyl amide (polyamine) at C-28 position led to DNA damage followed by apoptotic cell death and finally it contributes to the high toxicity. Besides the classical caspase dependent apoptosis, caspase independent cell death may occur maintaining key characteristics of apoptosis. Notably, Compound **5** bypassed the caspase dependent mechanism, evidenced by occurrence of floating cells showing morphological changes and weak DNA fragmentation despite no significant caspase activity observed during drug treatment.

Selectivity:

The selectivity of the compounds was assessed on human skin fibroblasts (WWO70327). It was observed that Compound **1** was 3 to 5 times more selective towards cancer cells than to fibroblasts, and similar selectivity was observed for Compound **2**. The most active Compound **3** and also Compound **5** were the least selective substances towards all types of tumours. The selectivity of **3(PtCl$_2$)** was between 2 and 3.5 times towards cancer cells than normal cells, and **5(PtCl$_2$)** was around 3 to 4 times more selective towards cancer cells. Different selectivity patterns were observed for **6(PtCl$_2$)**. The dose-response curves for the test compounds towards tumor (A549) and non-tumor (WWO70327) cells were determined (Appendix 1).

4.2 Chapter II: Biomolecules linked to transition metals complexes *(Appendix 3)*

The *in vitro* antitumor activity of Cisplatin (**CP, Pt1**) along with the corresponding ChAPt derivatives evaluated against a panel of five tumor cell lines of different histogenic origin. The cytotoxicity results revealed that all compounds showed potent anticancer properties with avg. IC_{50} values between 15-73 µM on all tested cell lines. From the results, it was observed that the cytotoxic activity was retained for the ChAPt conjugates. Compounds **Pt3** and **Pt4** showed less cytotoxicity than lead compound Cisplatin (**Pt1)** (Appendix 3).

The cell cycle and AnnexinV assay results clearly indicate that similar to **Pt1,** the compounds **Pt8** and **Pt9** induced apoptosis mediated by caspase cascade pathways along with the time dependent arrest of their cell cycle phases. The possible reason could be that the damage caused by compounds **Pt8** and **Pt9** to cells was unrepairable and they could follow the apoptosis. From the literature it was well known that **Pt1** exert its cytotoxic effects by covalent binding to genomic DNA[66]. Binding to mitochondrial DNA is discussed as controversial[66] but in general will be ascribed a subordinate importance for its activity. There is no direct correlation of drug accumulation, with respect to DNA platination and platinum induced toxicity is not entirely DNA dependent, and protein damage may be involved as well. **Pt5** bypassed the caspase dependent mechanism evidenced by occurrence of floating cells after treatment and showed strong SubG1peak from cell cycle analysis. This particular result is comparable with previous findings, where the THP(C11)Cis induced cell cycle independent and caspase dependent apoptosis[35, 134].

The present work revealed that among the panel of ChAPt(n)Carbo derivatives, **Pt9** has better cytotoxicity than others. The exact reason remains unclear. However it is possibly related to the lipophilic character of the long alkyl spacer $(-CH_2-)_{11}$, facilitating a more easy penetration into cellular membrane leads to its higher uptake into the cells. **Pt5** showed acute toxicity similar to that of **Pt9**. The cytotoxic effect of the **Pt5** similar to its parent drug **Pt1** at least in part due to its ability to interact with DNA and to inhibit DNA replication. However, certain differences between the two compounds would be expected to exist. **Pt1** is able to form monofunctional and bi-functional adducts with DNA. By contrast, the formation of ChAPt-DNA adducts may not necessarily be similar. The upregulation of all three caspases by all tested compounds was increased to maximum towards the end of the treatment, which strengthen the assumption that apoptosis induced by tested compounds traverse the mitochondrial or intrinsic pathway. The possible explanation would be that, the DNA damage caused by platinum conjugates act as a death signal which is conducted through mitochondria, increasing permeability that leads to the release of Cytochrome C. Cytosolic Cytochrome C binds Apaf-1 to activate the apoptosome and caspase 9 which ultimately leads to downstream activation of executioner caspase 3 (Appendix 3).

4.3 Chapter III: C-28 ester (NVX-207) and C-3 glucopyranoside (B10) derivatives of Betulinic acid *(Appendix 4)*

The main objective of this work was to test Betulinic acid (**1**) and its derivatives **B-10**, the Tris ester **NVX-207** and cyclodextrine encapsulated **NVX-207E** thereof on equine melanoma cell lines and to show that they induce apoptosis comparable to human cancer cell line A375 (Melanoma cancer). Additionally, the formulation with the highest prospects of clinical efficacy was evaluated for safety after intratumoral injection in two horses. In this *in vitro* study we could demonstrate that the derivative **NVX-207**, compared to the other tested compounds has the highest cytotoxicity on equine melanoma cell lines with an avg. IC_{50}-value of 5.6 µM and 6.5 µM (MelDuWi and MelJess). Hydroxyprolyl-β-cyclodextrine encapsulated **NVX-207E** resulted in a similar cytotoxic efficiency (avg. IC_{50} =7.7 µM and 8.3 µM for MelDuWi and MellJess cells respectively). A time dependent administration of **NVX-207E** revealed that the drug reaches and enter the cells or is attached to them within the first 24 h after treatment. The subsequent experiments were performed after 24 h of treatment and later time points. There was no substantial difference between the cytotoxic activities after 24 h and 96 h (Appendix 3).

Betulinic acid (**1**) is inhibiting cardiolipin desaturation leading to ultrastructural changes in the mitochondrial membrane, possible reason could be a direct interaction with cardiolipins in the mitochondrial membrane[59-60]. As a consequence, pores in the outer mitochondrial membrane are formed followed by release of Cytochrome C from pores into the cytoplasm. The formation of the pores is an essential step in apoptosis induction, as apoptosis can be prevented by co-treatment with α-D-Tocopherol. The subsequent activation of the initiator caspase 9 and the effector caspases e.g. caspase 3 was already demonstrated for Betulinic acid (**1**)[63, 135]. Betulinic acid (**1**) seems to activate also other pathways leading to controlled cell death in absence of caspase activity. Cell cycle results for both A375 and equine melanoma cell lines treated with all the four compounds show that the DNA fragmentation (SubG1) takes place early stages of after 48 h treatment and no cell cycle arrest was detected. The same time-dependent AnnexinV results were detected for all three melanoma cell lines treated with **1, B10, NVX-207** and **NVA-207E**. The AnnexinV-staining showed a huge increase of early and late apoptotic cells after 24 h and 48 h of treatment. Especially there is an immense amount of early apoptotic cells after 48 h. The time shift between the amount of apoptotic cells detected via AnnexinV staining and SubG1 cell measurement is explained by the subsequent occurrence of the visualized characteristics of the apoptosis induction pathway. Concurrently, the caspases 3, 8 and 9 get activated in a time-dependent manner. For the A375 cell line all three analysed caspases (caspase 3, 8 and 9) were activated in one third to two thirds of the cells after 24 h when treated with **1, NVX-207** and **NVA-207E** respectively. When treated with **B10** more than 90 % of the cells have activated caspases already after 24 h. Therefore, no

measurements were performed for **B10** after 48 h. For the equine melanoma cells (MelDuWi and MellJess) the activation of the caspases is time shifted except for treatment with **NVX-207E**. Treatment with **1, B10** and **NVX-207** result in a slight increase in the amount of cells with activated caspases after 24 h. After 48 h for MelDuWi cells treated with all tested drugs, the number of cells with caspase activation accounts to 90%. After 48 h the MellJess cells treated with **1, B10, NVX-207** and **NVA-207E** led to an activation of all three caspases was observed in two thirds, around 80% and more than 90% of the cells respectively (Appendix 4).

Further intratumoral administration of **NVX-207** found to be safe and well-tolerated in adult grey horses. Two adult female grey horses were administered with 0.06 mg/ml **NVX-207** (100 µM) every one week for 19 consecutive weeks. Both horses tolerated the injections well and did neither show local signs of irritation or inflammation nor clinical signs of systemic illness. Hematologic and blood-biochemical evaluations revealed only slight deviations from the reference ranges of the laboratory without any association to the application of the test substance. Clinical chemistry results unveiled no specific response to the weekly administration of **NVX-207** with slight alterations in liver specific enzymes Gamma-glutamyl dehydrogenase (GLDH) and Gamma-glutamyltransferase (GGT). One horse was euthanized after the 19th injection of **NVX-207** because of a colon torsion. At necropsy no specific findings associated with the administration of a chemotherapeutic agent were shown. In a first tolera-bility evaluation *in vivo* the formulation was administered every one week for 19 consecutive weeks and well tolerated in two adult melanoma affected horses. The whole set of results open up the possibility for first clinical *in vivo* studies on grey horses with melanoma.

4.4 Chapter IV: 2,4-dinitrophenylhydrazone (2,4-DNPH) derivatives of Betulinic acid induce caspase dependant apoptosis *(Appendix 5)*

In the present study, Caspases 3, 8 and 9 activation assays were carried out for two new Betulinic acid 2,4-DNPH derivatives**.** Previous investigations show that two 2,4-DNPH derivatives were more cytotoxic and selective towards different cancer cell lines than Betulinic acid **1**, for instance **2,4-DNPH1** (avg. IC_{50}= 1.76-2.51 µM) and **2,4-DNPH2** (avg. IC_{50}= 7.52-16.9 µM)[111]. **2,4-DNPH1** was the most cytotoxic compound and able to arrest cell cycle in G0/G1 phase, as well as inducing apoptosis as seen in AnnexinV and DNA laddering assays. while **2,4-DNPH2** was the most selective compound (5-7 times) towards cancer cells. In order to complement the previous results, caspase assays performed for **2,4-DNPH1** and **2,4-DNPH2** along with Betulinic acid (**1**) on A549 cells. After 24 h, **2,4-DNPH1** and **2,4-DNPH2** did not activate significantly any caspases. **2,4-DNPH1** was not able to activate caspases, when analysed after 48 h; a significant activation (50%) was observed after 72 h. **2,4-DNPH2** substantially activated caspases 3 and 8 after 48 h, and after 72 h the activation was up to 75%. The results showed that all caspases were activated during the experiment period, with

higher magnitude of caspases 8 and 3. Considering these results, it was inferred that the mode of action of **1** and its 2,4-DNPH derivatives against cancer cells is by caspase-dependent apoptosis induction (Appendix 5).

The interaction of lupane type pentacyclic triterpenes with phospholipids in cell membranes has been suggested as the most important step of their mechanisms of action [60, 136]. Other factors can also influence the passage of a substance through the cell membrane, such as lipophilicity, pH and pKa, molecular weight, stability, etc. More liposoluble substances have a higher partition coefficient (log P) and can cross the lipidic bilayers more easily. The optimal partition coefficient (log P_o) of the drugs is in general between 2 and 7; substances with log P_o higher than 7 could be retained in the lipidic membrane due to their high lipophilicity. Log P of **1** is 7.38, while **2,4-DNPH1** and **2,4-DNPH2** have respectively, 9.13 and 8.77 log P values, accordingly which were determined by ACD/ChemSketch® software. This shows that the 2,4-DNPH derivatives are more lipophilic, have higher molecular weight and bulky functional groups when compared to **1**. It is likely that 2,4-DNPH derivatives have difficulties to cross the membrane since they have affinity for lipidic substances, contributing with a retention process in the membrane. Another possibility is related to the size and volume of the derivatives, because during crossing of the membrane, the compounds could disorganize the lipidic bilayer and the 2,4-dinitrophenylhydrazone moiety could act like a "hook", retaining the molecules. These evidences could justify the slower mechanism of action of these derivatives in comparison to Compound **1**.

4.5 Chapter V: Sulfamate conjugates of Betulinic acid: Molecules with the double function-*in vitro* anticancer activity and Carbonic anhydrase IX inhibition *(Appendix 6)*

While probing the structural features responsible for the pharmacological effects of Betulinic acid **(BA**) and its sulfamate conjugates, interesting outcomes obtained and further helped to optimize their activity profile. The results show that the lead compound **BA** has the avg. IC_{50} of 8-15 µM against investigated tumours. **CAI1** and **CAI2** were slightly more cytotoxic than **BA** when tested on cancer cell lines; the sulfamate conjugate **CAI3** found to show high cytotoxic activity (avg. IC_{50}=5-10 µM) against all cancer cell lines. The presence of both acetyl and slufamate moieties at C-3 and C-28, respectively could be responsible for its high antitumor potency. The Compounds **CAI4**, **CAI5** and **CAI6** showed moderate to good cytotoxicity (avg. IC_{50}=8-20 µM) against used tumor cell lines. Based on the results obtained, we could summarize that derivatization at C-3 and C-28 positions of **BA** by sulfamates and alkyl amide derivatives leads to no loss of activity, despite a significant increase in the cytotoxic activity observed for the conjugates. The compounds were screened for their selectivity towards tumor cells. Interestingly, similar selectivity was observed for the compounds **CAI1**, **CAI3**, **CAI6** to

that of **BA** towards all tumors. The compounds **CAI2** and **CAI4** found to be least selective than **BA** towards all tested tumours (except A2780 with **CAI4**). When compared to **BA**, Compound **CAI5** is 3 times better selective towards 8505C and A2780 than to normal cells. Despite the different cytotoxicity and selectivity patterns of the investigated compounds, the cytotoxicity results revealed that **BA** and all of its sulfamate derivatives are potential lead compounds for newantitumor agents. Based on the promising cytotoxicity of the compounds, the compounds **BA, CAI1, CAI3 and CAI6** were selected and examined further to check whether they induced cell death, mediated by apoptosis or any other programmed cell death pathways[18]. MCF7 cell line was used here to investigate general anticancer mechanism of apoptotic induction. From the microscopic investigations it was observed that all tested compounds showed apoptosis features such as chromatin condensation, membrane blebbing and apoptotic bodies. The cell cycle results show that the tested compounds interfere with cell cycle of MCF7 cells; after 24 h treatment cell cycle G0/G1 phase arrest was recorded followed by increased SubG1 upon further treatment periods (48 h and 72 h). The cell cycle arrest by sulfamateconjugates correlated with activity of sulfonamide derivatives[90], which are known to be involved in the cell cycle perturbatios (G1 and G2 phases) with powerful anticancer activity (Appendix 6).

Further, cell death induction was assessed by the AnnexinV-FITC/PI staining assay. In comparison to controls an increase of the number of apoptotic cells was observed for all treated cells. During initial treatment period i.e., 24 h slight increase, around 10-15% apoptosis (Early apoptosis+Late apoptosis) was verified to all treated cells. After 48 h, more than 50% of the cells treated with **BA** were apoptotic, while the amount of 41% was observed to **CAI3**. For the cells treated with these two compounds, the rate of apoptosis is eventually increased upon further treatment period (72 h). In case of **CAI1** the rate of apoptosis relatively is lower than that of **BA** and **CAI3**, while for **CAI6** the minimum rate of apoptosis was observed throughout the treatment period. Except for cells treated with **BA** (during 72 h, necrotic cells=17.25%) no considerable amount of necrotic cells was exhibited for cells treated with **CAI1, CAI3, CAI6.** Despite **BA** being less active (higher IC_{50}=14 µM) than **CAI3** (IC_{50}=7 µM), it induced a higher magnitude of apoptosis in a shorter time than **CAI3**. This observation is in agreement with the cell cycle results. One critical hallmark of apoptosis is activation of caspases, which are responsible for the degradation process that eventually leads to the typical features of apoptosis[26]. The activation of the upstream caspases 8, 9 were tested by using fluorescent labelled inhibitors of caspases (FLICA) by flow cytometry. The results revealed that both of the caspases were activated during the experimental period, with higher magnitude of caspases 8 which strengthen the assumption that the caspases were involved and played a crucial role in the compound induced apoptotic cell death which is mediated by intrinsic pathway of mitochondrial apoptosis induced by tested compounds. The possible explanation

would be that, the G0/G1 arrest caused by sulfamate conjugates acting as a death signal which is conducted through mitochondria, increasing mitochondrial transition membrane permeability which leads to the release of Cytochrome C. Cytosolic Cytochrome C binds Apaf-1 to activate the apoptosome and caspase 9 which ultimately leads to apoptosis (Appendix 6).

The sulfamate conjugates of **BA** furthermore screened in an *in vitro* enzymatic assay using CA IX and, 4-nitrophenyl acetate (4-NPA) as a substrate; the inhibition constants (Ki) and the type of inhibition was determined for compounds **CAI1, CAI3, CAI6**. **CAI6** (Ki = 4.46 nM) seems to be moderately inhibits the CA IX enzyme activity. When compared to **CAI6, CAI1** (Ki = 2.62 nM) showed relatively better inhibitory effect and **CAI3** (Ki = 1.25 nM) showed superior inhibitory activity on CA IX. The Ki values of all three sulfamate conjugates are in single digit nano molar range which makes them act as the competitive inhibitors for binding substrate to CA IX. Different substitutions at **BA** skeleton influenced the overall inhibitory activity of the compounds against CA IX. Even though the C-28 sulfamate group ideally present in both of three compounds, different groups (sulfamate in **CAI1**, acetyl in **CAI3** and ethyl carbamate in **CAI6**) presented at C-3 positions could be the reason for their different inhibitory properties. It was assumed that the steric hindrance of C-3 acetyl group of **CAI3** is considerably small when it binds to the enzyme CA IX and efficiently inhibit its activity. While, the steric hindrance of sulphamate group of **CAI1** and the ethyl carbamate of **CAI6** relatively stronger (due to large sized groups) lead to loss of inhibitory effect. The enzyme assay results and the inhibitory constant values for the tested compounds strengthen this assumption.

5. Conclusions and Future Perspectives

The search for new candidates for anticancer agents is a very competitive field in research and many different approaches have been made. The preclinical development of bioactive natural products and their analogues as chemotherapeutic agents is a major objective of anticancer research programs. The present work not only tries to find new derivatives that are more potent than the existing ones but also intends to make a contribution to scrutinize the mechanism of action. The evaluation of the whole set of results would lead to new insights in the field of drug development. Based on the results obtained from each chapter the following conclusions were made,

1. In general, when two cytotoxic groups are covalently linked in one molecule, a more pronounced cytotoxic activity can be expected. But our results revealed that Betulinic acid-Cisplatin conjugates showed more or less similar cytotoxicity to that of Betulinic acid (**1**) and all conjugates found to be less cytotoxic than Cisplatin (**CP**) against all tumours. The property of double loading of two cytotoxic groups in conjugates doesn't contribute to exhibit improved cytotoxicity. On the other hand, among the panel of derivatives, Compound **3** (3-*O*-acetylbetulinic(2-(2-aminoethyl)aminoethyl)) amide was found to be highly cytotoxic with the average IC_{50} of 1.3-2.24 µM on all cancers and less selective towards tumours than to normal cells. The order of cytotoxicity of all compounds on investigated cancers from highest to lowest can be given below.

CP>3>2>1>5>4>3($PtCl_2$)>3(PtClS)>5($PtCl_2$)>5(PtClS)>6($PtCl_2$)>6(PtClS)

The structure of platinum conjugates influenced the ability to enter the membrane and to alter the mitochondrial membrane potential this could be the reason for the different cytotoxicities. Cisplatin **(CP**) is a DNA damaging agent (apoptosis-intrinsic pathway). To induce apoptosis it has to enter the nucleus followed by DNA damage takes place[66, 137]. In case of Betulinic acid-Cisplatin conjugates the Cisplatin **(CP**) ligand not directly linked to Betulinic acid **(1)** skeleton instead, it was covalently linked to the C-28 alkyl amide group (a polyamine spacer). When A549 cells treated with these conjugates inside the cells the ligands either may not be cleaved (there was no available enzyme to cleave ligands from spacer) then obviously the uncleaved ligands never enter the nucleus for DNA damage, or apoptosis caused by Betulinic acid (**1**) fragment is much quicker than by interaction of the platinum with DNA. In summary, the design of a more effective and more systematic structural variations of Betulinic acid-Cisplatin conjugates must be carried out in order to get more clarity and more reliable structure-activity relationship. Nevertheless, the results open up the possibility of double loading with two cytotoxic groups and the double loaded conjugates deserved to be proceed for further developmental strategies.

2. In case of Bile acid-Platinum conjugates, the increased order of IC_{50} values noticed for compounds **Pt6**, **Pt7**, **Pt8**, and **Pt9**, is clearly attributed to their spacer chain length. The magnitude of order of cytotoxicity of ChAPt(n)Carbo derivatives against all tested cancer cell lines from highest to lowest can be described as **Pt6<Pt7<Pt8<Pt9.** To summarize, the interesting outcome from our findings is that the ChAPt(11)Cis and ChAPt(11)Carbo induced programmed cell death with molecular features different from each other, suggesting that both drugs induce apoptosis through different initial pathways which was evidenced by the activation of caspases along with or without cell cycle arrest. In case of ChAPt(11)Cis the bi-oactive content (Cisplatin) after entering the cell may alter preferably the mitochondrial mem-brane potential and trigger the Intrinsic pathway of apoptosis [31, 32].The two carboxy leaving groups in the bioactive part (Carboplatin) of ChAPt(n)Carbo, enables the conjugate highly polar towards the nuclear DNA and ultimately promote cell cycle arrest followed by programmed cell death or apoptosis [33]. Even though the cytotoxicity potential is less than the lead compound, this ideal combination of ChAPt conjugates due to their possibility of targeted drug delivery with less side effects, the conjugates deserved to be proceeded for further pre-clinical (Kinetic studies) and *in vivo* investigations.

3. Betulinic acid and its two derivatives **B10** and **NVX-207**, both with an improved water solubility compared to Betulinic acid (**1**), were tested *in vitro* on two equine melanoma cell lines (MelDuWi and MellJess/HoMelZh) and human melanoma (A375) cell line. We could demonstrate that all three compounds especially **NVX-207** show high cytotoxicity on both equine melanoma cell lines. The treatment with these compounds lead to externalisation of phosphatidylserines on the cell membrane (AnnexinV staining), DNA-fragmentation (cell cycle analysis) and activation of initiator and effector caspases (Caspase assays). Our results indicate that the apoptosis is induced in the equine melanoma cells by all three compounds. Furthermore, we succeeded in encapsulating the most active compound **NVX-207** in 2-Hydroxyprolyl-β-cyclodextrine without a loss of its activity. To sum up, the *in vivo* results of repeated intratumoral administration of **NVX-207** in two adult grey mares show a very good tolerability. Neither clinical nor clinicopathological adverse effects of the compound were noted. This formulation can be used as a promising antitumor agent for treating grey horse melanoma.

4. The apoptotic mechanism of action of Betulinic acid 2,4-dinitrophenylhydrazone (2,4-DNPH) derivatives was evaluated. The biological results showed that the 2,4-DNPH moiety plays an important role for the cytotoxicity of these molecules and also for the activation of caspases and apoptosis induction. The structural features such as hydroxyl at C-3 or aldehyde at C-20 might influence the overall biological potential. Although the 2,4-DNPH derivatives are

highly cytotoxic and play a role in apoptosis cascade activation, it is noteworthy that the kinetics of their mechanism of action is slower than the precursor Betulinic acid (**1**).

5. The pentacyclic triterpenes (**BA**) combined with the strong CA IX inhibiting ability of sulfamates to develop more potent and selective inhibitors of CA IX. So far considerably less information is available on the biological activity and anticancer potential of triterpenoid sulfamates. The present investigation demonstrates that simple modifications of the parent structure of **BA** can produce a number of highly potent derivatives, which may improve the selective toxicity profile or to introduce general toxic effects. Studies performed here revealed that the Betulinyl sulfamates are fair to excellent cytotoxic agents against all tumor cell lines. The order of cytotoxicity (IC_{50}) of all tested compounds on investigated cancers from highest to lowest can be given below.

CAI3>CAI1>CAI2>BA>CAI6>CAI5>CAI4

Physical and pharmaceutical properties of the molecules, such as solubility in aqueous medium, intracellular penetration capability, stability, metabolic activation, etc., could explain the different rate of apoptosis between the sulfamate conjugates[95]. Particularly **CAI1, CAI3, CAI6** showed good cytotoxicity, induced cell cycle arrest, caused apoptosis mediated by activation of caspases and possess inhibitory activity against CA IX. Based on their structure, these sulfamate conjugates might also act as prodrugs, and their influence onto the integrity of the plasma membrane could be also further taken into consideration[138-139]. To sum up, Betulinyl sulfamates represent attractive candidates for further biological evaluations, especially for next pre-clinical (Kinetic studies) and clinical level investigations. The latter will contribute to the discovery of targets for cancer therapies that will aid in the design of novel antiproliferative agents.

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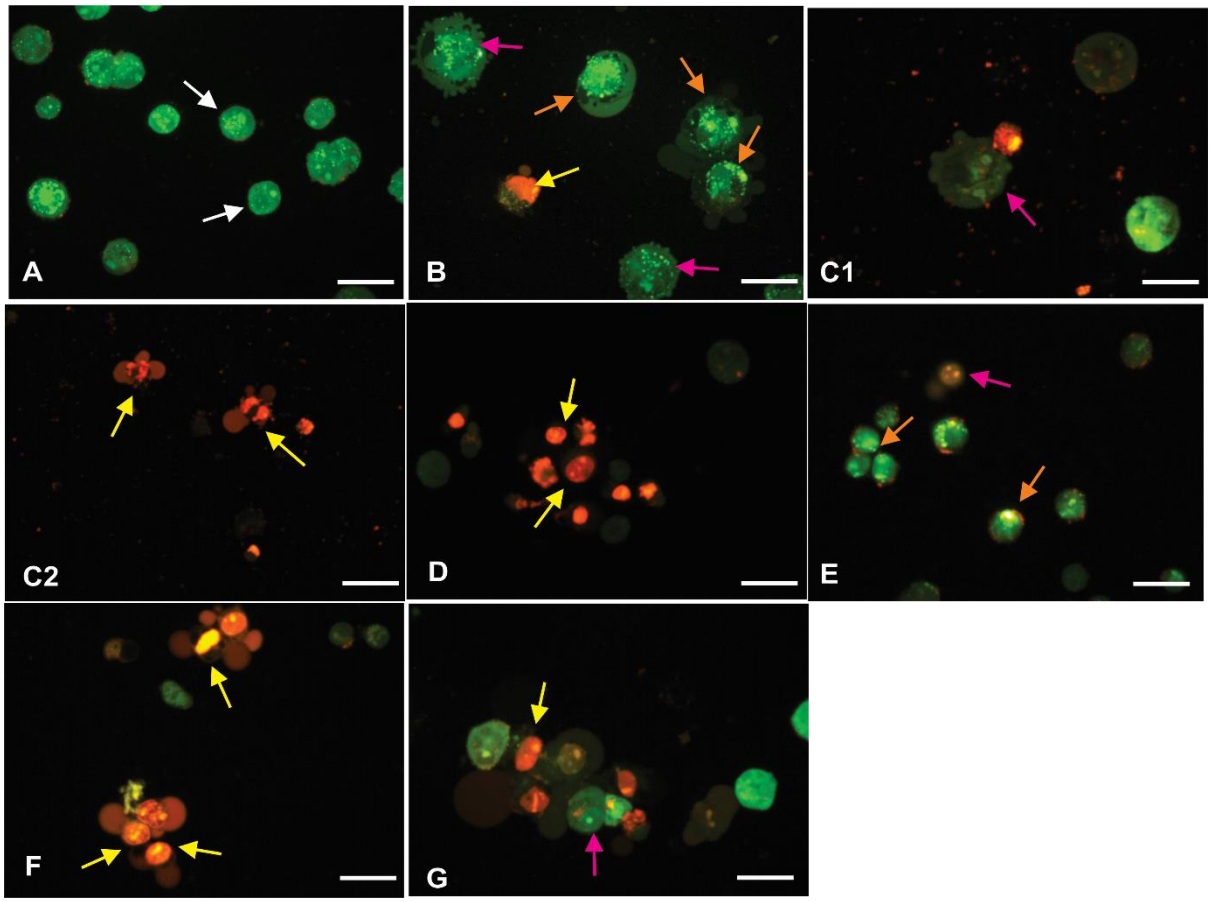
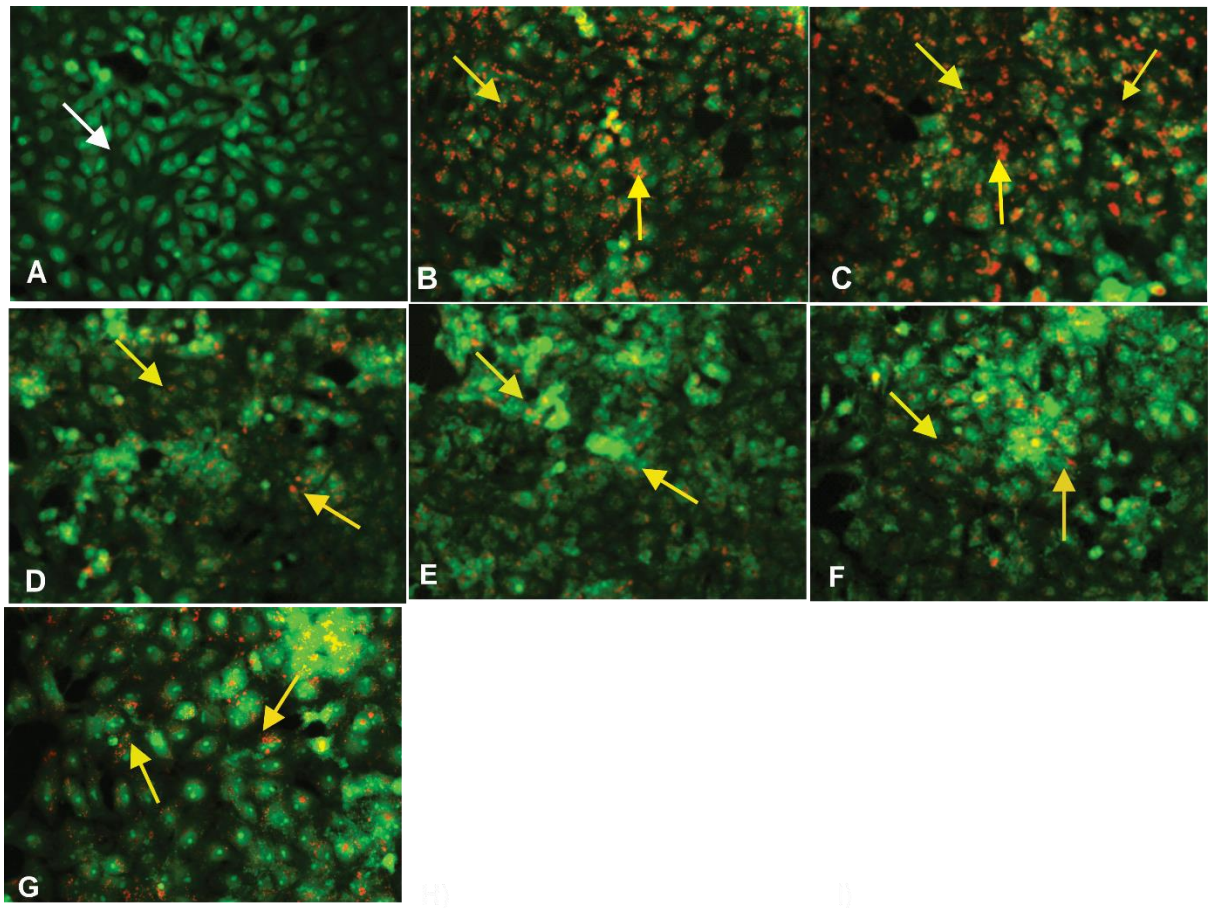
Supplementary Data

Figure S1. Identification of morphological behavior of apoptotic and necrotic properties of A.Control (untreated A549 cells) and cells treated with IC_{80} conc. of the compounds with B. **BA**, C1&C2.Compound **3**, D.Compound **3($PtCl_2$),** E.Compound **5**, F.Compound **5($PtCl_2$),** G.**CP.** The experiments were carried out on normal slides. All pictures were taken at 40x magnification. Labeling data of the pictures given below.



White arrows indicates untreated A549 cells round shaped with green uniform fluorescence. Orange arrows indicates early apoptotic cells with bright green non uniform fluorescence, normal cytoplasm and chromatin condensation. Pink arrows indicates late apoptotic cells with cytoplasmic and nuclear DNA fragmentation, membrane blebbing and formation of small apoptotic bodies. Yellow arrows indicates necrotic cells with red orange nuclear fluorescence. Scale bar-10 µM.

Figure S2. Characterization of A.Control (untreated A549 cells) and cells treated with IC_{80} conc. of the compounds B.**BA**, C.Compound **3**, D.Compound **3(PtCl$_2$),** E.Compound **5**, F. Compound **5(PtCl$_2$),** G.**CP** for autophagosomes (Autophagy). The experiments were carried out in chamber slides. All pictures were taken at 10x magnification. Labeling data of the pictures given below.



White arrows indicates untreated A549 cells grown as a dense monolayer. Yellow arrows indicates acidic autophagic vacuoles (bright orange fluorescent).

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.

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List of Publications:

D. Emmerich, **K. Vanchanagiri,** L. C. Baratto, H. Schmidt, R. Paschke.*"Synthesis and studies of anticancer properties of lupane type triterpenoid derivatives containing cisplatin fragment", European Journal of Medicinal Chemistry, 75 (2014), 460-466.*

K.Vanchanagiri, T.Mueller, R. Paschke. "*Elucidation of Anticancer Mode of action of Betulinic acid-Cisplatin Conjugates on Lung cancer A549 cells In vitro*". *Valley International Journals.* https://dx.doi.org/10.18535/ijmsci/v3i10.11.

Sebastian Paschke, Thomas Mueller, Hans-Joachim Schmoll, **Kranthi Vanchanagiri.** "*More insight into the mode of action of lipophilic antitumor drugs containing a platinum (II) fragment*". Submitted Manuscript.

G. Liebscher, **K. Vanchanagiri,** Th. Mueller, K. Feige, J.-M.V. Cavalleri, R. Paschke. *"In vitro anticancer activity of Betulinic acid and derivatives thereof on equine melanoma cell lines from grey horses and in vivo safety assessment of the compound NVX-207 in two horses", Chemico-Biological Interactions, 246 (2016), 20-29.*

Leopoldo Clemente Barattoa, Thomas Müller, Brás Heleno de Oliveira, Reinhard Paschke, **Kranthi Vanchanagiri**. "*Betulinic acid 2,4-dinitrophenylhydrazone derivatives induce caspases activation in A549 lung cancer cells*". *Valley International Journals.* https://dx.doi.org/10.18535/ijmsci/v3i11.05.

K. Vanchanagiri, Daniel Emmerich, Monique Bruschke, Matthias Bache, Franziska Seifert, Dirk Vormark, Reinhard Paschke. " *Synthesis and Biological Investigation of New Carbonic anhydrase (CA IX) Inhibitors*". Submitted Manuscript.

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