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Inhibition Akt by Tetracyclic Triterpenoids Induces Cell Cycle Arrest and Apoptosis in Prostate Cancer Cells

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Abbreviations

AKβBA	acetyl-11-keto-β-boswellic acid
Akt	protein kinase B, PKB
Akt inhibitor VIII	3-Dihydro-1-(1-((4-(6-phenyl-1H-imidazo[4.5-g]quinoxalin-7- yl)phenyl)methyl)-4-piperidinyl)-2Hbenzimidazol-2-one
Akt inhibitor X	10-(Diethylamino) butyl-2-chlorophenoxazine
αATA	3-α-acetyl-tirucallic acid
βATA	3-β-acetyl-tirucallic acid
APS	ammonium persulfate
ATCC	American Tissue Culture Collection
ATP	adenosine triphosphate
AR	androgen receptor
BSA	bovine serum albumin
DMSO	dimethyl sulfoxide
DNase	deoxyribonuclease
DNTP	deoxynucleoside triphosphate
DTT	dithiothreitol
EDTA	ethylenediamine tetraacetic acid
FCS	fetal calf serum
FACS	Fluorescence-Activated Cell-Scan
Fig	figure
FITC	fluorescein isothiocyanate
GSK-3β	glycogen synthase kinase-3β
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
HPLC	high pressure liquid chromatography
HRP	horseradish peroxidase
hr	human recombinant (protein)
h	hour
IKKα, β, γ	IκB kinase α, β, γ
IκB	inhibitor of NF-κB
l	liter
IAP	Inhibitor of Apoptosis

LDH	lactate dehydrogenase
LPLC	low pressure liquid chromatography
MAPK	mitogen-activated protein kinase
M-MLV RT	Moloney murine leukemia virus reverse transcriptase
Mol	mole
mdm2	murine double minute 2
NF-κB	nuclear factor κB
OTA	3-oxo-tirucallic acid
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PDK1	phosphoinositide-dependent protein kinase 1
pH	potential of hydrogen
PI3K	phosphatidylinositol-3-kinase
PKB	protein kinase B, Akt
PKC	protein kinase C
PVDF	polyvinylidene difluoride
Rel	reticuloendothelial protein
RIPA	radioimmuno-precipitation assay buffer
RNA	ribonucleic acid
RT-PCR	reverse transcription PCR
Rb	retinoblastoma protein
Oligo (dT)	oligo-deoxythymidine
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
SLB	sample loading buffer
Ser	serine
TAE	tris-acetate-EDTA buffer
TBE	tris-borate-EDTA buffer
TEMED	N',N',N',N'-tetramethylethylenediamine
Thr	threonine
Tricine	N-(2-hydroxy-1,1-bis(hydroxymethyl)ethyl)glycine
Tris	trishydroxymethylaminomethane

Tween 20	polyoxyethylene-20-sorbitan monolaurate
Tyr	tyrosine
UV	ultraviolet

1. Introduction

1.1. Cancer

Uncontrolled division of cells and the ability of these to spread, either by direct growth into adjacent tissue through invasion or by metastasizing into other tissues through the blood or lymph system, are characteristic for cancer (McKinnell et al, 2006).

Cancer is classified according to the original tissue. The most important factors that contribute to the cancer pathogenesis include failure of apoptosis, overactivation of oncogenes, inactivation of tumor suppressor genes, cell cycle activation of quiescent cells, acquisition of metastatic behavior by malignant cells, disordered responses to cellular growth factors and failure of the immune system surveillance (Long et al., 2005).

1.2. Prostate cancer

Prostate cancer is the most common type of cancer found in American men. It is the third leading cause of cancer death in men after lung cancer and colorectal cancer (Greenlee and Hill-Harmon, 2001). According to the American Cancer Society, prostate cancer is least common in South and East Asia, common in Europe, and most common in the United States. In the EU member countries deaths by cancer represent a quarter of the total number of annually deaths. In the case of premature deaths, the proportion is as high as 37%, with cancer representing the primary cause of death, ahead of cardiovascular mortality. For men, lung cancer represents 28% of total cancer mortality, followed by cancers of the intestine (11%) and prostate (10%) (Collective Expert Evaluation Reports, Cancer Institute, Germany 2005; Foster et al., 1999). The rate of prostate cancer deaths is declining because of the diagnosis in the early stages (Steliarova-Foucher et al., 2004).

The organ affected in prostate cancer is a gland of the male reproductive system. The size of the prostate is like the one of a chestnut. It is located below the bladder and in front of the rectum (Fig. 1). Prostate cells produce seminal fluid; this fluid

protects and nourishes the sperm. Prostate is divided into several zones: peripheral, central, transitional and periurethral. Clinically, these zones are important because 70% of adenocarcinoma develop from the peripheral zone (Amanatullah et al., 2000).

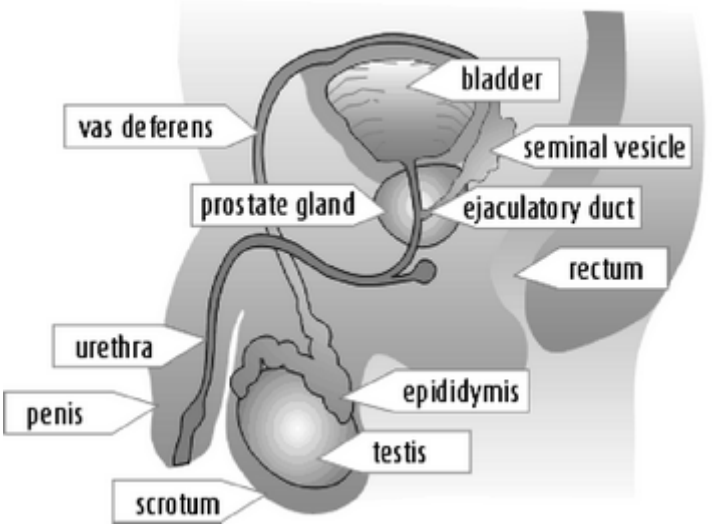


Figure 1. Prostate gland (www.prostate.com.ph/anatomy-p/ 2002).

Androgen steroids control the growth of the prostate gland. Testosterone is the most important androgen in men. It is produced mainly in the testes by Leydig cells located between the seminiferous tubules (Fig. 1). Small amounts of other androgens, such as androstenedione, dehydroepiandrosterone and their sulfates are produced by adrenal glands, small sized glands that sit on top of the kidneys (Huben and Perrapato, 1991). Free testosterone enters prostatic cells by passive diffusion. Cytosolic testosterone is converted by cytoplasmic membrane-bound enzyme 5-α-reductase to its active metabolite: dihydrotestosterone (DHT). DHT binds to intracellular androgen receptors, forming a receptor-steroid complex (Papatsoris and Papavassiliou, 2001). The complex translocates to the nucleus where it binds to the androgen responsive element (ARE). Binding to the ARE induces expression of ARE-dependent genes. The products of these genes as c-myc or cyclin D1 affect growth and function of hormone-dependent cancer cells (Feldmann and Feldmann, 2001).

Prostate cancer starts with small changes in the size and shape of the prostate gland cells. Grading of prostate adenocarcinoma is based on histological examination of prostatic tumor tissue according to the system developed by Gleason (Harding et al., 1999). The changes in the prostate are known as PIN (prostatic intraepithelial neoplasia) and can be either low-grade (almost normal) or high-grade (abnormal). The main symptoms of the prostate cancer are pain, difficulty in urinating, and sexual dysfunction.

Due to the changes in cellular environment and genetic alterations, enhanced proliferation leads to the initiation of cancer phenotype (Long et al., 2005). In the prostate cancer enhanced proliferation occurs also during the differentiation process from the androgen-dependent to the androgen-independent phenotype (Fig. 2). In the late stage of disease, most prostatic carcinomas become insensitive to androgen ablation and acquire the ability to activate different intracellular pathways that allow them to escape from apoptosis. Thus, the carcinoma becomes resistant to drug therapy.

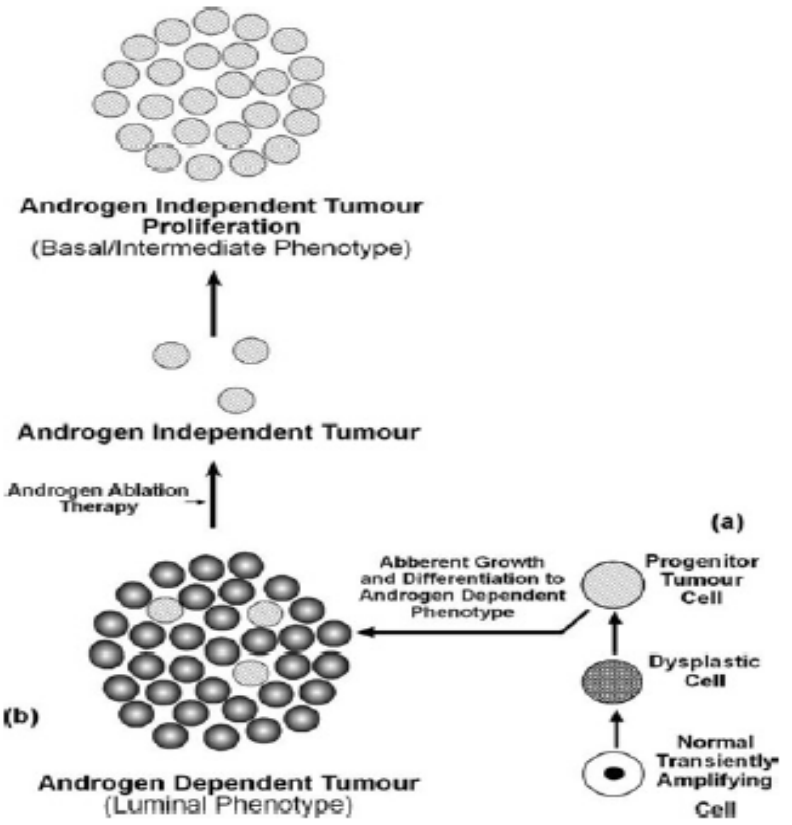


Figure 2. Development of prostate tumor (Long Morrissey, et al., 2005).

The prostate cancer cells, which do not undergo apoptotic cell death, display different chromosomal rearrangements or deletions in c-myc oncogene, androgen receptor (AR), tumor repressors E-cadherin, PTEN, p53 and Rb or cell-cell interaction regulators, such as β-catenin (Amanatullah et al., 2000).

1.3. Molecular pathways related to cancer development

The main signaling pathways that play an important role in the proliferation of cancer cells include mitogen activated protein kinase (MAPK)/Ras, the phosphatidylinositol-3-kinase (PI3K), and protein kinase C (PKC) (Faivre et al., 2006). These pathways depend on the activation of growth factor receptors (Henson and Gibson, 2006).

Ras family has either positive or negative effects on the regulation of apoptosis in tumor cells. Ras controls directly various effectors of different pathways, including PI3K and Raf. The Ras/Raf/MEK/ERK and Ras/PI3K/PTEN/Akt signaling cascades play critical roles in the transmission of signals from growth factor receptors which regulate gene expression and prevent apoptosis in tumor cells (Downward, 1998). Components of these pathways are often mutated or overexpressed in human cancer. Raf factors are associated with proliferation, and PI3K factors are associated with the long-term clonogenicity. In breast cells, activated Raf confers resistance to doxorubicin, tamoxifen and paclitaxel. The Raf-induced resistance is not related to p53/$p21^{cip1}$ expression (Hennessy et al., 2005), whereas deregulation of the Ras/PI3K/PTEN/Akt pathway was associated with altered levels of p53/$p21^{cip1}$ expression and with drug resistance in advanced prostate cancer cells (McCubrey et al., 2007). These observations demonstrate the importance of Ras/PI3K/PTEN/Akt pathway in the development of prostate cancer.

1.4. Activation of the Akt pathway

Ras is an activator of PI3K, which in turn activates Akt. PI3K is a heterodimeric lipid kinase activated in a number of human cancers, including prostate cancer (Woodgett, 2005). It consists of 85 kDa and 110 kDa regulatory and catalytic subunits,

respectively. Activated PI3K phosphorylates cellular inositol phospholipids at the position D3 of their inositol ring. The resultant phospholipid products, phosphatidylinositol 3,4,5-triphosphate (PIP_3) and 4,5-biphosphate (PIP_2), serve as second messengers to bind and regulate proteins containing pleckstrin homology domains (Chieffi and Cozzolino, 2006; Rajala et al., 2005).

Akt/PKB (protein kinase B) is a serine/threonine protein kinase that acts as signal collection node and a downstream target of PI3K. Deregulation of Akt kinase is frequently associated with human diseases including cancer and diabetes (Alessi et al., 1997). The Akt family comprises three highly homologous members known as Akt1/PKBα, Akt2/PKBβ and Akt3/PKBγ (Fig. 3). Binding of PIP_3 to the pleckstrin homology domain and subsequent recruitment to the inner surface of the plasma membrane activates Akt where it becomes phosphorylated. Akt activation depends on phosphorylation at two residues (Fig. 3); the Ser473 phosphorylation is dependent on mTOR kinase, whereas PDK1/2 phosphorylates Akt at Thr308 (Hill et al., 2001; Downward, 1997). The tumor suppressor PTEN, which is lost in a variety of human tumors including advanced prostate cancer, antagonizes the PI3K signaling by dephosphorylating PIP_3 (Madge and Prober, 2000; Maehama and Dixon, 1998). This results in constitutive activation of the PI3K/PTEN/Akt pathway in tumor cells.

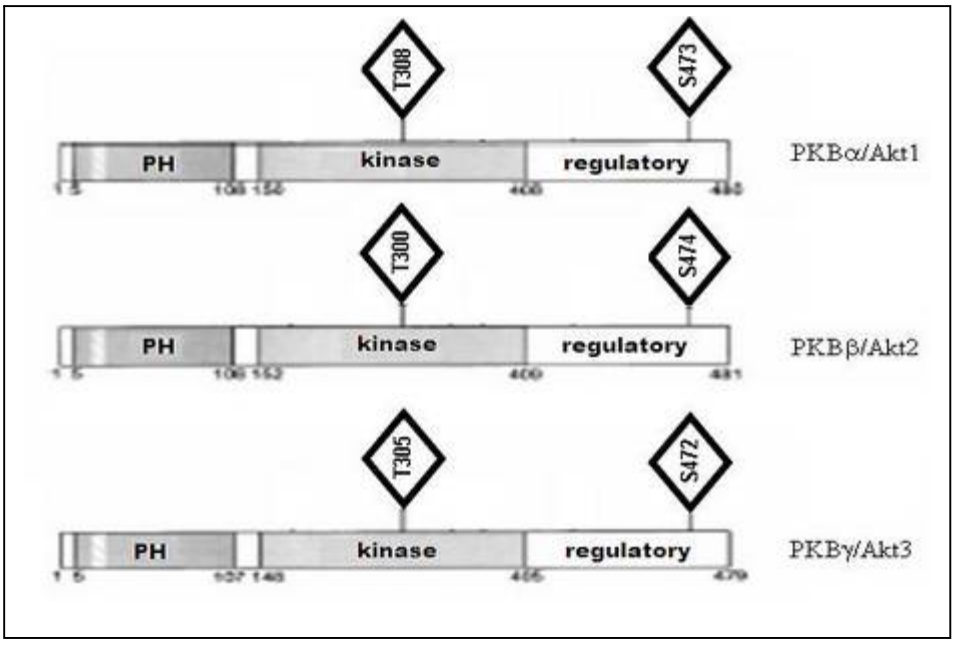


Figure 3. Domain structure of human Akt isoforms (Nicholson and Anderson, 2002).

1.5. Akt activity in tumor cells

Akt is a critical regulator of proliferation, nutrient metabolism and apoptosis in neurons, fibroblast, epithelial and endothelial cells (Faivre et al., 2006). Activation of Akt plays an important role in cancer cell survival in response to growth factors and other extracellular stimuli (Fig. 4). Akt induces phosphorylation and downregulation of the activity of proapoptotic proteins such as glycogen synthase kinase 3 (GSK-3) (Hennessy et al., 2005), it regulates degradation of cytosolic β-catenin, phosphorylates and inactivates proapoptotic BAD and caspase-9 (Majumder and Sellers, 2005). Activation of the transcription factor NF-κB through Akt promotes cell survival, because NF-κB is an important regulator not only of apoptosis but also inflammation in cancer cells (Aggarwal et al., 2004b). In addition, Akt can inactivate p53 through mdm2 promoting p53 degradation (Hernández-Vargas et al., 2007).

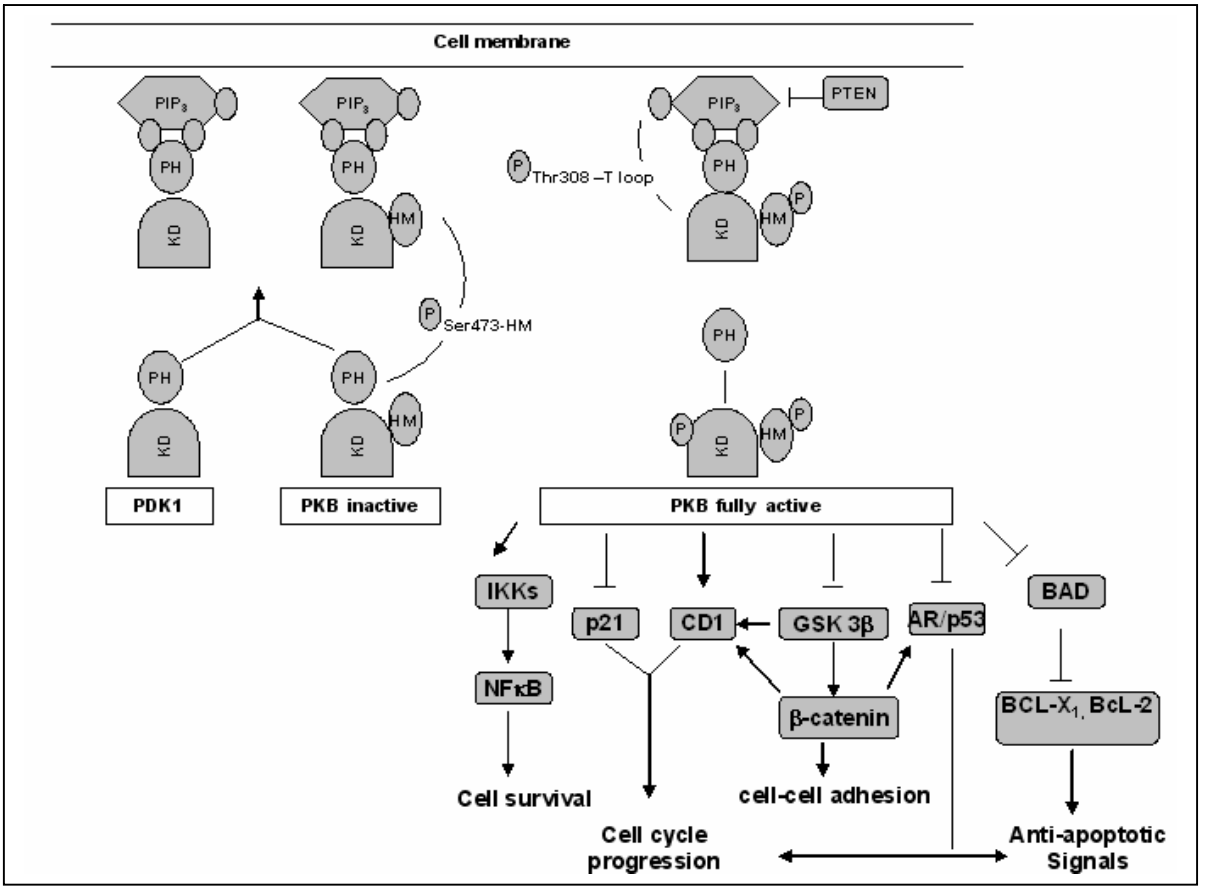


Figure 4. Role of Akt activation in cancer cell survival and apoptosis (modified after Faivre et al., 2006).

1.5.1. Akt/GSK-3/β-catenin pathway and regulation of cellular metabolism

A major physiological function of Akt is regulation of cell metabolism. High levels of insulin activate Akt, which inhibits GSK-3 that regulates the β-catenin/TCF/LEF transcriptional pathway. The inhibition of GSK-3 protects against apoptosis (Pap and Cooper, 1998).

During activation of the Wnt signaling pathway the cytoplasmic protein ß-catenin becomes stabilized and associates with transcription factors of the LEF/TCF family. In the absence of Wnt signaling the cytoplasmic ß-catenin is constitutively phosphorylated and degraded. ß-Catenin contains binding domains for the tumor suppressor APC (adenomatous polyposis coli), and GSK-3 and casein kinase 1a/e, which both can phosphorylate β-catenin (Behrens and Lustig, 2004; Meyer and Hart, 1998).

The phosphorylation sites of β-catenin are located in the N-terminal domain and are hotspots for mutations in tumors (Polakis, 2000; Seidensticker and Behrens, 2000) Phosphorylation of ß-catenin occurs in a two-step mechanism: first, Ser45 is phosphorylated by casein kinase 1, and then the Ser33, Ser37 and Thr41 are phosphorylated by GSK-3. Phosphorylated ß-catenin is recognized by the ubiquitin E3 ligase ß-TrCP, which induces its ubiquitination (Chesire and Isaacs, 2003). The consecutive phosphorylation mechanism implies that all four phosphorylation sites are essential for the efficient degradation of ß-catenin (Behrens and Lustig, 2004).

During activation of Wnt signaling pathway stabilized ß-catenin enters the cell nucleus and associates with TCF/LEF transcription factors, leading to the transcription of Wnt target genes (Sharma et al., 2002). The various interaction partners of ß-catenin in the nucleus are promising candidates to be tested for a putative role in tumorigenesis, regulating cell proliferation and developmental processes, as well as, tumor progression (Verras and Sun, 2006). β-Catenin/ LEF/TCF targets include c-myc and cyclin D1 genes, the promoters of which contain TCF binding sites and are controlled by ß-catenin/TCF complexes (Tetsu and McCormick, 1999). Through both targets ß-catenin/TCF promote progression of the cell cycle. Upregulation of c-myc leads to repression of the cyclin-dependent kinase

(CDK) inhibitor $p21^{CIP1}$ and thus stimulates G_1/S progression. Cyclin D1 can directly activate G_1 phase transition by CDK complexes. This signaling also interferes with apoptosis induced by chemotherapeutic drugs or might also affect cell proliferation by induction of expression of growth factors and their receptors (Behrens and Lustig, 2004).

1.5.2. Akt-mediated regulation of androgen receptor transcriptional activity

During androgen-dependent progression, prostate cancer cells depend primarily on the androgen receptor for growth and survival. As prostate cancer cells depend on androgens, androgen ablation is a standard treatment for patients suffering from prostate cancer. The androgen receptor protein (AR) is a member of the steroid nuclear receptor superfamily. AR in its inactive state is bound to a cytoplasmatic multi-protein chaperone (heat-shock) complex (Truica et al., 2000). AR characteristic structure-domains are (i) NH_2-terminal transcriptional domain (NTD) involved mainly in transcriptional activation of AR; (ii) DNA-binding domain (DBD) that is required for interaction with specific gene sequences, the so-called hinge region and (iii) the C-terminal ligand binding domain (LBD) that binds androgens.

Generally AR regulates gene expression through ligand-dependent mechanisms. The AR is activated by testosterone, the main circulating androgen in males that is secreted primarily by testes and circulates in the blood, where it is bound to sex hormone binding globulin. Androgen binding induces a conformational change in the AR that leads to dissociation from the chaperone complex and phosphorylation of the receptor. Once bound to androgen, The AR translocates from the cytosol to the nucleus. The conformation change also facilitates dimerization of the AR, which then binds to specific DNA sequences called androgen response elements (AREs) in the regulatory regions of target genes involved in cell growth (Jia et al., 2004). The NTD domain has an activation function domain 1 (AF-1), which functions in a ligand-independent manner (Lin et al., 2001).

AR activation can be also induced by growth factors in a ligand-independent manner (Culing et al., 1994). Deregulated growth factors and cytokines can activate AR

usually with the help of several androgen receptor co-activators, such as kinases (Akt), ATPases, acetylases, deacetylases and proteases or co-repressors, such as calreticulin, cyclin D1, GSK-3, PTEN and Smad 4 (Culing et al., 2000).

The crosstalk between β-catenin/LEF/TCF and PI3K/Akt pathways regulates the AR activity (Verras and Sun, 2006). Akt phosphorylates AR on Ser210 and Ser790 (Lin et al., 2001). This causes decreased transcriptional activity of AR on the $p21^{cip1}$ promoter and inhibition of apoptosis in prostate cancer cells expressing AR (Hanada et al., 2004; Nicholson and Anderson, 2002). In addition, binding to ß-catenin regulates AR. Through this interaction β-catenin augments the ligand-dependent activity of AR in prostate cancer cells. GSK-3 inactivation by Akt phosphorylation results in increased nuclear levels of β-catenin. This delineates novel mechanisms by which PI3K/Akt and PTEN regulate the androgen pathway during prostate cell growth and survival (Sharma et al., 2002).

1.5.3. Akt/NF-κB pathways and cell survival

NF-κB is an important regulator of apoptosis and inflammatory responses in the immune system (Xiao and Ghosh, 2005). It regulates expression of a wide variety of genes encoding cytokines, cytokine receptors, chemokines, cell adhesion molecules, cell-surface receptors, haematopoietic growth factors, acute phase proteins and other transcription factors (Greten and Karin, 2004). Transcriptional activation or repression of the NF-κB target genes requires binding of NF-κB dimers to the κB DNA binding sites (Aggarwal et al., 2004; Suh and Rabson, 2004).

NF-κB consists of homo- and heterodimers from the Rel/NF-κB family: NF-κB1 (p50/p105), NF-κB2 (p52/p100), RelA (p65), RelB and c-Rel. The most common heterodimer is p65/p50. In unstimulated cells binary complexes of these subunits are restricted to cytoplasm by interaction with members of a family of NF-κB inhibitors (IκBs). IκBs family members include IκBα, IκBβ, IκBγ and BcL-3 (Gustin A, et al., 2004).

In response to extracellular stimuli such as antigen-receptor crosslinking and exposure to cytokines (TNF-α, IL-1), bacterial components (LPS), viruses, ionizing radiation or oxidative stress, IκBα is phosphorylated, polyubiquitinated and then degraded by the 26S proteasome (Chen et al., 1995). Dissociation from IκBs releases the nuclear localization sequence of the NF-κB, permitting them to move into the nucleus, bind to the promoters of target genes and alter gene expression and cell function. Many studies have demonstrated that phosphorylation of Ser32 and Ser36 and subsequent polyubiquitination of IκBα are essential for the release and nuclear translocation of NF-κB dimers (Takada et al, 2003; Karin, 1999).

IκB is phosphorylated by IκB kinase complex (IKK), which is composed of IKKα, IKKβ and IKKγ (NEMO). IKKα and IKKβ are serine-threonine kinases, and IKKγ is a scaffolding protein essential for the function of IKKα and IKKβ. IKKα/β share a high degree of amino acid homology and domain organization (Gasparian et al., 2002). Catalytic activity of IKKα or IKKβ on IκBα phosphorylation is different and depends on the cell type. In addition, phosphorylation of RelA/p65 at Ser538 by IKK is required for the NF-κB transcriptional activation, where it upregulates the transcription of cell cycle regulators, such as cyclin D1, a target gene in cell proliferation (Takada et al., 2004).

IKKα and IKKβ can be activated by diverse kinases, among which are NF-κB-inducing kinase (NIK), MEKK1, Cot, NF-κB activating kinase (NAK/TBK), protein kinases C_1 and C_2, and MEKK3 (DiDonato et al., 1997). Another important kinase which affects IKK complexes and NF-κB activity is Akt. Once activated, Akt phosphorylates IKK inducing translocation of NF-κB to the nucleus (Ozes et al., 1999; Li and Sarkar, 2002) and activation of the NF-κB-dependent pro-survival genes, including Bcl-xL, caspase inhibitors and c-myc.

1.5.4. Role of Akt in the cell cycle progression

Cell cycle progression plays a critical role in malignant transformation and in the development of resistance to chemotherapy. The balance between the cell cycle regulators and their activation or inactivation determines whether cells proceed through the different cell cycle phases (Bali et al., 2004). The cell cycle is divided into four phases: generation of an identical copy of its genetic material in the synthesis (S) phase, and partitioning of all the cellular components between two identical daughter cells in mitosis (M) phase. The other two phases of the cell cycle G_1 and G_2 represent periods, during which cells prepare themselves for the successful completion of the S and M phase, respectively (Owa et al., 2001).

Some evidence supports a key role for the PI3K/Akt pathway in the cell cycle progression (Hanada et al., 2004). PI3K activity is required for G_1 to S phase transition in lymphocytes and in human mammary epithelial cells. Inhibition of PI3K by the specific inhibitor LY294002 has been shown to inhibit the G_2/M phase in Rat-1 cells with a blockade in S phase (Romero-Avila et al., 2002). Akt is activated during the synthesis of DNA in the cells. It promotes the cell cycle progression by (i) phosphorylation of CDK inhibitory proteins $p21^{cip1}$ and $p27^{kip1}$ causing their cytoplasmic accumulation, (ii) by increasing cyclin D transcription by β-catenin/GSK-3 pathway or by activation of downstream kinase mTOR/FRAP and (iii) by increasing cyclin D mRNA translation and subsequent phosphorylation of retinoblastoma protein (Rb). These events are crucial for the transition of cells through the G_1 checkpoint (Cooper and Shayman, 2001).

Perturbations of the cell cycle are common in carcinogenesis. Therefore, new types of anticancer therapeutics, which target cell cycle regulators, cyclin-dependent kinases and cyclin kinases inhibitors, are in clinical development today. New therapies targeting function of several cell cycle factors such as Rb, $p21^{cip1}$, p16 or p53 demonstrate encouraging results (Simons et al., 1999). Gene therapeutic approach to regulate the aberrant cell cycle has been also beneficial (Sternberg, 2005).

1.5.4.1. Regulation of CDK and CDKI

Results from several studies indicate a critical role for the cyclin-dependent kinases (CDK) in the temporal control of cell division and their relation to the different regulatory pathways that block progression through the cell cycle (Amanatullah et al., 2000). CDKs are a family of heterodimeric Ser/Thr protein kinases consisting of a catalytic CDK subunit and an activating cyclin protein subunit. Progression through the cell cycle is driven by activation and inactivation of CDK, which triggers the transition to the subsequent phase of the cycle (Fu et al., 2004).

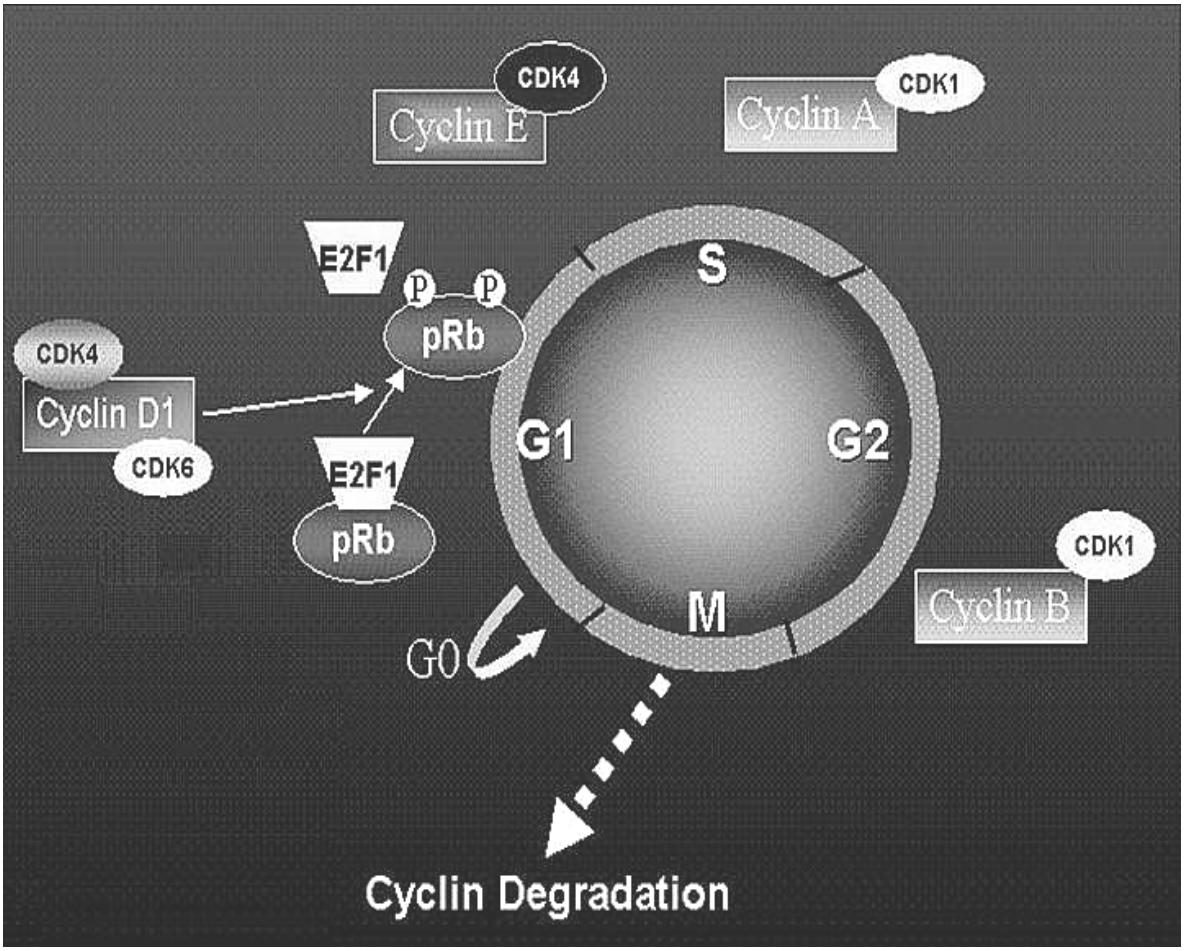


Figure 5. Cyclin - CDK regulation of the cell cycle (Tshilias et al., 1999).

CDK activities are specific for distinct phases of the cell cycle (Fig. 5). CDKs require association with a cyclin subunit for their activation. CDK4 and CDK6 complexes with D-type cyclins are responsible for the G_1 phase progression. A complex of CDK2-cyclin E is responsible for the G_1/S phase transition, CDK2-cyclin A is required for S

phase progression and a complex of CDK1 (or Cdc2)-cyclin B is needed for the G_2/M phase transition (Tshilias et al., 1999).

The CDK regulation involves controlled expression and degradation of cyclins, inhibitory phosphorylation and dephosphorylation of the CDKs themselves and expression or destruction of inhibitory proteins that associate with CDK or CDK /cyclin complexes (Amanatullah and Reutens, 2000). Cyclin/CDK complexes are kept inactive through association with CDK-inhibitory proteins (CKI) and inhibitory phosphorylation by Wee1/Myt1 kinases (Fig. 6). Activation requires ubiquitin-dependent proteolysis of the CKI, phosphorylation of the CDK at threonine by a CDK -activating kinase (CAK). Members of the Cdc25 family of dual-specificity phosphatases counteract Wee1/Myt1 phosphorylation and control the appropriate timing of CDK activation. Ubiquitin-dependent proteolysis of the cell cycle regulators in late G_1 and S phases involves E3 ligases such as SCF, while in M phase and early G_1 the anaphase-promoting complex (APC) is active (Boxen and van den Heuvel, 2002; Yanowitz and Fire, 2005).

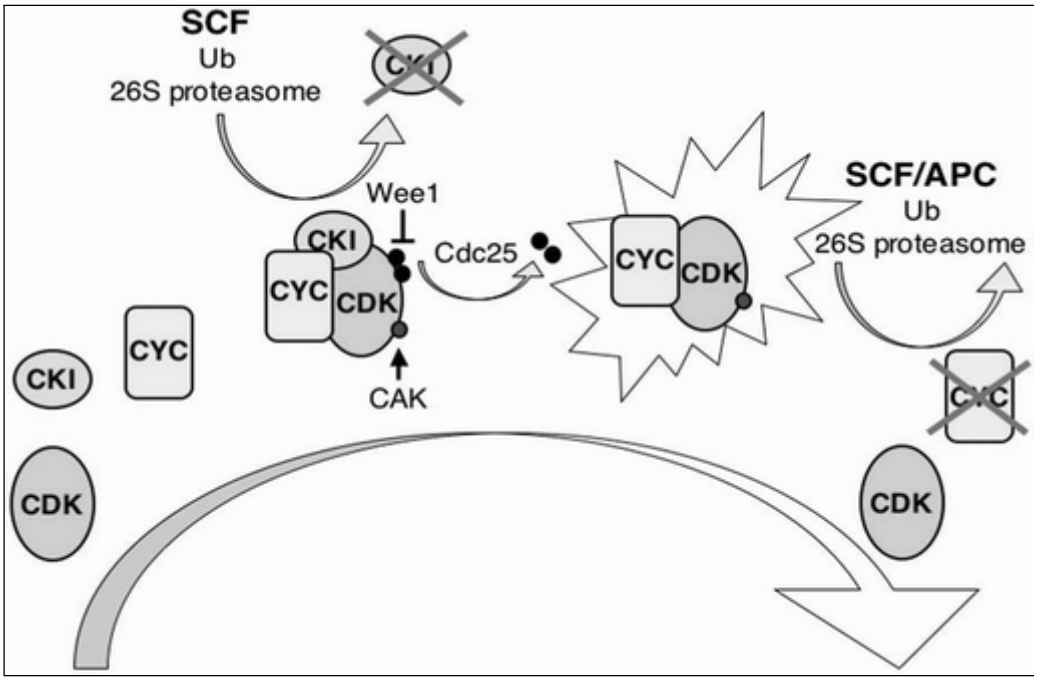


Figure 6. **Regulation of the cyclin- CDK activity** (http://www.wormbook.org/chapters/www_cellcyclereguln.html).

Control elements of cyclin/ CDK complexes, CDKI bind at ATP-binding sites of CDK and inhibit their activity. CDKI families are $p21^{Cip1}$, $p27^{Kip1}$, p57 and INK4 p15, p16, p18 and p19 (Fukuyama et al., 2003). $p27^{Kip1}$ usually regulates cell-cycle progression through the G_1/S checkpoint (Dai et al., 2003). $P21^{Cip1}$ is involved in all phases of the cell cycle and therefore known as a universal inhibitor of various CDKs. $P21^{Cip1}$ was shown to destabilize interaction between CDK4 and cyclin D, which induces inhibition of cell cycle progression in glioma and vascular smooth muscle cells (Besson and Yong, 2000). Upstream signals from Ras-ERK and Rho pathways reduce nuclear expression of $p21^{Cip1}$ (Schwartz and Shah, 2005). Different studies have shown that $p21^{Cip1}$ is a direct substrate for Akt and that this may regulate its subcellular localization (Nicholson and Anderson, 2002). Phosphorylation of $p21^{Cip1}$ by Akt reduces its association with CDK and interferes with the DNA replication in breast cancer cells (Zhou and Liao, 2001).

In prostate cancer cells $p21^{Cip1}$ kinase inhibitor is induced following stress in a p53-dependent manner, which results in the induction of cell cycle arrest (Gartel and Tyner, 2002). P53 is an important target for the Akt activity. Akt induces degradation of p53 via mdm2 phosphorylation. Human cancer cells expressing wild type p53 treated with DNA damaging agents undergo cell cycle arrest mediated by $p21^{Cip1}$, followed by caspase-3 cleavage and apoptosis (Zhan et al., 2000). Similarly, Akt decreases the AR-dependent transcriptional activity of $p21^{Cip1}$ gene (Hanada et al., 2004).

Dependent on the environmental conditions and the regulation by growth factors, cells in G_1 may temporarily or permanently leave the cell cycle and enter a quiescent or arrested phase known as G_0. While somatic cells show little response to genotoxic stress, DNA damage might induce both cell-cycle arrest and apoptosis in cancer cells during G_1 restriction point (Schwarz and Shah, 2005). The G_2/M checkpoint inhibits mitosis if the previous events are incomplete or if the DNA is damaged (Schumacher and Hofmann, 2001).

The cell cycle arrest also protects the cell from DNA damage. Thus, the cell cycle arrest represents a survival mechanism that gives the tumor cell the opportunity to repair damaged DNA. Any abrogation of the cell cycle checkpoints before the DNA

repair is complete can activate the apoptotic cascade, leading to cell death. Defects in the cell cycle machinery of prostate cancer cells might affect the cell cycle response to chemotherapeutic drugs. There are now agents available that abrogate the cell cycle checkpoints and make the tumor cell susceptible to apoptosis (Lanzi et al., 2001). These compounds can inhibit CDK by direct targeting the catalytic subunit. These inhibitors are silibinin (flavone), procyanidin B5, curcumin, grape polyphenols, indole-3-carbinol (indole), trans-resveratrol (phytoalexin) and flavopiridol (flavanol) (Singh et al., 2002).

1.5.4.2. Influence of the Rb phosphorylation and G_1/S transition

Rb regulates the transition of cells through the G_1/S checkpoint. Inhibition of the activity of CDKs and cyclin D1 complexes inactive Rb. Akt controls the expression and activity of cyclin D1, thus Akt is involved in the regulation of Rb phosphorylation and transition of cells through G_1/S phase (Nicholson and Anderson, 2002). Transition through the G_1/S phase is dependent on the regulation of Rb/E2F, which activates the transcription of genes responsible for the G_1/S transition and DNA synthesis including CDK2, cyclin A cyclin E, c-myc (Plath et al., 2002).

The tumor suppressor Rb comprises a family of transcriptional repressors, which inhibits genes involved in S-phase progression. The Rb family members are nuclear phosphoproteins. Rb-related proteins, p107 and p130 show considerable homology with Rb (p110) and are classified as a sub-family of the Rb family members (Mulligam and Jacks, 1998). All these proteins bind to and modulate the activity of E2F family of transcription factors that induce the transcription of genes needed for the cell cycle progression through S phase (Stevaux and Dyson, 2002). Rb-related proteins are regulated in a cell cycle-dependent manner by phosphorylation through the complexes cyclin D1/CDK4-6. Phosphorylation of Rb results in the liberation of E2F. The transcription of cyclin E/CDK2 complex promotes both progression from G_1 to S and more phosphorylation of Rb (Giacinti and Giordano, 2006).

1.6. Regulation of apoptosis in tumor cells

Multicellular organisms can activate a mechanism of self-destruction called programmed cell death or apoptosis to eliminate dangerous cells. Apoptosis plays an important role in tissue development, the immune system and in the development and treatment of cancer.

Caspases are proteases, which have cysteine residues at their active sites and cleave at aspartic acid residues of their substrate proteins (Budihardjo et al., 1999). Caspases are the ultimate effectors of the programmed cell death, inducing apoptosis by cleaving different cell target proteins. Key targets of the caspases include an inhibitor of DNase, responsible for the fragmentation of nuclear DNA. In addition, caspases cleave nuclear lamins, leading to fragmentation of the nucleus and cytoskeletal proteins, which results in disruption of the cytoskeleton, membrane blebbing and cell fragmentation (Zhang et al., 2000).

Caspases are synthesized as inactive precursors that are converted to the active forms by proteolytic cleavage catalyzed by other caspases. Therefore, the activation of initiator caspases starts a chain reaction leading to activation of effector caspases and subsequent cell death. Caspases are generally divided into two categories: the initiator caspases, which include caspase-2, -8, -9, 10 and the effector caspases, consisting of caspases-3, -6 and -7 (Zhang et al., 2000).

1.6.1. P53 tumor suppressor

The major role of p53 in normal cells is to induce cell cycle arrest or apoptosis in response to genotoxic or cytotoxic cellular stress, particularly DNA damage. P53 is an important cell cycle checkpoint regulator of both G_1/S and G_2/M checkpoints but seems to be not important for the mitotic spindle checkpoint during mitosis. P53 activates $p21^{Cip1}$, which is a key regulator of G_1 arrest and contributes to the G_2/M arrest. P53 also acts by inhibiting Cdc2 or by inhibiting binding of Cdc2 to cyclin B. The p53 tumor suppressor gene is the most frequently mutated gene in human cancer, indicating its important role in conservation of normal cell cycle progression.

In response to massive DNA damage, p53 triggers the apoptotic cell death pathway. Thus, p53 has direct relevance to therapeutic strategies (Ko and Prives, 1996; Giaccia and Kastan, 1998).

P53 contains an N-terminal transactivation domain, a core DNA binding domain and a C-terminal multifunctional regulatory domain (Ko and Prives, 1996). Highly conserved residues in its N- and C-terminal domains are targets for potential post-translational modification via phosphorylation, dephosphorylation or acetylation (Giaccia and Kastan, 1998). Phosphorylation of p53 at Ser15 and Ser37 contributes to its regulation by affecting the binding of co-activators and the negative regulator mdm2, which induces p53 degradation. Recent reports demonstrated that after DNA damage Akt phosphorylates mdm2 at Ser166 or Ser186 and contributes to its nuclear localization (Hanada et al., 2004; Kaustov and Yi, 2006).

New data suggest that p53 is involved in AR activation and inhibition of apoptosis. Mutation of the p53 gene in prostate tumor cells correlates inversely with the AR activity (Feldman and Feldman, 2001). Regulations of AR and wild type p53 expression during androgen-dependent cell growth highlight the importance of p53 as a modulator of AR signaling in prostate cells (Cronauer et al., 2004).

After p53 accumulates in the nucleus, it binds to DNA as a tetramer and regulates transcription of different target genes including $p21^{Cip1}$, GADD45, cyclin G, 14-3-3-sigma, and thrombospondin 1, mdm2, IGF-BP3, BAX and others. However, depending on the stress and cell type, p53 activation can lead to responses as different as a reversible cycle arrest, induction of a senescent-like state or apoptosis (Ko and Prives, 1996; Giaccia and Kastan, 1998).

1.6.2. Bcl-2 family

Bcl-2 family members that promote cell death act by inducing mitochondrial damage, cytochrome c release and caspase activation. The Bcl-2 family helps to regulate the activation of procaspases. An important proapoptotic Bcl-2 related protein is BAD. Akt phosphorylates it on Ser136, thereby inhibiting its proapoptotic activity (Fulda and

Debatin, 2002). Phosphorylated BAD is released from a complex with Bcl-2/Bcl-x that is localized on the mitochondrial membrane and forms a complex of 14-3-3-proteins in the cytosol. By this, its proapoptotic function is inactivated (Song et al., 2005).

1.7 Anticancer therapy

In 1909 Paul Ehrlich introduced chemotherapy as a term. In 1942 nitrogen mustard (mechlorethamine), an inhibitor of mitosis, which was originally developed as a poison gas, was the first substance used as a cytostatic drug in tumor therapy. Since then, synthetic products or modified naturally occurring substances have been used to treat cancer.

Established prostate cancer therapies include a combination of surgery, chemotherapy, radical retropubic prostatectomy and radiation therapy (Lance et al., 2001). At the beginning, prostate cancer is hormone-dependent and can be treated by hormone ablation. It aimes to prevent the biosynthesis of new circulating androgens, such as testosterone, dihydrotestosterone, androstenedione and dehydroepiandrosterone in testes and to block their action at target tissue sites (Papatsoris and Papavassiliou, 2001). Up to 80% of the patients with local or metastatic prostate cancer will respond partially or completely to androgen deprivation as measured by (i) cessation of disease progression; (ii) tumor regression or (iii) rarely, disappearance of metastatic foci (Carnero, 2002). Hormone deprivation therapy can be used in neoadjuvant or adjuvant settings. However, it creates an environment for the selection of androgen-independent cancer cells, leading to hormone-refractory prostate cancer (Jaruja-Killeen and Rayford, 2004). Due to advances in understanding of molecular mechanisms governing androgen-independent progression of prostate cancer, novel therapeutic approaches to treat prostate cancer involving gene therapy, manipulation of active and passive immunity, apoptosis induction and differentiation therapy have been investigated (Small et al., 2000).

Agents dominating the anticancer pharmacotherapy have limited selectivity for cancer cells versus healthy cells. Anticancer drugs, such as alkylating agents,

antimetabolites, antracyclines, plant alkaloids, topoisomerases inhibitors affect DNA synthesis in tumor cells and thus induce cell death (Boyle, 2000). In general, the selectivity only refers to the rapid rate of cell growth. This approach also targets haematopoietic progenitor cells in bone marrow, epithelial cells of the gastrointestinal tract and hair cells (Jaruja-Killeen and Rayford, 2005). A variety of drugs such as paclitaxel, epirubicin, adriamycin, 5-fluorouracil (5-FU), hydroxyurea and cisplatin have been used for treatment of prostate cancer (Zalcberg et al., 2000). Due to the strong adverse effects of these drugs, development of new therapeutic approaches is necessary.

Cancer cells are resistant to mechanisms that cause programmed cell death in normal cells because of the constitutive activation of the specific survival signaling pathways. Therefore, survival-signaling pathways provide novel molecular targets for a new type of drugs (Dai and Grant, 2003b). Modern cancer drugs specifically or selectively interact with molecules important for cancer development or progression. Therapeutic agents recently employed for the treatment of solid tumors inhibit the activity of growth receptors. Some of these therapeutic agents do not directly interfere with the DNA function, such as the tyrosine kinase inhibitor imatinib, Gleevec®, which directly targets Abl tyrosine kinase in chronic myelogenous leukemia and gastrointestinal stromal tumors (Tannock et al., 2004).

1.8. Plant derivatives as antitumor drugs

Secondary metabolites from plants are a rich source of antitumor compounds. Isolation, identification, characterization, quantification of phytochemicals and evaluation of their potential benefits in treatment of human cancers have become an important area of pharmaceutical sciences. To date, approximately more than 30 classes of phytochemicals, such as isothiocyanates, indoles, flavonoids, isoflavonoids, dithiolthiones, coumarines, isoprenoids and organosulphides have been isolated and tested for their anticancer potential (Singh at al., 2002).
Alkaloids and triterpenoids are used as drugs. For example, taxanes and vinca alkaloids have been approved as antitumor drugs (Tannock et al., 2004). They block cell division by preventing either assembly or disassemble of microtubules during

mitosis. Vinca alkaloids bind to tubulin during M phase. Vincristine, vinblastine, vinorelbine and vindestine are alkaloids from *Catharanthus roseus* (Lanzi et al., 2000). Etoposide and teniposide are derivatives of podophyllotoxin from *Podophyllum peltatum*. These compounds prevent G_1/S phase progression (Guo et al., 2007). Taxanes derived from *Taxus brevifolia*, such as paclitaxel, enhance stability of microtubules, preventing the separation of chromosomes during anaphase (Sachdev, 2007).

Some phytochemicals inhibit the expression of cyclin D1 and cyclin E, downregulate CDK4/6 and CDK2 levels, as well as their kinase activity, and induce cell cycle arrest in G_1. Upregulation of CDKI INK4, $p21^{Cip1}$, $p27^{Kip1}$ and hypophosphorylation of Rb by phytochemicals are always associated with G_1 arrest and inhibition of cell proliferation, which could be a valuable strategy against prostate cancer growth and progression (Aggarwal et al., 2004).

The anticancer efficacy of natural compounds has been linked to the modulation of mitogenic signaling; such as growth factor receptor mediated activation of mitogen-activated protein kinases (MAPK) and stress-activated protein kinases (SAPK); as well as, an alteration in cell survival signaling, such as PI3K/Akt and NF-κB. These effects of phytochemicals on most of the cell signaling cascades are ultimately linked to the modulation of cell cycle regulatory molecules leading to growth inhibition and/or apoptotic death.

1.8.1. Compounds from *Boswellia* species

The genus *Boswellia* (Burseraceae), consisting of 10 species of trees and shrubs, is distributed in the tropical parts of Asia and Africa. The most important species are *Boswellia sacra*, *Boswellia serrata* and *Boswellia carterii*. The tree stem has an oleogum called frankincense (olibanum resin). The gum resin of *Boswellia* species is known to contain a mixture of oil, terpinole, ß-sitosterin, arabinose, xilise, galactose and pentacyclic and tetracyclic triterpenoids. The triterpenoids are a large group of natural isoprenoids present in different plants. Squalene is the biosynthetic precursor

of these compounds; the cyclization of squalene produces a characteristic triterpenoid configuration with a C_{30} skeleton.

Boswellia oleogum resins have been used in medicinal preparations for the treatment of amenorrhoea, menorrhagia, polyuria, rheumatism, ulcers, scrofulous affections, syphilis, sores, and nervous diseases. It is also used in diarrhea, asthma and bronchitis (Tawab et al., 2001). *Boswellia* extracts are used for medicinal purposes in many Asian countries. Some compounds, such as ursolic and oleanolic acids, are known to be antiinflammatory, anticarcinogenic and antioxidant agents (Atta-ur-Rahman et al., 2005). Acetyl-11-keto-β-boswellic acid (AKβBA), a pentacyclic triterpenoid from frankincense was recently identified as a cytotoxic compound (Syrovets et al., 2005).

Little is known about the biological activity of the tetracyclic triterpenoids. The tetracyclic triterpenoids 3-oxo-tirucallic acids were shown to induce mobilization of intracellular Ca^{2+} in neutrophils and inhibit activity of 5-lipoxygenase (Boden et al., 2001).

1.9. Aim of the study

Prostate cancer is one of the most common cancers in men. Inhibition of pathways that interfere with tumor cell proliferation provides a new treatment option for prostate cancer patients. One of the most important pathways related to cell proliferation, survival and inhibition of apoptosis in prostate cancer cells is the Akt pathway.
The main goal of the present study is isolation and purification of tetracyclic triterpenoids from the oleogum resin of *Boswellia carterii* with activity against hormone-dependent and hormone-independent prostate cancer cells. In addition, the pharmacological mechanism related to their bioactivity.should be elucidated.

2. Materials and methods

2.1. Equipment

Centrifuges

Sigma 4K15 (Sigma, Osterode, Germany)

Rotina 48RS (Hettich, Tuttlingen, Germany)

Eppendorf 5415 (Eppendorf, Hamburg, Germany)

Spectrophotometer

Gene Quant Pro RNA/DNA calculator (Amersham, Freiburg, Germany)

Cell culture incubator

Queue (Nunc, Wiesbaden, Germany)

Electrophoresis apparatus

Mini Protean 3 Electrophoresis Cell (BioRad Laboratories, Munich, Germany)

Agargel Mini (Biometra, Goettingen, Germany)

Agargel Maxi (Biometra, Goettingen, Germany)

Thermomixer

HTM 130L (HCL Bio Tech, Bovenden, Germany)

Microplate reader

Dynatech MR7000 (Dynex Technologies, Berlin, Germany)

PCR machines

GeneAmp PCR System 2400 PE (Applied Biosystems, CA, USA)

GeneAmp PCR System 9700 (Applied Biosystems, CA, USA)

Sonicator

Sonopuls GM70 (Bandelin Electronics, Berlin, Germany)

Sonorex RK 100 SH (Bandelin Electronics, Berlin, Germany)

Semidry protein transfer apparatus

TransBlot SD (BioRad Laboratories, Munich, Germany)

Fluorescence activated cell scan (FACS)

FACScan (Becton Dickinson, Franklin Lakes, USA)

Microscope

Olympus CK2 (Olympus Europa GmbH,Hamburg, Germany)

Scanner for autoradiography

FLA-3000 (Fuji Film, Duesseldorf, Germany)

Vacuum centrifuge

SpeedVac Plus SC210A (Thermo Savant, NY, USA)

UV Transilluminator

Herolab E.A.S.Y. 429 K (Herolab, Wiesloch, Germany)

Vortex

Genie (Bender & Hobein, Bruchsal, Germany)

Phosphoimager

(Molecular Dynamics, Sunnyvale, CA)

Flourescent Microscope

Photoshot (Nikon, Duesseldorf, Germany)

Microwave

(Siemens, Munich, Germany)

Instrumentation for UV, MS and NMR Spectroscopy

Soxhlet (VWR International, Darmstadt, Germany)

Low pressure pump S1121 (Sykam, Fuerstenfeldbruck, Germany)

Fraction collector 201 (Abimed, Langenfeld, Germany)

UV detector SP-6 (Gynkotek, Germering, Germany)

High pressure pump LC-9A (Shimadzu, Kyoto, Japan)

Automatic sample injector Aspec XL (Abimed, Langenfeld, Germany)

UVD 340S photodiode-array detector (Dionex, Idstein, Germany)

NMR AMX 500 spectrometer (Bruker, Bremen, Germany)

Finnigan MAT SSQ-7000 spectrometer (Thermo Finnigan MAT, Bremen, Germany)

Chromeleon software (6.11) (Dionex, Idstein, Germany)

Column Oven IWN CH100 (Junedis, Groebenzell, Germany)

Software Valoo-statistical calculations (Applica, Bremen, Germany)

2.2. Extraction, fractionation and isolation of triterpenoids

The biologically active tetracyclic triterpenes: 3-oxo-tirucallic acid (OTA), 3-α-acetoxy-tirucallic acid (αATA) and 3-β-acetoxy-tirucallic acid (βATA) were isolated from the oleogum resin of *Boswellia carterii* Birdwood. The fractionation procedure was developed using a modified method of Winterstein and Stein (Winterstein and Stein, 1932)**.** This includes a Soxhlet extraction with methanol (MeOH), specific precipitation with Ba^{2+} salts and fractionation by column chromatography. After fractionation, the highest amount of triterpenoids was found in the 90% MeOH (3) fraction. For this reason, the 90% MeOH (3) fraction was selected for further isolation and purification of the compounds. The purification procedure included semi-preparative and analytical HPLC, as described (Büchele et al., 2003).

The isolated and purified compounds (>99%) were characterized by mass spectrometry, one- and two-dimensional nuclear magnetic resonance spectrometry (Department of Nuclear Magnetic Resonance, Ulm University, Germany).

2.2.1. Materials and method

60 g oleogum resin of *Boswellia carterii* (frankincense) Heinrich Klenk, Schwebheim, Germany was extracted with n-hexan for 5 h at 80°C, followed by biphasic extraction with water (pH 14)/dichloromethane (pH 2-3).

The extract was further fractionated with SiO_2 column (200x20mm, Merck KG, Darmstadt, Germany) under hydrostatic flow using ethyl acetate: hexane (v/v) as eluent as indicated in Tab.1.

Table 1. Fractionation of the oleogum resin extract.

Fraction (No.)	Ethyl acetate (%)	Hexane (%)	Elution vol. (ml)
1	5	95	720
2	10	90	720
3	20	80	720
4	30	70	720
5	50	50	720
6	100	0	720

Fraction No. 3 was collected and fractionated further by HPLC on a LiChrospher 100 RP-18 column(40 µm, 200x20 mm, Merck KG, Darmstadt) under hydrostatic flow using methanol: water (v/v, see Tab. 2) as eluent.

Table 2. Fractionation of the extract by HPLC.

Fraction (No.)	Methanol (%)	Water (%)	Elution vol. (ml)
1	70	30	100
2	80	20	100
3	90	10	100
4	100	0	100

Fraction No. 3 was collected and fractionated further by LPLC on a LiChrospher 100 RP-18 column (12 µm, 200x20 mm, Merck KG, Darmstadt, Germany) under 30 ml/min using acetonitrile: water: acetic acid (v/v/v, see Tab. 3) as eluent, detection was performed at 215 nm.

Table 3. Fractionation of the extract by LPLC.

Fraction (No.)	Acetonitrile (%)	Water (%)	Acetic acid (%)
1	75	25	0.2
2	80	20	0.2
3	97	3	0.2

Further purification of the individual compounds was performed by HPLC on a Reprosil-Pur ODS-3 column (250x8 mm, 5 µm, Merck KG, Darmstadt, Germany) with a flow rate of 4 ml/min. For the washing steps methanol: water: acetic acid (v/v/v, 80:20:0.2) and methanol: acetic acid (100:0.2, v/v) were used as eluent. Fraction No. 1 (OTA) was eluted with methanol: water: acetic acid (96:14:0.2, v/v/v) and the fractions No. 2 and 3 (βATA and αATA) were eluted with methanol: water: acetic acid (94:16:0.2, v/v/v). Detection was performed at 210 and 220 nm.

Table 4. Retention times of the compounds purified by HPLC chromatography.

Fraction (No.)	Compound	Retention time (min)
1	**OTA**	12.54
2	**βATA**	13.93
3	**αATA**	14.62

2.3. Cell culture

2.3.1. Materials

The androgen-dependent LNCaP and the androgen-independent PC-3 cell lines were purchased from ATCC (Teddington, UK); media were obtained from Gibco (Karlsruhe, Germany).

2.3.2. Method

The androgen-dependent LNCaP cell line was cultured in RPMI 1640 supplemented with 1% L-glutamine, 1% glucose, 100 U/ml penicillin, 100 µg/ml streptomycin and 10% FCS; the androgen-independent PC-3 cell line was cultured in F-12K with 100 U/ml penicillin, 100 µg/ml streptomycin and 10% FCS.

2.4. Determination of cytotoxic activity

2.4.1. Materials

AKβBA	B. Büchele, Inst. of Pharmacology of Natural Products and Clinical Pharmacology, Ulm University, Germany
Akt inhibitor VIII	3-Dihydro-1-(1-((4-(6-phenyl-1H-imidazo[4.5-g]quinoxalin- 7-yl)-4-piperidinyl)--2-one, Calbiochem, San Diego, USA
Akt inhibitor X	10-(Diethylamino) butyl-2-chlorophenoxazine, Calbiochem, San Diego, USA
Triton X100	Serva Feinbiochemicals, Heidelberg, Germany
Cell Proliferation Kit (XTT)	Roche Diagnostics, Mannheim, Germany
Cytotoxicity Detection Kit (LDH)	Roche Diagnostics, Mannheim, Germany

Table 5. Compounds analyzed.

Substance	Formula	Molecular weight	Characteristic
OTA 3-oxo-tirucallic acid	$C_{30}H_{46}O_3$	474.7	White solid powder, soluble in DMSO
αATA 3-α-acetoxy-tirucallic acid	$C_{22}H_{50}O_4$	498.7	White solid powder, soluble in DMSO
βATA 3-β-acetoxy-tirucallic acid	$C_{22}H_{50}O_4$	498.7	White solid powder, soluble in DMSO
AKβBA Acetyl-11-keto-β-boswellic acid	$C_{32}H_{48}O_5$	512.0	White solid powder, soluble in DMSO

2.4.2. Method

Evaluation of cell growth was analyzed by XTT assay based on the cleavage of the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) (XTT) to purple formazan dye by metabolically active cells. Additionally, the LDH (lactate dehydrogenase) released from dead cells with leaking membranes was analyzed. Cells treated with 0.1% triton X100 were used as positive control (100% dead cells).

The cell cultures were treated with different concentrations of AKβBA, OTA, αATA, βATA (Tab. 5) or Akt-inhibitors. $5x10^3$ cells/well in 300 µl medium were incubated with different concentrations of substances in 96 well microtiter plates for a total of 24 and 48 h at 37°C in 5% CO_2. Formazan was measured photometrically by scanning the plates at multiwell spectro-photometer ELISA reader at 450 nm for the XTT assay and at 490 nm for the LDH assay (Wenk et al., 2004). The assays were always performed in triplicate. At least three different cell preparations were tested. The IC_{50} data values for each individual cell line were evaluated from concentration-response curves using Microsoft Excel and SigmaPlot software.

2.5. Analysis of apoptotic parameters

2.5.1. Expression of phosphatidylserine on the cell membrane surface

2.5.1.1. Materials

Annexin V	Roche Diagnostics, Mannheim, Germany
Paraformaldehyde (4%)	Merck, Darmstadt, Germany
PBS (with $CaCl_2$)	Gibco, Karlsruhe, Germany
Propidium iodide	Sigma-Adlrich, Steinheim, Germany
Mounting medium	Biomeda, Burlingame, USA
Vectashield	Vector Laboratories, Burlingame, USA

2.5.1.2. Method

Early or intermediate stages of apoptosis, were analyzed by phosphatidylserine expression on the outer membrane leaflet. Phosphatidylserine was detected by fluorescence microscopy of cells stained with FITC-labelled annexin V. Annexin V is a Ca^{2+}-dependent phospholipid-binding protein (Jaruga-Killeen and Rayford, 2005) that binds phosphatidylserine in the membrane. The cells were pretreated with 10 µM AKβBA, OTA, αATA or βATA for 24 h, then washed with PBS, fixed with 4% paraformaldehyde (100 µl) for 30 min and stained with 1 µl annexin V (10X stock solution) and propidium iodide (PI) (250 µg/ml). FITC-labeled annexin V (green) and PI (red) were detected by microscopy.

2.5.2. Measurement of DNA fragmentation

2.5.2.1. Materials

Suicide Track™ DNA Ladder Isolation Kit	Calbiochem, San Diego, USA
Photo apparatus EASY 429	Herolab, Wiesloch, Germany
UV-Photometer	Herolab, Wiesloch, Germany
Electrophoresis apparatus	Biometra, Goettingen, Germany

2.5.2.2. Method

This experiment is based on the measurement of DNA degradation in the cells as a biochemical marker of apoptosis (Lin et al., 2006). Endonucleolysis is characterised by DNA strand breaks. The endonuclease mediated nucleosome excision at 180-200 base pairs can be detected as a DNA ladder by electrophoresis. After treatment with 10 µM AKβBA, OTA, αATA or βATA of LNCaP and PC-3 cells for 72 and 96 h, respectively, non-adherent cells were harvested by centrifugation, washed with ice-cold PBS and the cells were incubated in lysis buffer. DNA ladder fragments were extracted from the cells by separating apoptotic DNA from chromatin. After that, DNA was precipitated with isopropanol and analyzed by electrophoresis (1.5% agarose gel). After staining with ethidium bromide the presence of DNA ladders was visualized under UV light.

2.5.3. Cell cycle analysis

2.5.3.1. Materials

Ethanol	Merck, Darmstadt, Germany
PBS	Gibco, Karlsruhe, Germany
Propidium iodide	Sigma-Aldrich, Steinheim, Germany
RNase A	Sigma-Aldrich, Steinheim, Germany
CellQuest software	Becton and Dickinson, NJ, USA
FACScan	Becton and Dickinson, NJ, USA

FACS buffer
1% BSA
0.01% NaN_3
PBS

2.5.3.2. Method

The DNA content of the cell can provide information about the cell cycle and consequently the effect on the cell cycle of added stimuli e.g. drug treatment. The most commonly used DNA dye is propidium iodide (PI), which intercalates into the DNA helix and fluorescens strongly red. PI is excited at 488 nm and it can be used on most common flow cytometers. PI also stains double-stranded RNA, therefore it has to be removed with ribonucleases.

The cells ($1x10^6$) were treated with 10 µM AKβBA, OTA, αATA or βATA for 24 and 48 h at 37°C in 5% CO_2 atmosphere. After that, the cells were washed, pelleted and stained with PI/RNase A buffer. Reduced cellular DNA content was determined by flow cytometric analysis of the sub-G_0/G_1 formation. Twenty thousand cells were analyzed using the CellQuest acquisition and ModFit analysis software to determine the ratio of cells in the G_0/G_1 phase and the G_2 phase (Syrovets et al., 2005). The results are expressed as mean ± SEM. Statistical significance was analyzed using the Newman-Keuls test. Differences were considered significant for $p<0.05$.

2.6. Analysis of protein expression

2.6.1. Preparation of cell extracts for western blot analysis

2.6.1.1. Materials

PVDF membrane	Amersham Biosciences, Freiburg, Germany
Hyperfilm	Amersham Biosciences, Freiburg, Germany
ECL substrate reagent	Amersham Biosciences, Freiburg, Germany
Rainbow marker RPN800	Amersham Biosciences, Freiburg, Germany
BCA Protein Assay Kit	Pierce, Rockford, USA
Protease inhibitor cocktail	Sigma-Aldrich, Steinheim, Germany
Electrophoresis apparatus	BioRad Laboratory, Munich, Germany
Sonorex RK 100 SH	Bandelin, Berlin, Germany

Table 6. Composition of SDS-PAGE gel (16 ml end volume).

	10%	**12%**	**15%**
3XGB	5.31 ml	5.31 ml	5.31 ml
AB 30%	5.33 ml	6.40 ml	8.00 ml
Glycerol 50%	3.36 ml	3.36 ml	2.24 ml
H_2O	1.86 ml	0.77 ml	0.35 ml
APS	100 µl	100 µl	100 µl
TEMED	10 µl	10 µl	10 µl

Table 7. Concentration of polyacrylamide used for the different molecular weight of proteins.

Molecular weight (kDa)	% polyacrylamide
100-200	5-7.5
35-90	8-10
13-30	12-15

Table 8. Antibodies.

Antibody	Company	Cat No.	Dilution	Source Expected size	Storage °C	Exposure time
Actin	Chemicon	MAB1501	1:1000 5% milk	Mouse 42 kDa	-20	1' / 5'
Akt1	Upstate	05-796	1:500 5% milk	Rabbit 64 kDa	-20	Overnight
Akt2	Cell Signaling	2964	1:1000 5% BSA	Rabbit 60 kDa	-20	10' / 3 h
p-Akt (Ser473)	Upstate	05-736	1:5000 5% milk	Rabbit 60 kDa	-20	10' / 3 h
AR	Cell Signaling	3202	1:1000 5% BSA	Rabbit 110 kDa	-20	Overnight
p-AR (Ser213/210)	Imgenex	IMG-561	1:1000 5% BSA	Mouse 110 kDa	-20	Overnight
p-BAD (Ser136)	Cell Signaling	9295	1:500 5% BSA	Rabbit 23 kDa	-20	Overnight
β-catenin	Cell Signaling	9562	1:1000 5% BSA	Rabbit 92 kDa	-20	10' / 3 h
Caspase-3	Cell Signaling	9662	1:1000 5% milk	Rabbit 17,19,35 kDa	-20	Overnight
CDK4	Santa Cruz	SC 749 (H 303)	1:1000 5% BSA	Rabbit 34 kDa	4	10' / 3 h
c-myc	Cell Signaling	9402	1:1000 5% BSA	Rabbit 57-70 kDa	-20	10' / 3 h
Cyclin D1	Dianova	11696 BcL1/Ab3	1:1000 5% milk	Rabbit 36 kDa	4	10' / 3 h
Cyclin E	Santa Cruz	SC 2503	1:1000 5% BSA	Mouse 53 kDa	4	10' / 3 h
p-GSK-3β (Ser9)	Cell Signaling	9336	1:1000 5% BSA	Rabbit 46 kDa	-20	Overnight
IKKα	Becton and Dickinson	556532	1:500 5% milk	Maus 64 kDa	-20	10' / 1 h
NF-κB p65	Santa Cruz	SC-109	1:1000 5% milk	Rabbit 65 kDa	-20	3 h
NF-κB p-p65 (Ser538)	Cell Signaling	3036	1:2000 5% milk	Mouse 65 kDa	-20	3 h
p-β-catenin (Ser45)	Cell Signaling	9564	1:1000 5% BSA	Rabbit 92 kDa	-20	3 h
$p21^{Cip1}$	Pharmingen	65951A	1:250 5% milk	Mouse 21 kDa	4	Overnight
p53	Chemicon	MAB4040	1:1000 5% milk	Mouse 53 kDa	-20	3 h
p-p53 (Ser15)	Cell Signaling	9286	1:2000 5% milk	Mouse 53 kDa	-20	Overnight
p-ERK (Thr202/Tyr204)	Cell Signaling	9106	1:2000 5% milk	Mouse 42,44 kDa	-20	Overnight
p-Rb (Ser807/811)	Cell Signaling	9308	1:1000 5% BSA	Rabbit 110 kDa	-20	10' / 3 h
Rb	Cell Signaling	9309	1:2000 5% Milk	Mouse 110 kDa	-20	Overnight
Topoisomerase I	Santa Cruz	SC-10783	1:500 5% milk	Rabbit 100 kDa	4	Overnight

Solutions and buffers for immunoblotting:

30% acrylamide solution

30% acrylamide

0.8% N-N´-methylenebisacrylamide

3X gel buffer (GB)

3 M Tris HCl, 0.3% SDS, pH 8.45

TBST

10 mM Tris HCl (pH 8.0)

150 mM NaCl

0.05% Tween 20

RIPA lysis buffer

1X PBS

1% Nonidet P40

0.5% sodiumdeoxycholate

0.1% sodiumdodecylsulfate

Phosphatase inhibitors (100X)

125 mM NaF

250 mM β-glycerophosphate

25 mM Na_3VO_4 (sodium orthovanadate)

Blotting buffer

250 mM glycine

25 mM Tris (pH 8.3)

10% or 20% methanol

Blocking buffer

5% milk in TBST

or 5% BSA in TBST

2.6.1.2. Method

Aliquots of whole cell lysates were prepared in lysis buffer. Equal amounts of proteins were separated by SDS-PAGE gel electrophoresis as described in Current Protocols in Molecular Biology (Ausubel et al., 2007). Samples were transferred onto PVDF membranes for 1 h. To reduce unspecific protein binding the PVDF membranes were blocked for 1 h in blocking buffer. Afterwards the membranes were washed with TBST and probed with specific primary antibodies (specific conditions, see Tab. 8). Next the secondary antibodies were added for 45 min at RT. After incubation with antibody the membranes were washed with TBST. The proteins were visualized using enhanced chemiluminescense detection (ECL) and subsequent exposure to Hyperfilm. For all western blots, actin was used as control for loaded protein. Western blot results are representative of at least three independent experiments.

2.6.2. Nuclear extracts for western blot analysis

2.6.2.1. Materials

Buffers for nuclear extraction

Buffer A
10 mM HEPES, pH 7.9
1.5 mM $MgCl_2$
10 mM KCl
0.5 mM DTT
0.1% Nonidet P40
Phosphatase and protease inhibitors (1:100 / see sec. 2.6.1.1.)

Buffer B
20 mM HEPES, pH 7.9
1.5 mM $MgCl_2$
0.5 mM DTT
0.2 mM EDTA
25% glycerol
Phosphatase and protease inhibitors (1:100 / see seccion 2.6.1.1.)

2.6.2.2. Method

LNCaP and PC-3 cells were scraped on ice and washed twice with ice cold PBS. The cell pellet was incubated with approximately 100 µl cold buffer A (about 3X pellet cell volume) for 10 min on ice. The cells were transferred in a cold glass Dounce homogenizer and homogenized by repeated strokes. After centrifugation of the homogenate, the supernatant (cytoplasmic extract, CE) was shock-frozen in liquid nitrogen and stored at -70°C. The pellet was resuspended in 50 µl cold buffer B and extracted for 30 min with shaking, followed by centrifugation. The supernatant (nuclear extract, NE) was shock-frozen in liquid nitrogen and stored at -70°C**.** Inhibitors (1:100 / see secction 2.6.1.1.) and DTT were added to all buffers just before use.

2.7. RT-PCR

2.7.1. Materials

Primers

GAPDH, 598 bp (BiomersNet, Ulm, Germany)
F: 5'-cca ccc atg gca aat cc atg gca-3'

R: 5'-tct aga cgg cag gtc agg tcc acc-3'

Cycles 22; Tm: 55°C

Cyclin D1, 231 bp (Thermo Electron, Hamburg Germany**)**
F: 5'-gaa caa aca gat cat ccg caa aca-3'

R: 5'-tgc tcc ggc agg ccc gga ggc agt -3'

Cycles 23; Tm: 60°C

Cyclin E, 213 bp (Thermo Electron, Hamburg Germany**)**
F: 5'-gcg att tcg cat cag tgg tgc gac ata gag -3'

R: 5'-gcg att tcc gct ttg ctc ggg ctt tgt cca -3'

Cycles 23; Tm: 55°C

Buffers and reactive for RT-PCR

5X first-strand buffer

250 mM Tris-HCl (pH 8.3)

375 mM KCl

15 mM $MgCl_2$

0.1 M DTT

10X PCR buffer

200 mM Tris-HCl (pH 8.4)

500 mM KCl

50 mM $MgCl_2$

10 mM EDTA

PCR mix, 25 µl volume

2.5 µl 10X PCR buffer

0.75 µl 50 mM $MgCl_2$

1 µl 10 mM dNTP-Mix

1 µl Primer 5 pM (F)

1 µl Primer 5 pM (R)

0.35 µl *Taq* DNA polymerase

2 µl cDNA

16.4 µl distilled water

TE buffer for PCR-electrophoresis

10 mM Tris-HCl (pH 7.5)

1 mM EDTA

10X TBE buffer PCR-electrophoresis

880 mM Tris (pH 8.3)

880 mM boric acid

25 mM EDTA.

10X TAE buffer PCR-electrophoresis

400 mM Tris (pH 8.3)

1.1% acetic acid

PCR loading buffer

50% glycerol

0.1% bromphenol blue

0.1% xylenecianol

2.7.2. Method

Total RNA was extracted using TRIzol as described in standard protocol from Invitrogen. 1 µg RNA was transcribed with oligo $d(T)_{12-18}$ primers and M-MLV reverse transcriptase, followed by PCR. Primers for cyclin D1 and cyclin E were as described (Hosokawa et al., 1999). Conditions were such that the PCR reactions did not reach the saturation phase. Control experiments showed no DNA contaminations. Normalization was carried out using GAPDH as internal standard (Syrovets et al., 2005b). The PCR products were fractionated by electrophoresis (1.5% agarose gel) and visualized by ethidium bromide staining. All shown RT-PCRs are representatives of at least three independent experiments.

2.8. Determination of kinase activity

2.8.1. Immunoprecipitation of IKKα/β and Akt1/2

2.8.1.1. Materials

Triton lysis buffer

25 mM Tris pH 8.0

100 mM NaCl

10 mM NaF

25 mM β-glycerophosphate

2 mM EDTA

2 mM EGTA
10% glycerol
1% triton 100X
1 mM Na_3VO_4
Protease inhibitors (1:100)

2X kinase buffer

20 mM Hepes, pH 7.5
20 mM β-glycerophosphate
100 µM Na_3VO_4
0.5 mM NaF

Table 9. Compounds used for the determination of kinase activity.

	Company	Stock solutions	Vol. for 10 µl
GSK-3β	Cell Signaling, Denver, MA	129 µg/120 µl	1 µl (0.5 µg)
IκBα	Santa Cruz, Santa Cruz, CA	50 µg/50 µl	1 µl (0.5 µg)
ATP	Merk KG, Darmstadt, Germany	100 µM	2.5 µl (10 µM)
$MgCl_2$	Gibco, Karlsruhe, Germany	250 mM	1 µl (25 mM)

2.8.1.2. Method

The assay was performed as previously described (Syrovets et al., 2005). PC-3 cells ($1.5x10^7$) were washed once with PBS. Pellet was resuspended in lysis buffer containing a protease inhibitors mixture. After centrifugation the supernatants were collected and incubated with protein A-Sepharose beads for 30 min to eliminate nonspecific binding. Afterwards the cell lysates were incubated with 2 µg of anti-IKKα or anti-Akt1 polyclonal antibodies and precipitated with protein A-Sepharose beads at 4°C for 2 h. The precipitated kinases were washed and then preincubated with the respective triterpenoids (30°C, 20 min). IKKα and Akt1 activity was determined by incubation (30°C, 20 min) with GST-tag fusion proteins IκBα or GSK-3β as substrates (0.5 µg) in the presence of 10 µM ATP containing 5 µCi ^{32}P-γATP. Proteins were separated by 10% SDS-PAGE gel and these were blotted onto PVDF membranes. Phosphorylated IκBα or GSK-3β were detected by autoradiography

using a phosphor imager. To check loading and for confirmation of the IKK and Akt immunoprecipitation, the membranes were analyzed by immunoblotting with IKKα/β or Akt1/2 antibodies.

2.8.2. Analysis of kinase activity using human recombinant IKK and Akt

2.8.2.1. Materials

Table 10. Compounds used for the determination of kinase activity of hrIKK and hrAkt.

	Company	Stock solutions	Volume for 25 µl
Kinase buffer	-	2X	6 µl
GST-IKKα	Upstate, Lake Placid, NY	5 µg / 50 µl	2.8 µl (100 nmol)
GST-IKKβ	Biaffin, Kassel, Germany	10 µg / 42 µl	1.1 µl (100 nmol)
His-Akt1	Biaffin, Kassel, Germany	20 µg / 33.3 µl	0.25 µl (100 nmol)
His-Akt2	Upstate, Lake Placid, NY	10 µg / 100 µl	1.2 µl (100 nmol)

2.8.2.2. Method

100 nmol of active recombinant His-Akt1, GST-IKKβ, His-Akt2 and GST-IKKα were treated with 10 µM of either triterpenoids or Akt inhibitor VIII (Calbiochem). The kinase activity was analyzed as indicated above. Proteins were separated by 10% SDS-PAGE. Phosphorylated IκBα and GSK-3β were detected by phosphor imager analysis.

3. Results

3.1. Fractionation, isolation and purification of tetracyclic triterpenoids from oleogum resin of *Boswellia carterii*

This study was aimed to determine the in vitro biological activity of triterpenoids isolated from oleogum resin of *Boswellia carterii.* The mono- and triterpenoids present in *Boswellia* species are derivatives of squalene. This class of compounds, with C_{30} skeleton posses a keto or acetyl group in position C_3, which is held responsible for most of their important activities (Glaser et al., 1999).

3.1.1. Extraction

60 g of freshly ground *Boswellia carterii* oleogum resin, was extracted with n-hexane as a solvent for 5 h at 80°C by Soxhlet extraction method (Büchele et al., 2003). Dry extract (~5 g) was submitted to basic extraction with water and organic solvent (pH 14), followed by acid-extraction with dichloromethane (pH 2-3). The obtained dichloromethane extract was denominated ED. The analysis of different ED fractions by normal phase TLC chromatography on silica gel indicates that the extraction method was efficient to obtain fraction with great amounts of OTA and related compounds. TLC chromatography of different ED extracts is shown below (Fig. 7).

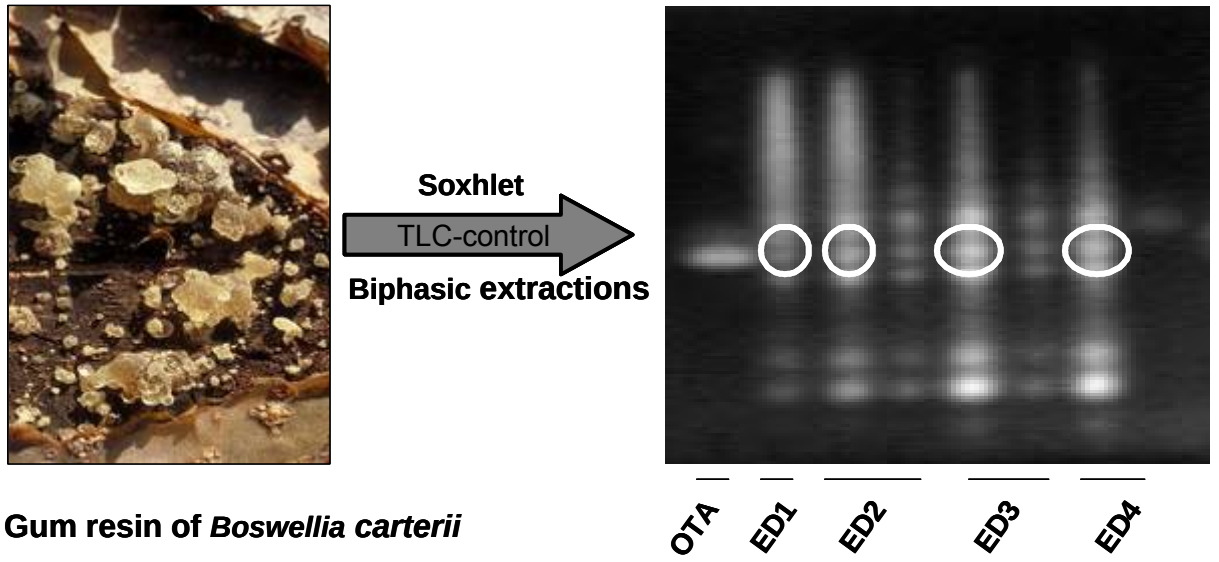


Figure 7. Preparation of dichloromethane extract (ED) from olegum resin *of Boswellia carterii*: ED was prepared by Soxhlet and biphasic extractions. The different dichloromethane extracts (ED) were analyzed by normal phase TLC chromatography (ethyl acetate: hexan, 7:3, v/v). OTA was used as standard for the TLC chromatography.

3.1.2. Fractionation

Different amounts of dichloromethane extract (ED) were loaded onto silica gel column (200 x 20mm) with ethyl acetate : hexane (v/v) as eluent. The eluted fractions were controlled by RP-18 TLC (methanol: water, 90:10, v/v). Deacetylated boswellic acid mixture (n=6, N1), acetylated boswellic acids mixture (n=6, N2) obtained as described above (Büchele et al., 2006) and OTA were used as standards for the RP-18 TLC. Control by TLC indicated that with up to 40 mg the column was not overloaded. The fraction eluted from normal phase chromatography with 20% ethyl acetate was selected as the fraction with the highest amount of OTA and structurally related compounds (Fig. 8).

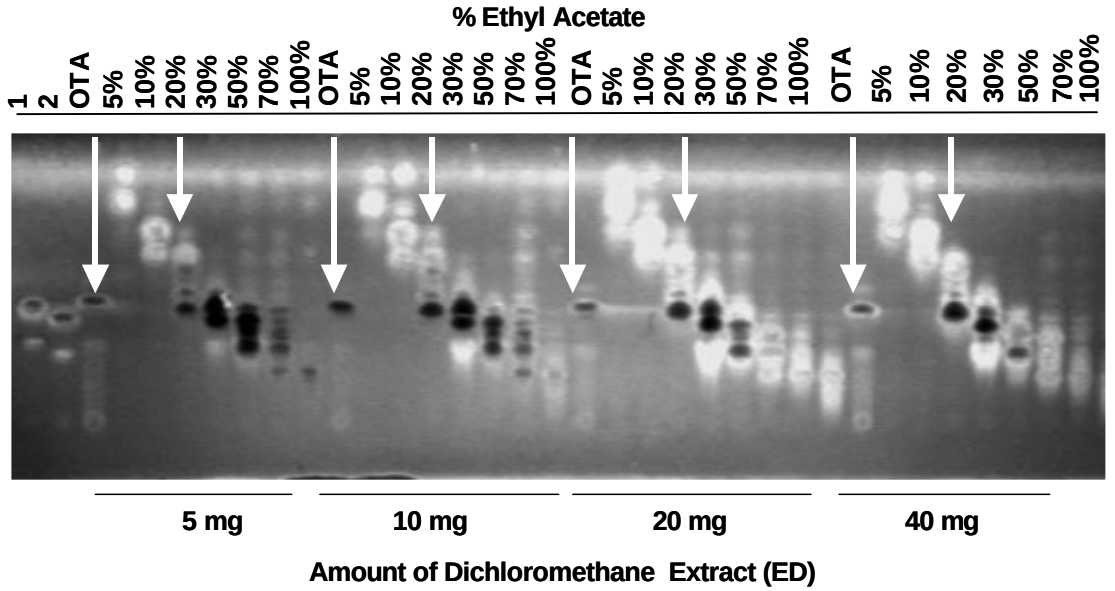


Figure 8. RP-18 thin layer chromatography. TLC RP-18 (methanol: water, 10:90, v/v) loading and fractionation control of normal phase chromatography from different amounts of the dichloromethane extract (ED). (1) Deacetylated boswellic acid mixture, (2) acetylated boswellic acid mixture and OTA were used as TLC RP-18 standards. The fraction 20% ethyl acetate obtained by normal phase chromatography was selected as the fraction with the highest amount of OTA and structurally related compounds.

For the next fractionation, the 20% ethyl acetate fraction from normal phase chromatography was separated on a LiChrospher 100 RP-18 (40 µm, 200x20 mm) column. The mobile phase velocity was controlled by gravity flow of methanol: water (v/v). The eluted fraction No. 3 (90% methanol) from this fractionation was selected for further separation with a LiChrospher 100 RP-18 (12 µm, 200x20 mm) column. The fraction No. 3 (90% methanol) was eluted at a rate flow of 30 ml/min and fractions were detected at wavelength λ=215 nm (Fig. 9A). The highest amount of OTA and structurally related compounds were recovered in fraction No. 2 (80% acetonitrile) (Fig. 9B). TLC RP-18 (methanol: water, 90:10, v/v) was used to control the fractionation (Fig. 9B).

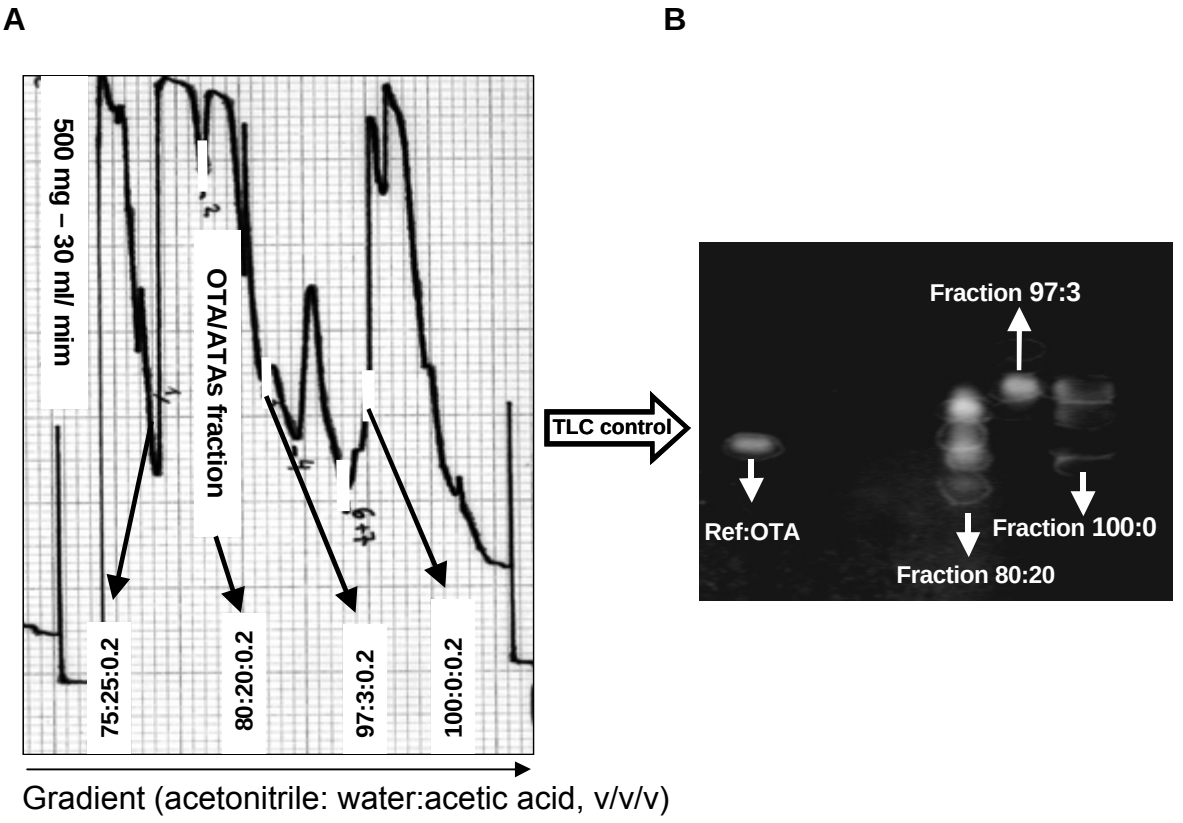


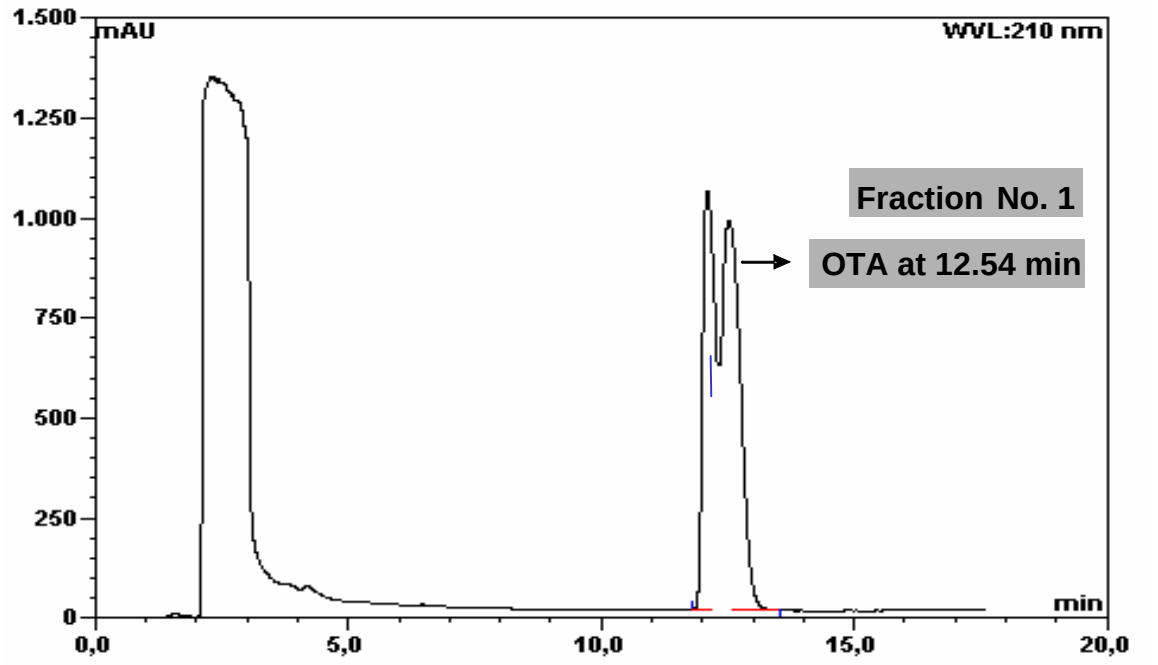
Figure 9. Further fractionation of the fraction No. 3 (90% methanol). (A) Fraction No. 3 (90% methanol) from LiChrospher 100 RP-18 (40 µm, 200x20 mm) column was selected for further fractionation with a LiChrospher 100 RP-18 (12 µm, 200x20 mm) column. Detection wavelength was λ=215 nm, flow rate 30 ml/min, acetonitrile: water: acetic acid: (v/v/v) as indicated mobile phase. (B) TLC RP-18 control (methanol: water, 90:10, v/v) of LPLC fractions.

3.1.3. Purification

To separate OTA and structurally related compounds from the fraction No. 2 (80% acetonitrile), a semi-preparative HPLC chromatography method with a Reprosil-Pur ODS-3 (3 µm, 250x8 mm) column was selected. The detection of compounds was performed at the wavelengthes λ=210 nm and 220 nm. A flow rate of 4 ml/min and 28°C were used for the best resolution of the peaks. Mobile phases were: (A) methanol: water: acetic acid (80:20:0.2, v/v/v) and (B) methanol: acetic acid (100:0.2, v/v). Isocratic separation of fraction No. 1 (Fig. 10A) was at 96% methanol and for fractions No. 2 and No. 3 (Fig. 10B) was at 94% methanol. The gradient used allowed separation of OTA (fraction No. 1) at 12.54 min, βATA (fraction No. 2) at 13.93 min and αATA (fraction No. 3) at 14.62 min. Furthermore, the recovered semi-

preparative HPLC fractions were processed to purification step by analytical HPLC according to the literature (Büchele et al., 2003).

A



B

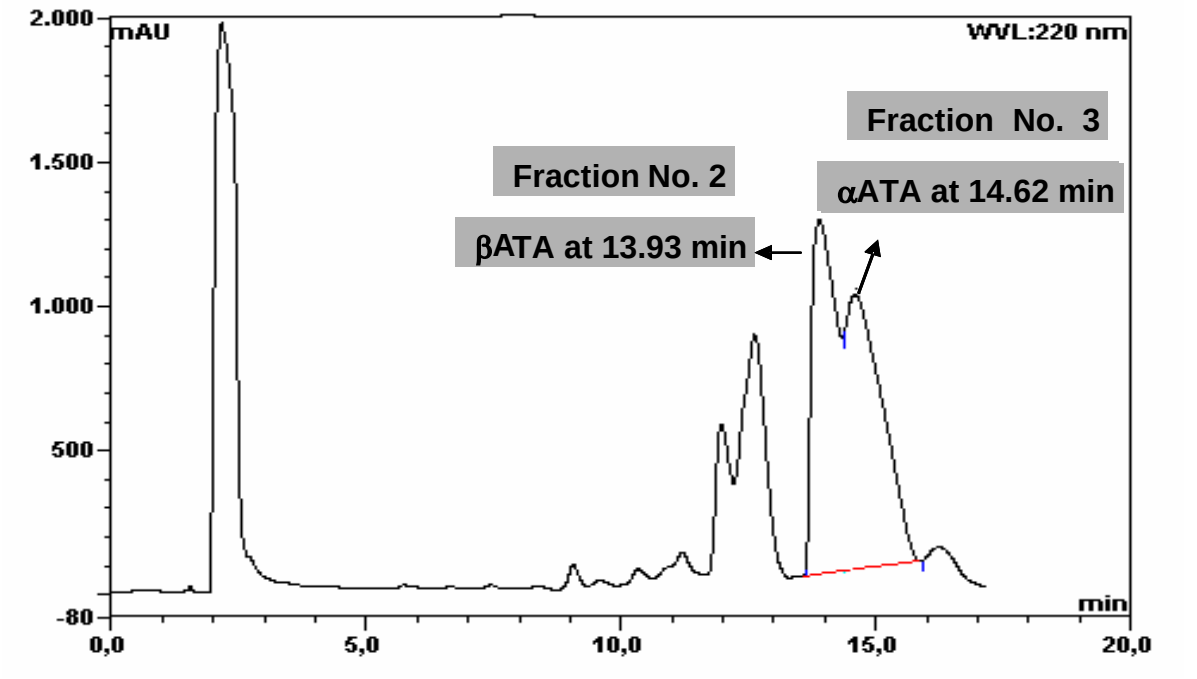


Figure 10. LPLC of fraction No. 2 (80% acetonitrile). To separate OTA and αATA and βATA present in the LPLC fraction No. 2 (80% acetonitrile) a semi-preparative HPLC chromatography was used. 5 mg/50 µl DMSO of sample were loaded onto a Reprosil-Pur ODS-3 (5 µm, 250x8 mm) column. Detection was at the wavelengthes λ=210 nm and 220 nm. Flow rate was 4 ml/min and the temperature 28°C. Mobile phases were (A) methanol: water: acetic acid (80:20:0.2, v/v/v) and (B) methanol: acetic acid (100:0.2, v/v). Isocratic separation of fractions was at 96% methanol (fraction No. 1) and at 94% methanol (fractions No. 2 and No. 3). OTA (fraction No. 1) was recovered at 12.54 min, βATA (fraction No. 2) at 13.93 min and αATA (fraction No. 3) at 14.62 min.

3.1.4. Determination of the structures of OTA, αATA and βATA

As estimated by HPTLC and HPLC, the purity of compounds was 99%. Chemical structures of OTA, αATA and βATA were characterized by UV spectroscopy, mass spectrometry as well as by 1D and 2D nuclear magnetic resonance spectroscopy (Fig. 11).

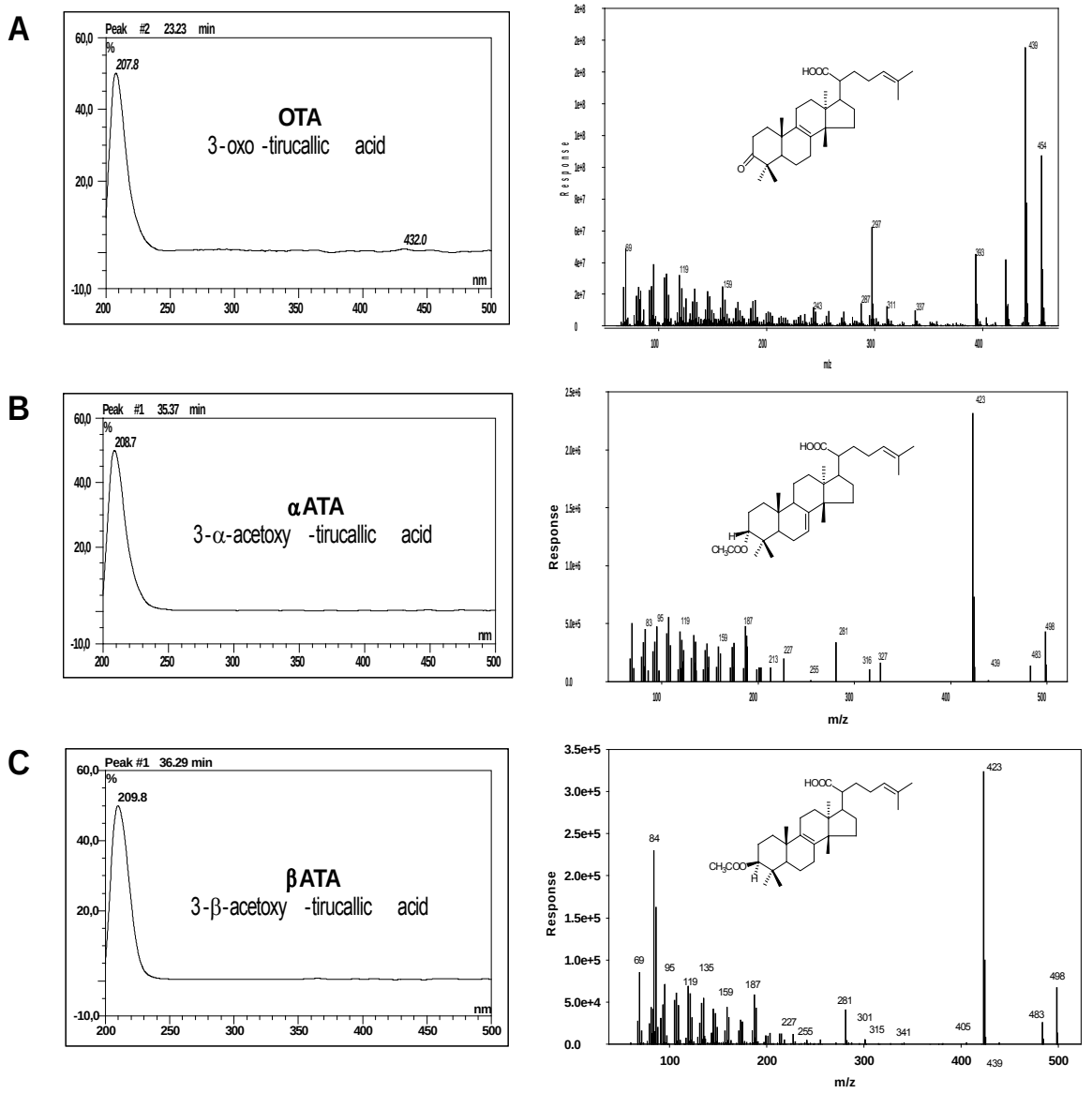


Figure 11. Spectroscopy of triterpenoids. UV-spectra and mass-spectroscopy for: (A) 3-oxo-tirucallic acid (OTA), (B) 3-α-acetoxy-tirucallic acid (αATA), (C) 3-β-acetoxy-tirucallic acid (βATA). Mass-spectroscopy was performed using a Finnigan MAT SSQ-7000 in the electrospray ionisation mode.

The biological activity of the pentacyclic triterpenoid AKβBA (Fig. 12) has been demonstrated in previous studies (Syrovets et al., 2000). In preliminary experiments AKβBA was used as reference compound to compare the biological activities of penta- and tetracyclic triterpenoids on prostate cancer cells.

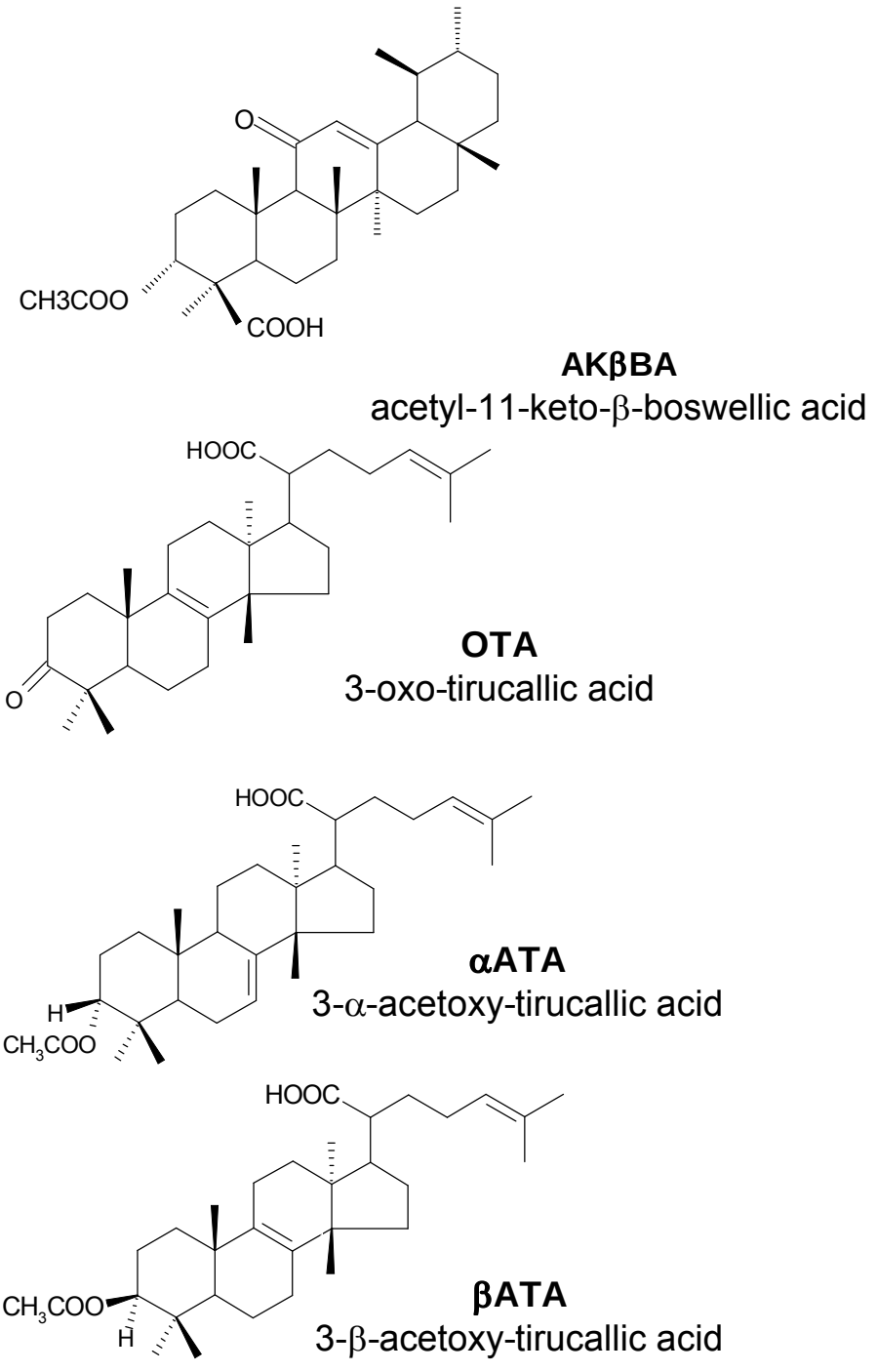


Figure 12. Structure of triterpenoids from *Boswellia carterii*.

3.2. Determination of cytotoxic effects of triterpenoids on prostate cancer cells

To investigate if triterpenoids have an influence on cell proliferation, PC-3 and LNCaP cells were treated with the compounds at different concentrations for 24 and 48 h. the effects on the cell proliferation was monitored by mitochondrial reduction of XTT salts by viable cells. After 24 h treatment AKβBA was the more active compound and after 48 h treatment αATA and βATA showed more activity than OTA or AKβBA with IC_{50} values between 3 and 10 µM (Fig. 13).

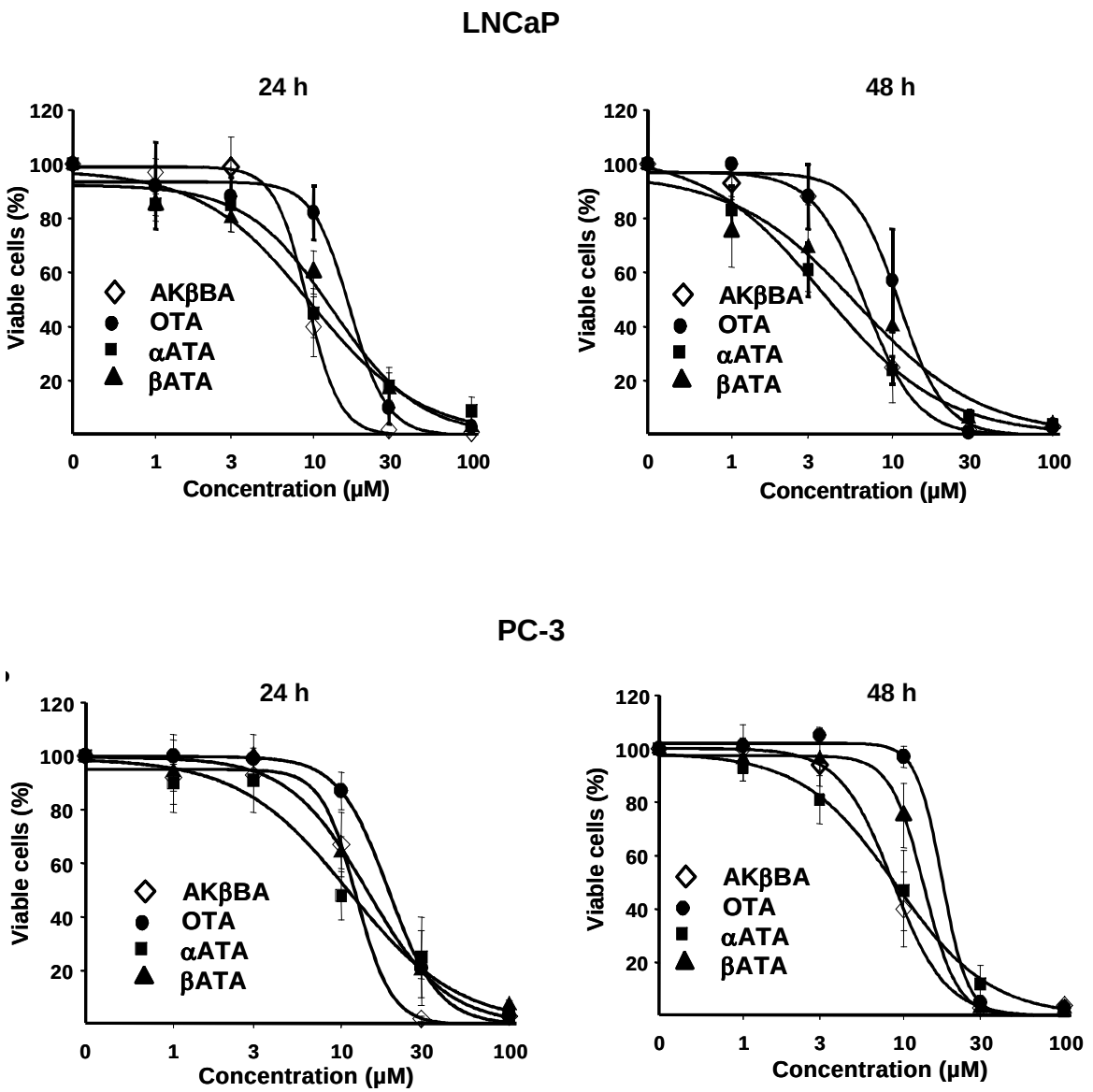


Figure 13. Triterpenoids exert antiproliferative effects on LNCaP and PC-3 cells. Cells were treated with triterpenoids for 24 and 48 h. The amount of cells in control samples treated with DMSO solvent (0.5%) was set 100%. The assay is based on the measurement of the metabolization of tetrazolium salts (XTT) to water-soluble formazan salt by viable cells. The data are mean ± SEM from three independent experiments each performed in triplicate.

The enzyme LDH is released from the cytoplasm after membrane damage. The amount of enzyme in the supernatant depends on the proportion of lysed cells. Analysis of LDH release was used as an indicator of cell viability in PC-3 and LNCaP cells. Treatment with AKβBA OTA, αATA or βATA indicated a higher reduction of viable cells after 48 h than after 24 h at the same conditions (Fig. 14). These results are in agreement with those obtained in XTT experiments.

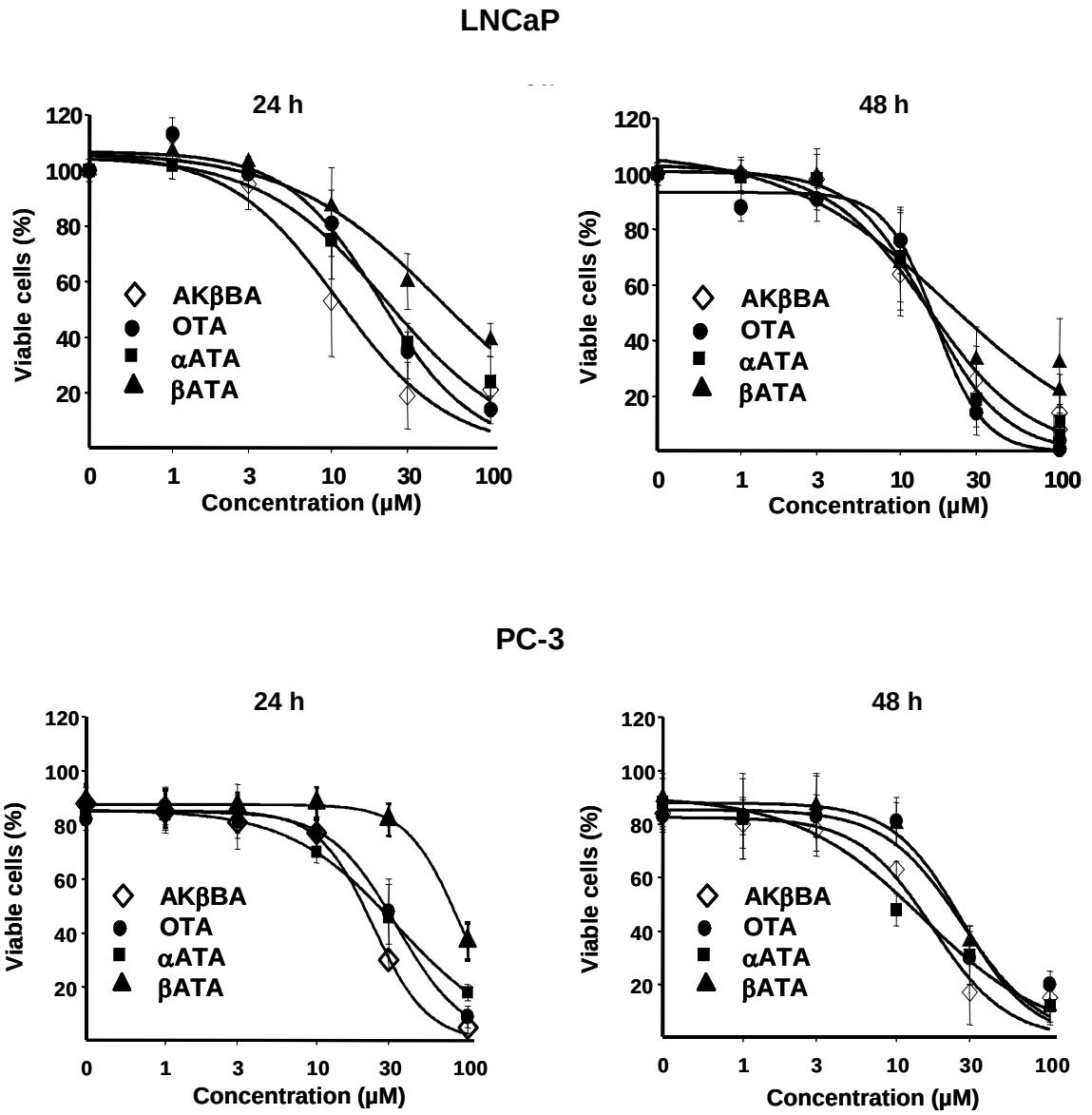


Figure 14. Triterpenoids exert cytotoxic effects on LNCaP and PC-3 cells. Cells were treated with (triterpenoids for 24 and 48 h. The assay is based on the measurement of the release of LDH from the cytosol of the damaged cells into the medium. The amount of LDH released by 0.1% triton-treated cells was set as 100% dead cells. The data are mean ± SEM from three independent experiments each performed in triplicate.

Triterpenoids exerted concentration-dependent cytotoxic effects on LNCaP and PC-3 cells. We have further calculated the concentrations necessary to inhibit 50% of prostate cells proliferation and viability (IC_{50}). αATA and βATA were more active than OTA in both cell lines (Tab. 11). The cell proliferation was analyzed by the XXT assay, cell death by the LDH release assay of cells treated for 24 and 48 h with triterpenoids. The data are mean from three independent experiments each performed in triplicate.

Table 11. Concentrations of triterpenoids required to induce 50%inhibition of prostate cell proliferation and cell death.

Substance	IC_{50} (μM) XTT Assay				IC_{50} (μM) LDH Assay			
	24 h		48 h		24 h		48 h	
	LNCaP	PC-3	LNCaP	PC-3	LNCaP	PC-3	LNCaP	PC-3
AKβBA	9 ±1	11 ± 2	7 ± 2	9 ± 3	26 ± 1	24 ± 2	15 ± 2	23 ± 3
OTA	18 ± 3	19 ± 4	9 ± 2	14 ± 1	28 ± 4	33 ± 4	17 ± 4	22 ± 4
αATA	12 ± 1	17 ± 2	11 ± 4	14 ± 2	22 ± 2	31 ± 3	13 ± 1	28 ± 3
βATA	10 ± 3	13 ± 3	4 ± 1	8 ± 1	21 ± 3	28 ± 4	10 ± 4	11 ± 3

Additionally, the results indicated that concentrations above 30 μM are fully toxic for both cell lines. For this reason, 10 μM was selected as a concentration of triterpenoids used to analyze the molecular mechanisms of cell death in the following experiments.

3.3. Triterpenoids induce apoptosis in prostate cancer cells

To define the cytotoxicity of AKβBA, OTA, αATA or βATA as proapoptotic activity, we analyzed different apoptosis parameters. One of the membrane changes in the early-intermediate stages of apoptosis is the translocation of phosphatidylserine (PS)

from the inner cell membrane to the outside. It is possible to detect PS by using FITC-labelled annexin V and combining staining of annexin V-FITC with propidium iodide (Fig. 15). Treatment of LNCaP and PC-3 cells with AKβBA, OTA, αATA or βATA (10 μM) for 24 h, triggered expression of phosphatidylserine on the outer cell membrane, a definite sign of apoptosis is DNA fragmentation by endonucleases activated by caspases. Using the DNA laddering assay we have analyzed DNA fragmentation in tumor cells. Triterpenoids induced DNA laddering in prostate cancer cells (Fig. 16). DNA laddering was observed in cells treated for 72 h (LNCaP) or 96 h (PC-3) with 10 μM OTA, αATA or ßATA.

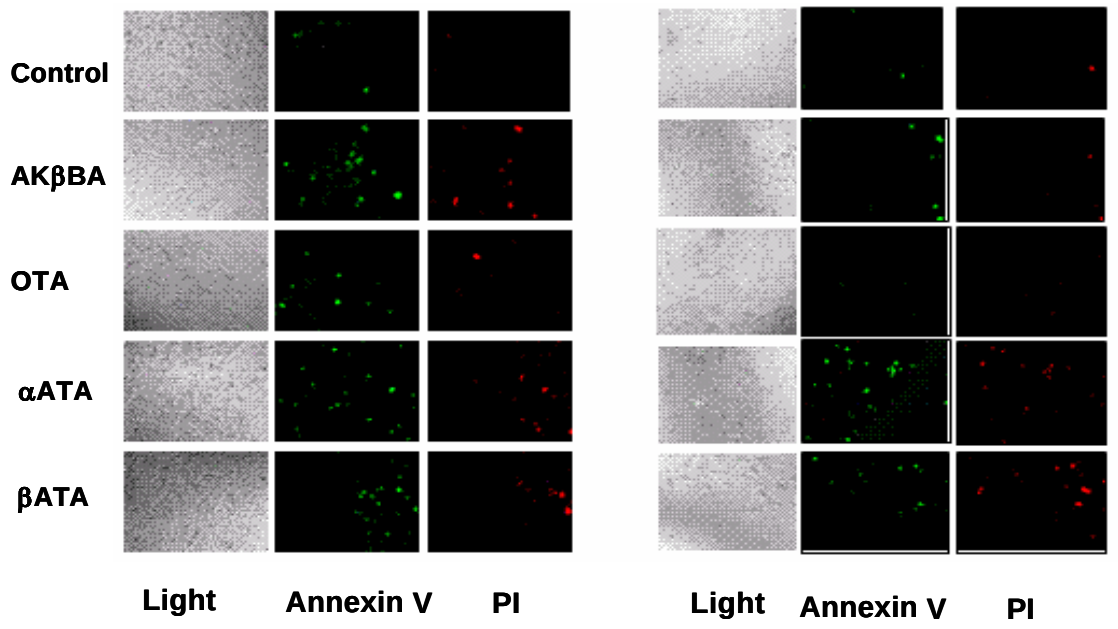


Figure 15. Triterpenoids induce phosphatidylserine expression on the surface of LNCaP and PC-3 cells. The cells were treated with 10 μM of triterpenoids for 24 h. Expression of phosphatidylserine on the cell surface was analyzed by binding of FITC-labelled annexin V (green). Necrosis was measured by propidium iodide counter-staining (red). Data are representative out of three independent experiments.

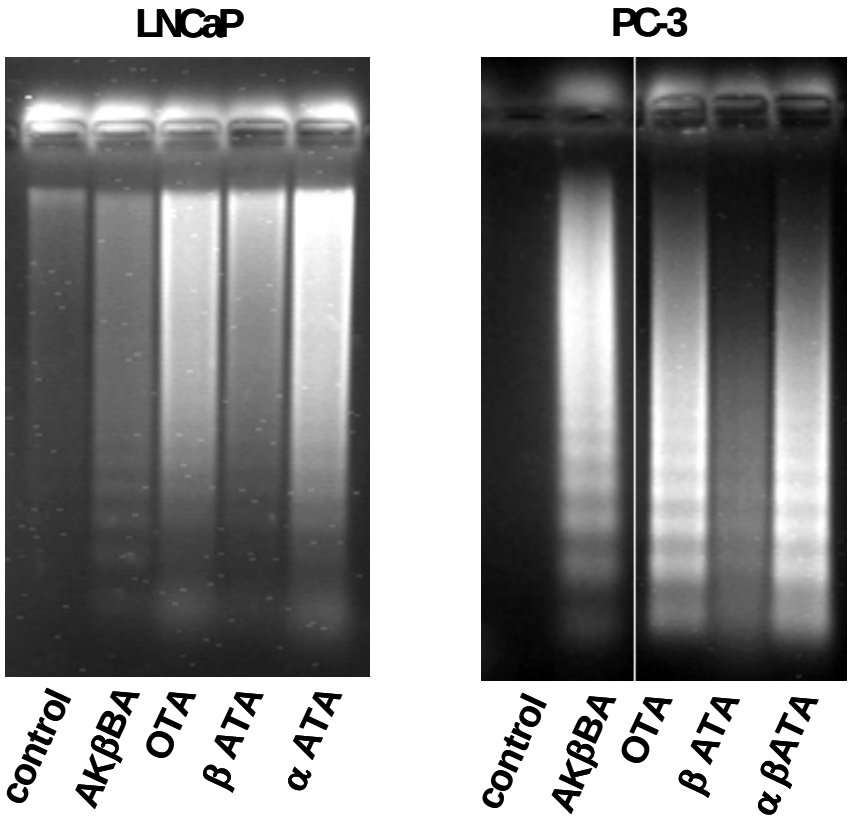


Figure 16. Triterpenoids induce DNA laddering in PC-3 and LNCaP cells. DNA laddering was analyzed in the cells treated with AKβBA, OTA, αATA or βATA (each 10 μM) for 72 h (LNCaP) and 96 h (PC3). Data are representative out of three experiments.

Caspases are the ultimate effectors or executioners of programmed cell death. Caspase-3 is present in the cytosol as an inactive 32 kDa proenzyme that is activated by proteolytic cleavage during apoptosis. Activation of Bcl-2 family members such as BAD promotes caspase cleavage. This process is an indicator of activation of the caspase cascade responsible for the fragmentation of nuclear DNA. In previous experiments DNA fragmentation was observed in LNCaP and PC-3 cells (Fig. 16). The compounds also induced cleavage of caspase-3 in PC-3 after 48 h of treatment and in LNCaP after 72 h of treatment (Fig. 17).

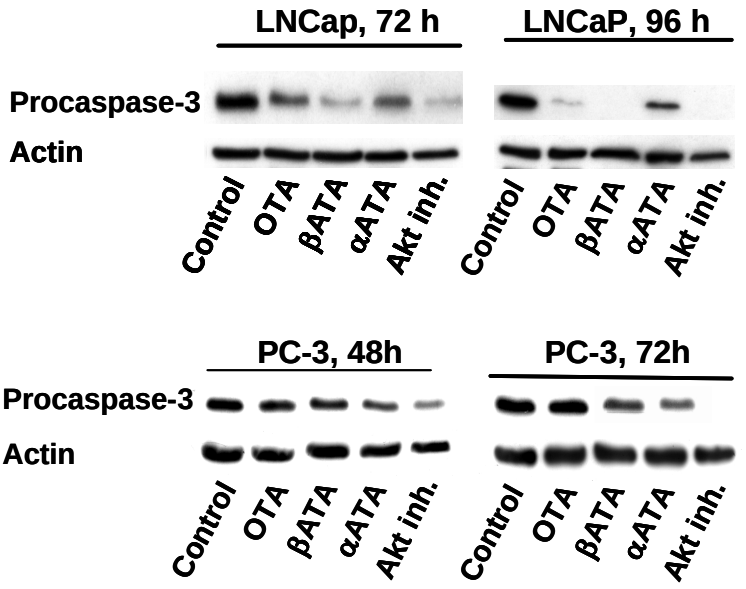


Figure 17. Triterpenoids trigger cleavage of procaspase-3 in prostate cancer cells. Analysis of the procaspase-3 cleavage was performed by immunoblotting. LNCaP cells were treated for 72 h and PC-3 cells for 48 h with OTA, αATA or βATA (each 10 μM). The proteins were visualized using enhanced chemiluminescence detection. Rrepresentative experiments out of three are shown.

3.4. Triterpenoids induce cell cycle arrest in prostate cancer cells

The DNA content of the cell can provide information about the cell cycle and, consequently, about the effect of added stimuli e.g. drug treatment on the cell cycle. Cellular DNA was stained with propidium iodide and analyzed by flow cytometry. The results of the cell cycle analysis are presented as % of cells in different phases of the cell cycle. Treatment with the triterpenoids resulted in strong reduction of the number of cells in the S phase. More PC-3 cells were arrested in the G_1 phase, whereas the LNCaP cells accumulated in the G_2 phase (Fig. 18). These results correlate with the induction of apoptosis, which was demonstrated by expression of phosphatidylserine and formation of the DNA laddering as a result of treatment of LNCaP and PC-3 cells with AKβBA, OTA, αATA or β-ATA.

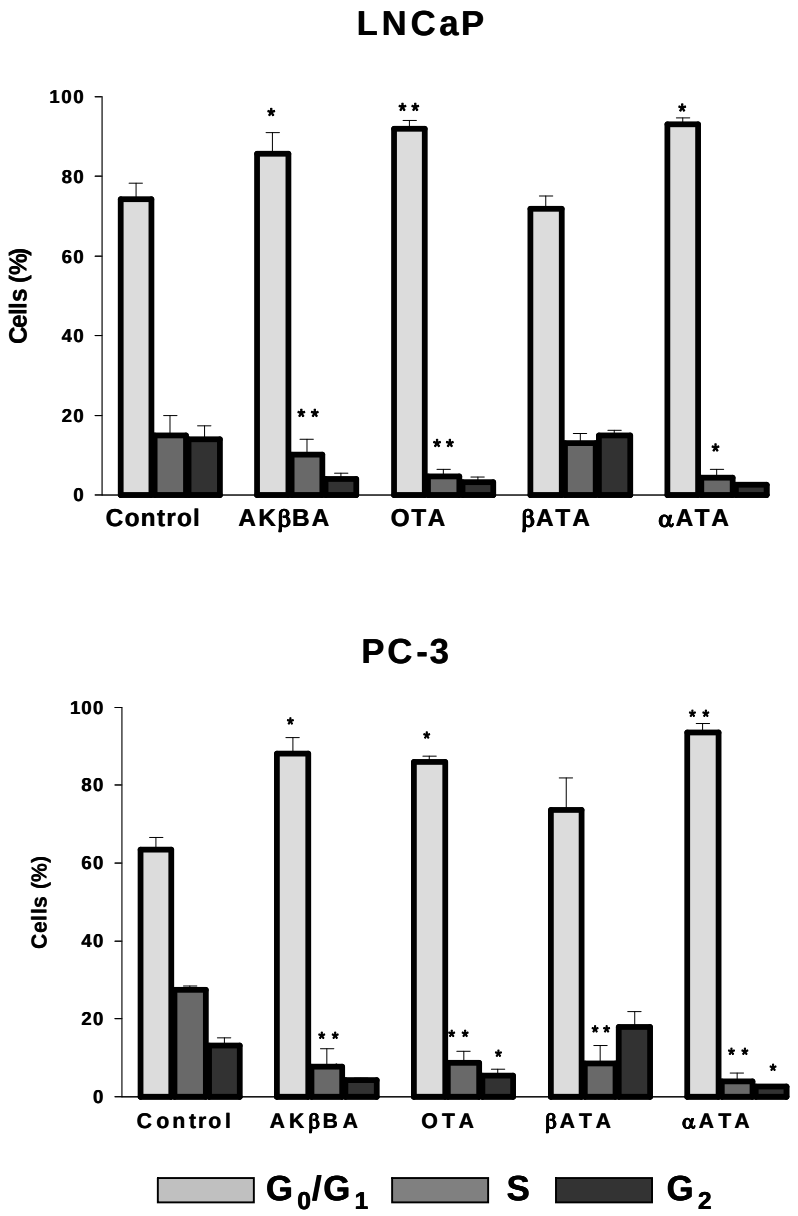


Figure 18. Triterpenoids induce cell cycle arrest of the prostate cancer cells. The LNCaP and PC-3 cells were treated for 48 h with 10 μM AKβBA, OTA and αATA or βATA. Cells were subsequently stained with propidium iodide and analyzed by FACS. Cell cycle analysis was performed using ModFit software. Data are mean ± SEM from three independent experiments. Statistical analysis was performed using the Newman-Keuls test, * $p < 0.05$, ** $p < 0.01$.

3.5. Inhibition of the expression of cell cycle regulators by AKβBA and OTA

Preliminary analysis of expression of different cell cycle regulators was carried out only with pentacyclic triterpenoid AKβBA and tetracyclic triterpenoid OTA. The obtained results were used as a basis for the following experiments that included analysis of the activity of other tetracyclic triterpenoids. We performed RT-PCR analysis of the mRNA expression and western blot analysis of the protein expression of cyclin D1, one of the central internal regulators of a transition between G_1 and S phase.

The mRNA expression of cyclin D1 was reduced after treatment of cells for 3, 6 and 12 h with AKβBA or OTA. Reduction of cyclin D1 mRNA expression correlated with the cell cycle arrest of tumor cells in G_1. The data indicate that OTA is more active than AKβBA in LNCaP cells whereas in PC-3 cells AKβBA was the more active compound (Fig. 19). In PC-3 cells, NF-κB, a transcriptional regulator of cyclin D1 expression, is constitutively active and AKβBA inhibits the NF-κB activity (Syrovets et al., 2005).

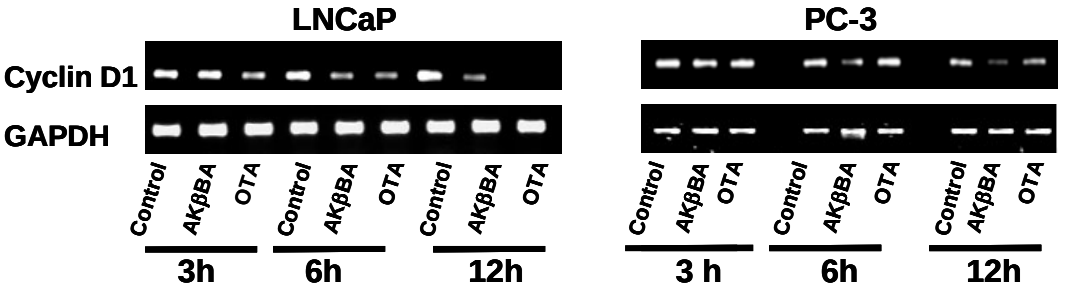


Figure 19. AKβBA and OTA downregulated cyclin D1 in PC-3 and LNCaP cells. Cells were treated for 3, 6 and 12 h with AKβBA or OTA (each 10 μM). RNA was isolated by TRIzol reagent, reverse transcribed using M-MLV and oligo $d(T)_{12-18}$ primers. The PCR products were separated by electrophoresis and visualized by ethidium bromide staining. Representative experiments out of three are shown.

Expression of cyclin D1 protein was also reduced after treatment with AKβBA or OTA (Fig. 20A). Association of cyclin D1 with cyclin-dependent kinases CDK4 and CDK6 results in phosphorylation of Rb. It maintains control of the G_1 to S phase transition through the release of E2F transcription factor. Cyclin E expression can greatly accelerate G_1 progression on prostate cancer cells. Cyclin E is also necessary for the

Rb phosphorylation. Reduction of CDK4 expression after 24 h treatment was observed in both cell lines. Under the same conditions, the expression of cyclin E shows no significant changes (Fig. 20A). A decrease in cyclin D1 levels is sufficient to induce hypophosphorylation of Rb after treatment with AKβBA or OTA in both cell lines at the indicated times (Fig. 20B). This data correlate with the observed induction of cell cycle arrest in prostate cancer cells treated with triterpenoids.

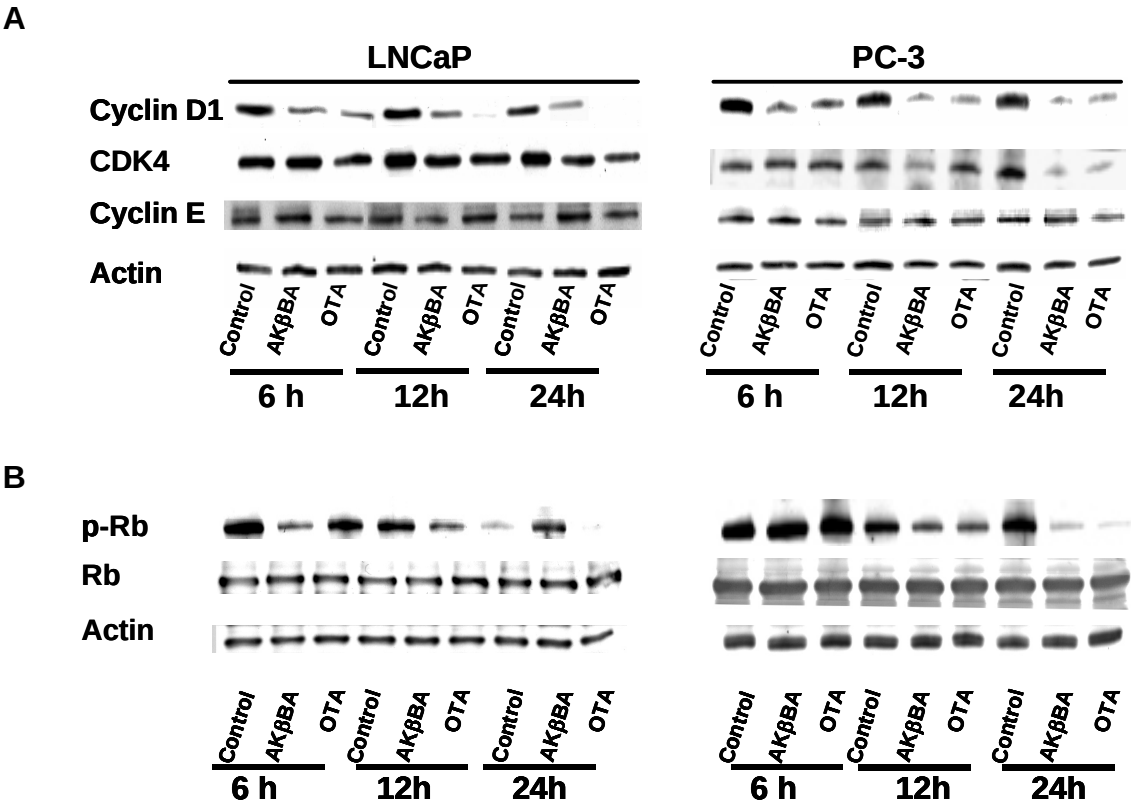


Figure 20. Triterpenoids Inhibit the protein expression of regulators of the G_1 phase transition in prostate cancer cells. (A) Analysis of cyclin D1, cyclin E, CDK4 and (B) Rb expression and phosphorylation were performed by immunoblotting. Cells were treated for 6, 12 and 24 h with AKβBA or OTA (each 10 µM). The proteins were visualized using enhanced chemiluminescence detection. Representative experiments out of three are shown.

The transcription of cyclin D1 is regulated by Ras, mitogen-activated FRAP/mTOR kinase, NF-κB and β-catenin (Fu et al., 2004; Pavletich et al., 1999; Morgan, 1997). To define, which signaling pathway was affected by triterpenoids, analysis of nuclear translocation of β-catenin and NF-κB was carried out. For this, the nuclear extracts from LNCaP and PC-3 cells treated with AKβBA or OTA were prepared. Both transcription factors are important for the regulation of transcription of cyclin D1. The

results show a rapid reduction of the p65 translocation into the nucleus in both cell lines and a decrease in nuclear β-catenin in the PC-3 cells (Fig. 21).

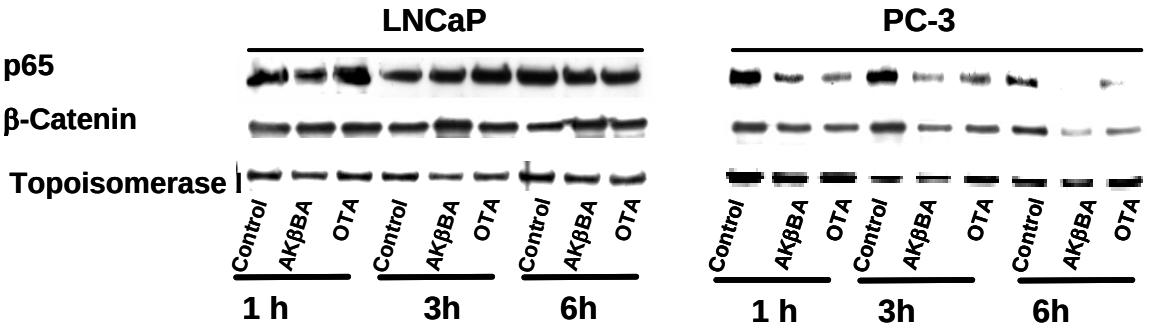


Figure 21. Triterpenoids Inhibit the nuclear translocation of β-catenin and p65. The cells were treated with AKβBA or OTA (each 10 μM) for 1, 3 or 6 h and nuclear extracts were prepared. Equivalent amount of proteins were electrophoretically resolved and detected by immunoblotting. The proteins were visualized using enhanced chemiluminescence detection. Representative experiments out of three are shown.

Components of the PI3K/Akt pathway are activated more frequently than any other pathway in cancer patients, indicating that Akt activation is directly related to cell survival in cancer cells (Hennessy et al., 2005). Promotion of cell survival by PI3K/Akt pathway is interconnected with the regulation of activation of NF-κB and β-catenin.

Based on this information, we hypothesized that the Akt pathway might be directly affected by the triterpenoids in LNCaP and PC-3 cells. Next we determined the levels of expression of Akt isoforms and Akt activity in LNCaP and PC-3 cells.

3.6. Expression and role of Akt in prostate cancer cells

High activity of Akt has been detected in many types of human cancer including prostate cancer. Akt facilitates the growth factor mediated cell survival and blocks apoptotic cell death. Expression of Akt isoforms, such as Akt1, Akt2 and Akt3, is cell-type dependent.

The mRNA and protein levels of three isoforms of Akt were compared in LNCaP, PC-3, DU 145 prostate cancer cell lines and monocytes as non-tumor control cells. 24 cycles of PCR product amplification were used for all three isoforms to enable the

comparison of mRNA expression levels of the Akt isoforms. Akt1 and Akt2 are present in all prostate cancer cell lines. Expression of Akt3 was observed only in DU 145. In PC-3 cells, Akt3 is present at low levels (Fig. 22A). The same pattern of Akt isoform expression was observed at the protein level (Fig. 22B). Akt activation was analyzed by immunoblotting as Akt phosphorylation on Ser473 residue. Activation of Akt was observed in LNCaP and PC-3 cells, which are PTEN deficient.

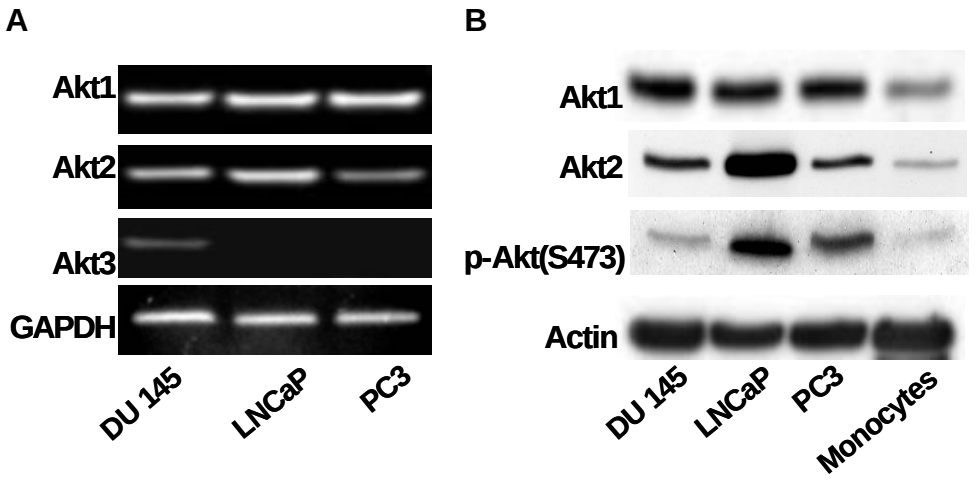


Figure 22. Analysis of Akt isoforms expression in prostate cancer cell lines. (A) For determination of mRNA expression of Akt isoforms, RNA was isolated by TRIzol reagent. All three isoforms were amplified with 24 cycles. PCR reactions were performed to avoid the saturation phase. Normalization was carried out using GAPDH as internal standard. Equal amounts of PCR products were separated by electrophoresis and revealed by ethidium bromide staining. (B) Expression of Akt isoforms was determined by immunoblotting. The proteins were visualized using enhanced chemiluminescence detection. Monocytes served as unstimulated non-tumor control. Representative experiments out of three are shown.

In order to analyze the correlation between Akt activation, proliferation and survival in LNCaP and PC-3 cells, we performed an XXT assay of the cells treated for 48 h with two commercial Akt inhibitors. The inhibitors are structurally and functionally different. Akt inhibitor VIII, inhibits Akt in pleckstrin homology domain-dependent manner. Akt inhibitor VIII is a cell-permeable quinoxaline compound, a more selective inhibitor of Akt1/Akt2 compared to Akt3. Akt inhibitor X is not pleckstrin homology domain dependent and is not isoform specific. Akt inhibitors VIII and X induce inhibition of cell proliferation and cell death in a concentration-dependent manner (Fig. 23). The results confirmed that the Akt pathway is indispensable for the proliferation and survival of these prostate cancer cells. The Akt inhibitor VIII was selected as a control parameter of inhibition of Akt activity by triterpenoids in prostate cancer cells.

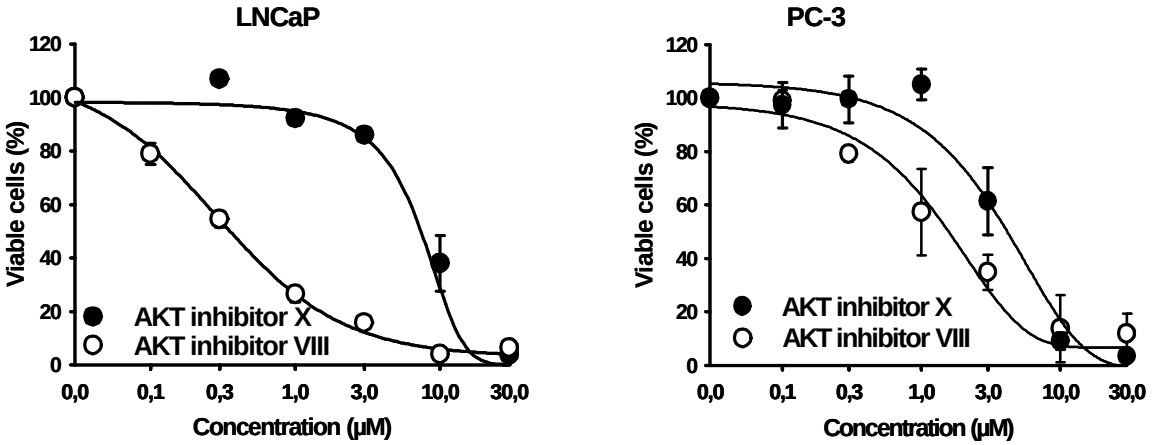


Figure 23. Akt activation is important for the survival of prostate cancer cells. LNCaP and PC-3 cells were treated with different concentrations of structurally different Akt inhibitors VIII and X for 48 h. Cell viability was measured by XTT assays and is expressed as % compared to control cells. Results are mean ± SEM of three independent experiments each performed in triplicate.

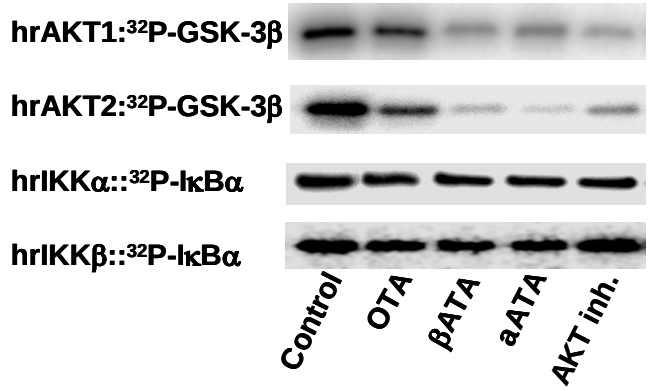
3.7. Tetracyclic triterpenoids inhibit Akt activity

To demonstrate the effect of triterpenoids on Akt activity, we performed in vitro kinase assay using either immunoprecipitated Akt or active human recombinant Akt1, Akt2 and GSK-3β as Akt substrate. IκBα was used as a substrate for the IκB kinases (IKKα and IKKβ), which were analyzed for comparison (Fig. 24A).

The results demonstrated that GSK-3β phosphorylation induced by human recombinant Akt1 and Akt2 was inhibited by the tetracyclic triterpenoids. On the contrary, the activities of recombinant IKKα and IKKβ in terms of IκBα phosphorylation was not affected.

The compounds also inhibited activity of immunoprecipitated Akt, but they did not influence the activity of immunoprecipitated IKKα (Fig. 24B). hrAkt1 was then treated with different concentrations of OTA, αATA or βATA. αATA and βATA are the more potent inhibitors of hrAkt1 activity compared to OTA (Fig. 24C).

A.



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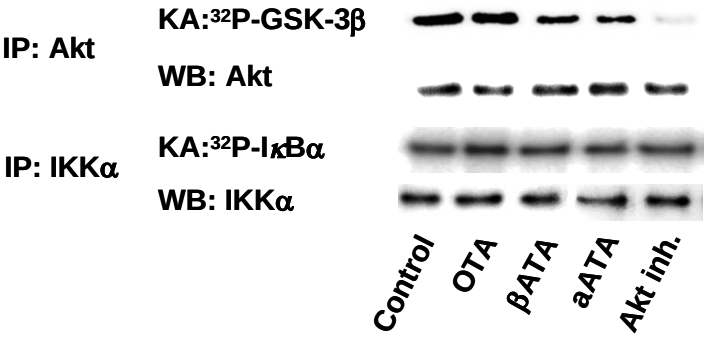


C

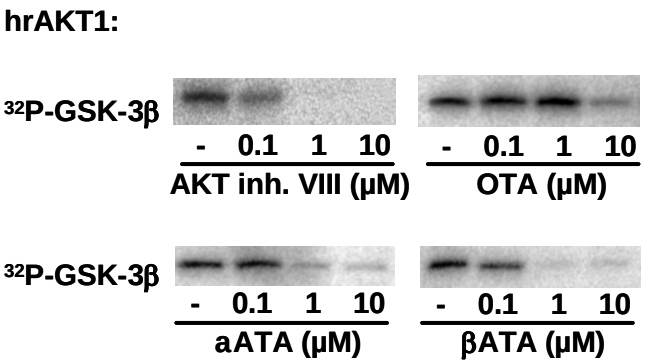


Figure 24. Tetracyclic triterpenoids inhibit the activity of immunoprecipitated and human recombinant Akt. (A) In vitro kinase assay. Active human recombinant (hr) Akt1, Akt2, IKKα, IKKβ (100 nmol) were pretreated for 20 min at 30°C with triterpenoids or Akt inhibitor VIII (each 10 μM). GSK-3β or IκBα were used as substrates. (B) Constitutively active Akt and IKK were immunoprecipitated from PC-3 cells, preincubated for 20 min at 30°C with the triterpenoids or the Akt inhibitor VIII (each 10 μM) and analyzed in a kinase assay using GSK-3β or IκBα as substrates. (C) Alternatively, Akt1 was pretreated with different concentrations of the triterpenoids or the Akt inhibitor VIII (0.1-10 μM) and analyzed as above. Results of the kinase assays and immunoblots are representative out of three.

3.8. Triterpenoids inhibit signaling pathways dependent on Akt activation

Inhibition of phosphorylation of Akt at Ser473 was used as an indicator for Akt activity in prostate cancer cells. Because Akt activation might affect activation of MAP kinase signaling pathways (Rennefahrt et al., 2005), the activity of ERK1/2 was also analyzed. Treatment with triterpenoids for 3 h inhibited Akt activity. βATA and αATA were the most active compounds. ERK1/2 activity was increased upon treatment with the tetracyclic triterpenoids in both cell lines (Fig. 25A).

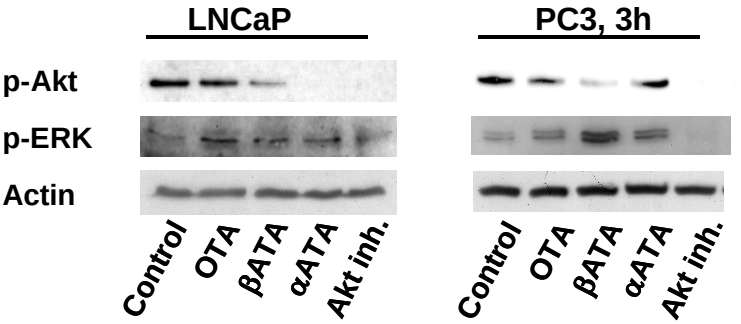
Inhibition of Akt activity by triterpenoids in both cell lines was confirmed by downregulation of phosphorylation of GSK-3β at Ser9. Akt signaling inhibits progression through the cell cycle by different mechanisms. Expression of cyclin D1 was inhibited whereas no important changes in the expression of cyclin E were observed after 6 h treatment with triterpenoids (Fig. 25B). Phosphorylation of Rb protein is regulated in a cell cycle-dependent manner by complexes cyclin D1/CDK4-6 resulting in the release of E2F from Rb, an important event for the G_1/S check point regulation. After downregulation of the cell cycle regulator cyclin D1, subsequent inhibition of phosphorylation of Rb protein by treatment with OTA, αATA or βATA was observed (Fig. 25C).

Inactivation of GSK-3β by phosphorylation on Ser9, results in increased nuclear levels of β-catenin. This protein is involved in TCF-mediated gene transcription of cell cycle regulators such as cyclin D1 and c-myc (Dang, 2006). β-Catenin also augments activity of the androgen receptor (AR) in androgen-dependent LNCaP cells. Akt Inhibition by tetracyclic triterpenoids induces inhibition of nuclear translocation of β-catenin and expression of c-myc and AR in LNCaP cells after 6 h treatment (Fig. 26A).

NF-κB activation and translocation of p65/RelA subunit into the nucleus, is essential for the transcription of the cell cycle regulator cyclin D1. Akt can regulate this NF-κB activation. The nuclear accumulation of p65/RelA was inhibited within 6 h treatment

of LNCaP and PC-3 cell with triterpenoids (Fig. 26A). The Akt VIII inhibitor did not reduced the expression of c-myc in PC-3 cells at these conditions. The inhibitor was active in these cells only after 24 h treatment (Fig. 26B).

A



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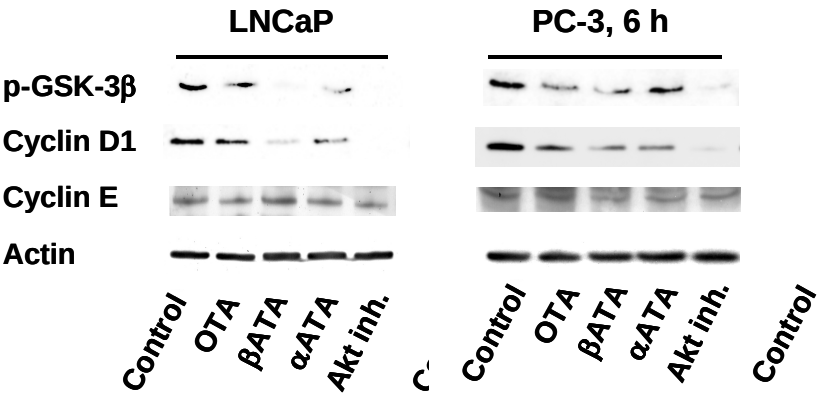

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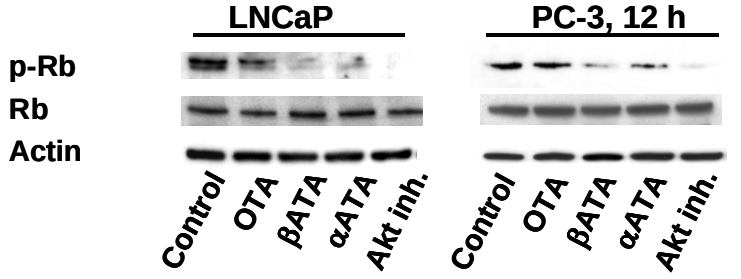
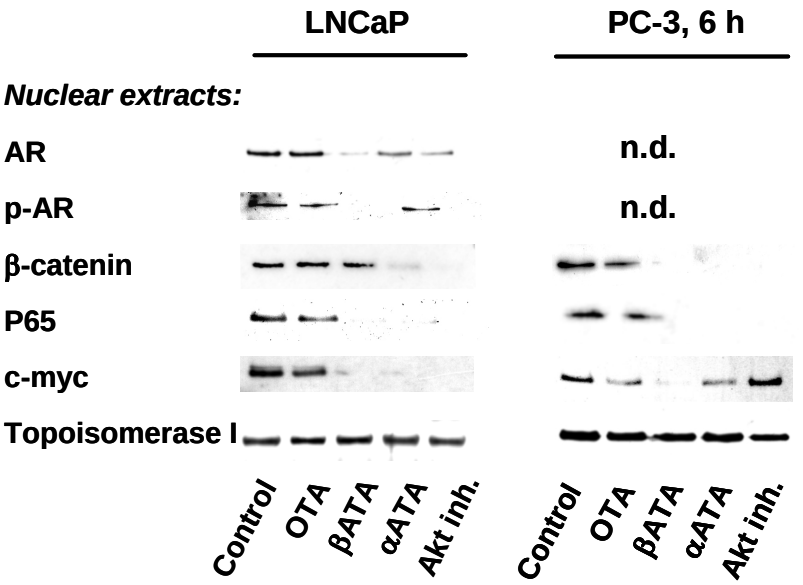


Figure 25. Triterpenoids inhibit signaling pathways dependent on Akt activation. Analysis of cyclin D1, cyclin E and p-Rb expression was performed by immunoblotting. Cells were treated for 3, 6 and 12 h with OTA, αATA or βATA (each 10 μM). The proteins were visualized using enhanced chemiluminescence detection. Representative experiments out of three are shown.

A



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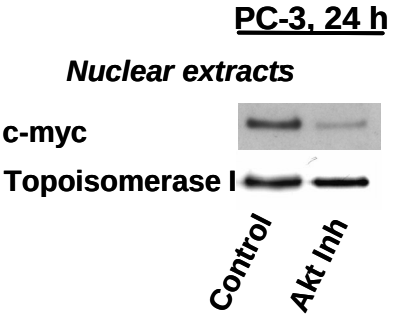

Figure 26. Inhibition of nuclear accumulation of transcriptions factors important for the cell cycle progression. The cells were treated with OTA, αATA or βATA (each 10 μM) for (A) 6 and (B) 24 h and nuclear extracts were prepared. Equivalent amounts of proteins were electrophoretically resolved and detected by immunoblotting. The proteins were visualized using enhanced chemiluminescence detection. Representative experiments out of three are shown.

The p53 tumor-supressor is expressed in LNCaP cells. The PC-3 cells are lacking p53 (Wang et al., 2004). This tumor-supressor plays an important role in maintenance of normal cell cycle progression in androgen-dependent LNCaP cells. During androgen-dependent cell growth, p53 is also essential as a modulator of AR signaling. Under physiological conditions p53 is maintained at low levels by the cellular protein mdm2. The Akt-dependent phosphorylation of mdm2 on Ser166 or Ser186 facilitates it nuclear localization and thus, indirectly contributes to p53 degradation (Hanada et al., 2004). Treatment with triterpenoids results in nuclear stabilization of p53 in LNCaP cells (Fig. 27A). Upregulation of a cyclin kinase inhibitor

and negative regulator of the cell cycle transition, $p21^{Cip1}$ contributes in a p53-dependent manner to the G_2/M cell cycle arrest in LNCaP cells. At variance, in PC-3 cells which do not express AR and p53, $p21^{Cip1}$ expression and localization is not changed after treatment either with tetracyclic triterpenoids or Akt inhibitor (Fig. 27B).

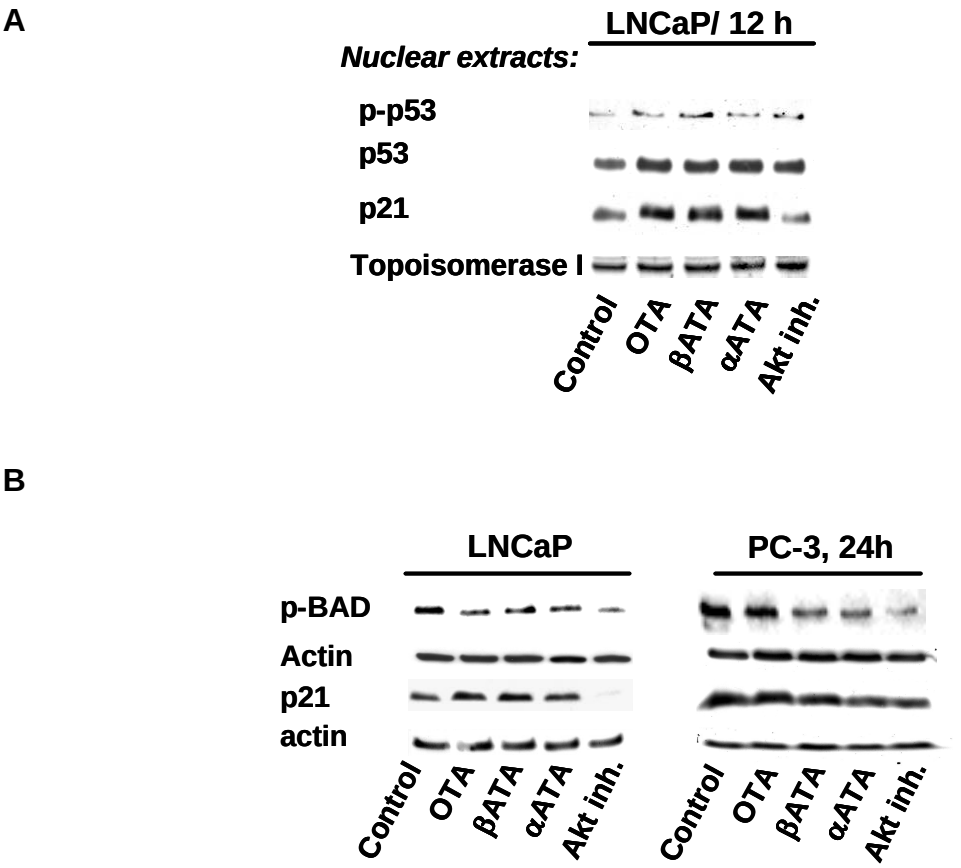

Figure 27. Triterpenoids inhibit signaling pathways dependent on Akt activation. The cells were treated either with OTA, αATA or βATA (each 10 μM) for 12 (A) and 24 (B) h. Whole cell lysates or nuclear extracts were prepared. Equivalent amounts of proteins were electrophoretically resolved and detected by immunoblotting. The proteins were visualized using enhanced chemiluminescence detection. Representative experiments out of three are shown.

Bcl-2 family and inhibitors of apoptosis (IAPs) are the main intracellular regulators of the cell death program. BAD belongs to the Bcl-2 family and it is a direct target of Akt in promoting cell survival. Akt phosphorylates BAD on Ser136 and inhibits its pro-apoptotic activity. Inhibition of Akt activity in LNCaP and PC-3 cells by triterpenoids induced reduction of BAD phosphorylation after 24 h of treatment (Fig. 27B).

The effects of pentacyclic triterpenoids on the expression and phosphorylation of the proteins, which are known targets of Akt, was similar to those of the commercially available Akt inhibitor. These data are in agreement with the results of the kinase assays and demonstrate that the tetracyclic triterpenoids are selective inhibitors of Akt in prostate cancer cells.

4. Discussion

Detection of prostate cancer in early stages has a direct impact on survival. In a study (Brenner et al., 2005), 70% of total 1816 cases of prostate cancer diagnosed in Germany between 2000 and 2002 had a relative survival of almost 10 years. Androgen ablation therapies in early stages of disease prevent development of clinical manifestations (Lance et al., 2001). The most common drugs used in ablation therapy are progestational agents (megesterol acetate, cyperone or ketoconazole), steroidal anti-androgen agents, nonsteroidal anti-androgens (flutamide, nilutamide and bicalutamide), gonadotropin and estrogens (diethylstilbestrol) (Papatsoris and Papavassiliou, 2001). Nevertheless, patients who underwent ablation therapy and potentially curative surgery are in at substantial risk for relapse of the tumor. Almost 20-40% of patients in early stage of the disease who underwent radical prostatectomy and radiotherapy suffered recurrence (Davies et al., 2006).

The relapsed tumors are hormone-insensitive. Androgen deprivation leads to apoptosis in androgen-dependent cells; but at the same time it may facilitate selection of androgen-independent cells, thus making the endocrine therapy less effective. Recurrent androgen-independent prostate cancer is lethal (Long et al., 2005). Therefore, it is important to pursue investigations that would allow development of new drugs for the treatment of prostate cancer in early and in advanced stages of the disease.

The prostate cancer cell lines LNCaP, PC-3 and DU 145 have been used extensively to examine the direct and indirect effects of different therapies. In this study, LNCaP and PC-3 cell lines were used. These prostate cancer cells exhibit constitutive activation of distinct signaling pathways, which are important in determination of the activity of antitumor drugs. Thus LNCaP, but not PC-3 cells, express wild type p53 and their growth depends on activation of the androgen receptor (Wang et al., 2004). Both cell lines, however, harbor mutations in PTEN, rendering them PTEN null (Sulis and Parsons, 2003). As a result, the PI3K/Akt pathway is constitutively active in these cells (Woodgett, 2005).

Alterations in a limited number of signaling pathways, which normally regulate cell proliferation, differentiation, survival, senescence and cell-cell interactions in tissues, are regarded as fundamental for the development of human cancers (Hanahan and Weinberg, 2000). Thus, proliferation signals from the autocrine growth factors, overexpressed tyrosine kinase receptors, mutated RAS or overexpression of growth factor-dependent transcription factors such as c-myc, or cyclin D1 and mutations in Wnt signaling pathway are important targets in advanced prostate cancers (Chesire and Isaacs, 2003). With the discovery that Ras/PI3K/Akt activation increases cell survival, it rapidly became evident that this pathway could be an effective target for therapies in advanced prostate cancer (Faivre et al., 2006).

Control of the Akt activation depends on specific expression of its three isoforms: Akt1, Akt2 and Akt3 (Datta et al., 1999). The two first Akt isoforms are ubiquitously expressed in all cell types and tissues. The expression of Akt3 is relatively tissue restricted (Li et al., 2005). Akt1 knockout mice exhibit growth defects and Akt2 knockout mice exhibit mainly defects in glucose homeostasis. These differences have yet to be correlated to differences at the level of substrate selectivity or signal functions of these two Akt isoforms. Activated Akt isoforms are overexpressed in a variety of malignancies, including colon, pancreatic, prostate, testis cancer and non-Hodgkin's lymphoma (Takahashi et al., 2003). Akt2 is the predominant form that is amplified in breast and ovarian tumors. Akt2 mutations have been detected in colon cancer and the expression of Akt2 increases invasion of cancer cells in vitro (MacKeigan et al., 2005). Recent data using a siRNA approaches pointed to differential roles of Akt1 and Akt2 in the cell cycle regulation as well. Thus, Akt1 was indispensable for proliferation of cells, whereas Akt2 promoted cell cycle exit utilizing $p21^{cip21}$ (Heron-Milhavet et al., 2006). The oncogenic effects of Akt1, Akt2 and Akt3 as analyzed *in vitro* and in animal models appeared to be indistinguishable (Mende, et al., 2001).

In our study we found that mRNA expression of Akt2 in PC-3 and DU 145 cells was weaker compared with LNCaP. Akt1 was highly expressed in all cells and Akt3 expression was observed only in DU 145 cells.

The Akt activation depends on PI3K activation. This results in the localization of PDK1 via its pleckstrin homology domain to the cellular membrane. Akt isoforms are also recruited to the lipid-rich plasma membrane, where they are phosphorylated at the residues Thr308 by PDK1 and Ser473 by a complex of mTOR-kinase (McCubrey et al., 2007). Thr308 phosphorylation activates Akt partially. For the full activation of Akt an additional phosphorylation at Ser473 is required (Li et al., 2005). We have shown that Akt activation at Ser473 is significantly higher in LNCaP and PC-3 prostate cancer cells than in PTEN positive DU 145 or unstimulated human monocytes.

The relationship between deregulated PI3K activity and the onset of cancer is well documented. For example, a mutated version of the p85 subunit of PI3K has recently been isolated from a Hodgkin's lymphoma-derived cell line (Jücker et al., 2002). Other reports directly implicate PI3K activity in a variety of human tumors including breast cancer, lung cancer, melanomas and leukaemia among others (McCubrey et al., 2007). Further evidence also supports the involvement of Akt in malignant transformation of cells (Nicholson and Anderson, 2002). It has been shown that PI3K is the predominantly activated growth-factor dependent pathway in LNCaP human prostate cancer cells (Lin et al., 1999).

Constitutive activation of the Akt signaling pathway found in LNCaP and PC-3 cells is the result of loss of expression of the tumor suppressor gene PTEN. This tumor suppressor encodes a lipid phosphatase that negatively regulates signaling pathways downstream from PI3K. Some authors have demonstrated that knocking out the PTEN gene in the mouse prostate is sufficient to trigger the development of metastatic prostate cancer (Wang et al., 2003). On the contrary, in DU 145 cells the activity of Akt was reduced in comparison to Akt activity in LNCaP and PC-3 cells, because the DU145 cells express constitutive PTEN. These results confirm that the differences in isoforms expression and activation of PTEN are cell-type specific. In addition, PTEN loss and Akt activation correlate with a high Gleason grade in advanced stage of prostate cancer (Schmitz et al., 2007). Therefore, these targets may provide additional options for the treatment of prostate cancer patients.

In the present study, we observed that triterpenoids were effective inducers of apoptosis as indicated by caspase-3 activation and formation of DNA ladder in LNCaP and PC-3 cells. Therefore, we aimed to investigate the mechanism of action of tetracyclic triterpenoids. Our results demonstrated that the incubation of cells with tetracyclic triterpenoids affects negatively several components of the PI3K/Akt pathway, such as GSK-3β, β-catenin, p65 and AR. This occurred because triterpenoids induce dephosphorylation of Akt at Ser473 and thus, reduce the Akt kinase activity.

As a result of the intensive studies on the molecular mechanisms implicated in the development of androgen independence and of prostate cancer invasion, an interesting variety of specific targets for prostate cancer gene therapy has been discovered. These include immunotherapy and anti-invasion therapy, along with different small-molecule compounds, which inhibit or stimulate some of the signaling pathways. Several investigations have been directed to develop ATP-competitive kinase inhibitors. These non-selective small molecules showed antitumor activity in xenograft animal models and in clinical trials (Luo et al., 2005). In addition, allosteric inhibitors that require pleckstrin homology domain of Akt and induce apoptosis in LNCaP cells have been described (Lindsley et al., 2005). There have been also developed Akt inhibitors such as calmodulin-like inhibitors (Kau et al., 2003), myo-inositol derivatives and phosphatidylinositol ester analogues (David-Beabes et al., 2000), which inhibit the Akt phosphorylation. There is no significant clinical experience for the latter inhibitors (Majumder and Sellers, 2005). Several studies describe inhibitors such as rapamycin and everolimus inhibiting mTOR in clinical phase I-II studies for prostate cancer (Armstrong and Carducci 2006). Perifosine, a synthetic alkyl-lysophospholipid and Akt inhibitor is currently in phase II trials for patients of several cancers with refractory solid tumors (Armstrong and Carducci, 2006; Amaravadi and Thompson, 2005).

Small molecules isolated from natural sources coupled with advances in combinatorial chemistry have been used successfully to probe biological mechanisms; these molecules are occasionally more potent and specific than synthetic compounds (Eggert et al., 2006). Extracts from *Boswellia* oleogum resins, commonly called frankincense, have been used in traditional medicine and in clinical

pilot studies for the treatment of chronic inflammatory diseases (Ammon, 2006). The tetracyclic triterpenoids isolated from oleogum resin of *Boswellia carterii* exert a cytotoxic effect on prostate cancer cells with αATA and βATA more active than OTA. Moreover, the tetracyclic triterpenoids are potent inhibitors of Akt activity. The inhibitory efficacy of the tetracyclic triterpenoids was the following: βATA>αATA>OTA. Particularly βATA inhibited the activity of human recombinant Akt at concentrations as low as 0.1 µM. The triterpenoids also inhibited the activity of Akt immunoprecipitated from proliferating PC-3 cells, but showed no effect on immunoprecipitated IKKα, which is also constitutively active in PC-3 cells. The effects of the triterpenoids were detected as early as 3 h after their addition to the cells. In addition, the activity of Akt in prostate cells was reduced after 3 h, which indicates that triterpenoids are rapidly taken up by the cells.

Analysis of the relationships between Akt activity and proliferation of prostate cells has shown, that Akt Inhibitor VIII, which interacts with the pleckstrin homology domain, and inhibitor X, that has no pleckstrin homology domain-dependent mode of action, both reduced in a concentration-dependent manner proliferation and viability of the cells. These data are in line with the literature (Defeo-Jones et al., 2005; Thimmaiah et al., 2005) and allow to conclude that Akt activity is essential for the proliferation of prostate cancer cells. An Akt inhibitor VIII concentration sufficient to inhibit all three Akt isoforms (Defeo-Jones et al., 2005), was selected for future experiments.

MAP kinase signaling pathways play central roles in the regulation not only of cell growth and differentiation but also of cell survival (Rennefahrt et al., 2005). At variance to Akt, ERK1/2 phosphorylation was increased in prostate cancer cells after treatment with the triterpenoids. Activation of ERK1/2 kinase observed in LNCaP and PC-3 cells might be a result of the inhibition of Akt by the triterpenoids. Previous studies have demonstrated that inhibition of Akt1 in breast epithelial cells resulted in activation of ERK1/2 (Irie et al., 2005). It has been demonstrated that Akt might negatively regulate the Raf/ERK pathway through phosphorylation of B-Raf (Guan et al., 2000). Activation of Akt and inactivation of ERK in high Gleason-grade prostate cancer has also been demonstrated. For this reason, the increased Akt activation, together with decreased ERK activation, can be an excellent predictor of poor clinical

outcome in prostate cancer (Li et al., 2007). Interestingly, the commercial Akt inhibitor VIII (Akti 1,2) (Lindsley et al., 2005) inhibited the activity of Akt and ERK1/2, suggesting that this compound might also target ERK1/2 or another kinase upstream of ERK.

In addition to the analysis of the effects of triterpenoids on Akt, we determined the consequences of the inhibition of Akt activity in prostate cancer cells in terms of expression of important effectors of survival and cell proliferation. One of the targets of Akt activity is the NF-κB pathway. Activation of this pathway is cell-type specific. Activation of NF-κB is dependent on IKK (Yamamoto et al., 2000). Activated Akt phosphorylates IKKα, which promotes activation of its partner, IKKβ (Ozes et al., 1999). IKK phosphorylation promotes NF-κB dissociation from the inhibitors IκB, followed by nuclear localization of p65 and other NF-κB subunits. Nuclear NF-κB binds DNA and activates genes that promote cell survival such as c-myc in PC-3 and epithelial cells (Gustin et al., 2001; Thomas et al, 2002). We observed inhibition of nuclear translocation of p65 after treatment of LNCaP and PC-3 cells with triterpenoids for 6 h. These data indicate that the inhibition of Akt activity by OTA, αATA and βATA blocks downstream targets of Akt essential for the proliferation in prostate cancer cells.

A major physiological function of Akt is regulation of cell metabolism. High levels of insulin promote glucose incorporation into glycogen via inhibition of GSK-3β by Akt-mediated phosphorylation (Truica et al., 2000). Akt and GSK-3β are also involved in regulation of β-catenin/TCF/LEF transcriptional pathway. Inhibition of GSK-3β by Akt also destabilizes short living cyclin D1 and c-myc in prostate cancer cells. Moreover, Akt regulates cyclin D1 and c-myc additionally at the level of translation (Nicholson and Anderson, 2002; Song et al., 2005). Accordingly, Akt activation by growth factors induces rapid expression of c-myc. Ectopic expression of c-myc is sufficient for the induction of the S phase entry in quiescent cells, indicating that c-myc plays a central role in G_1/S transition. C-Myc induces a number of genes involved in G_1/S transition, such as CDK4, Cdc25A, Id2 and activates cyclin E/CDK2 complexes (Guo, 2007). Mice lacking c-myc show an embryonic lethal phenotype accompanied by a drastic reduction of CDK2/4/6 activities (Matsumura et al., 2003).

Cyclin D-dependent kinases initiate phosphorylation of Rb, whereas c-myc induces expression of D cyclins and inhibitors of $p27^{Kip}$, an inhibitor of cyclin-dependent kinases (Dang et al., 2006). Akt also induces gene expression of various internal cell cycle regulators such as cyclin D1, Rb and c-myc. Furthermore, recent data indicate that Akt can directly phosphorylate $p21^{Cip1}$ and $p27^{Kip}$, inhibitors of cyclin-dependent kinases, which results in their inactivation, nuclear export and proteolysis (Fukuyama et al., 2003). Both, the D and E cyclin complexes are necessary for the entry into progression through the G_1 phase of the cell cycle. In cultured cells, the enforced overexpression of D cyclins can shorten the G_1 interval with attenuation of activity of $p21^{Cip1}$/$p27^{Kip}$ inhibitors (Jaruga-Killeen and Rayford, 2005). Treatment with CDK-selective drugs results in G_1 phase arrest and cell cycle exit (Tetsu and McCormick 2003). Ras-dependent cyclin D1 synthesis and its stabilization in the G_2 phase allow division of cells. Conversely, abrupt discontinuation of cyclin D synthesis and its rapid degradation, lead to a precipitous decay in kinase activity (Guo et al., 2002). Our results demonstrate a reduction of cyclin D1 and c-myc expression and Rb protein phosphorylation induced by triterpenoids and the Akt inhibitor in LNCaP and PC-3 cells. These data correlate with the induction of cell cycle arrest and induction of apoptosis observed in LNCaP and PC-3 cells. Recent work has demonstrated that inactivation of cyclin dependent kinase CDK4 completely protects transgenic mice overexpressing c-myc from developing epithelial tumors (Miliani de Marval et al., 2004). Similarly, inactivation of cyclin D1 confers resistance to breast cancers, induced by oncogenic Ras transgenes targeting the mammary gland, and to intestinal tumors (Misra et al., 2006).

The effect of Akt on the different internal cell cycle regulators supports its role in the cell cycle transition. Triterpenoids induced a drastical reduction of S phase in both cell lines and accumulation of PC-3 cells in G_1. These results suggest that interfering with cyclin D1 function and with other cell cycle regulators and targets of Akt activity might be of clinical benefit in cancer treatment.

Initially most patients respond favorably to androgen ablation and anti-androgen therapy, which has become a standard treatment of prostate cancer. However, the majority of patients will relapse to androgen-independent cancer. Prostate cells survive and grow in the low androgen environment by bypassing and sensitizing the

AR pathway (Jenster, 2000). From the studies on AR function in prostate cancer, it is clear that AR plays an important role in cancer development and progression. The vast numbers of AR abnormalities observed in prostate tumors from patients treated with hormonal therapy suggest that many cells exhibit changed AR pathway (Burnstein, 2005). To maintain activation of this pathway in a low androgen environment cells can mutate the AR and activate it in a ligand-independent manner by growth factors and cytokines, or coactivators such as Akt (Wang et al., 2005; Jenster, 1999). Activation of oncogenes and autocrine growth factor stimulation are two mechanisms which contribute to the development of complete androgen-independence. It has been reported that Akt enhances AR transactivation either directly through its interaction with AR or indirectly by regulating GSK-3β and β-catenin (Sharma et al., 2002). LNCaP are androgen dependent cells and the nuclear translocation of AR was inhibited by triterpenoids confirming that the inhibition of Akt activity blocks the activation of AR, one of the most important effectors of proliferation in this type of tumor cells.

P53 is a major regulator of cell death in response to stress involving DNA damage. The level and function of the p53 protein is mediated by mdm2 ubiquitination (Mayo and Donner, 2001). Akt regulates mdm2 by phosphorylation, which leads to mdm2 binding to p53, followed by degradation of p53. It has been shown that Akt/mdm2/p53 pathways have implications on the regulation of apoptosis in cancer cells (Gottlieb et al., 2002). An increase in the centrosome amplification, decreased p53 expression and downstream $p21^{Cip1}$ levels in breast cells (McCubrey et al., 2007) characterize drug-resistance induced by Akt. Additionally, Akt-mediated phosphorylation of AR correlates with cellular resistance to tamoxifen (Nicholson and Anderson, 2002).

LNCaP cells contrary to PC-3 express wild type p53 (Wang et al., 2004). Analysis of the DNA content of LNCaP cells treated with triterpenoids showed cell accumulation in G_2/M phase. A complex consisting of CDK1 and cyclin B regulates the progression through G_2/M phase. Akt can directly phosphorylate and inhibit the formation of this complex (Pavletich, 1999). Furthermore, it has been shown that $p21^{Cip1}$ is a direct substrate for Akt activity and that this may regulate subcellular localization of $p21^{Cip1}$ (Zhou et al., 2001). P53 also regulates cell cycle progression of tumor cells by

induction of $p21^{Cip1}$ expression (Lu et al., 2006). We have shown that triterpenoids induce inhibition of Akt activity, p53 nuclear accumulation and $p21^{Cip1}$ expression in LNCaP cells. No overexpression of $p21^{Cip1}$ was observed in p53 null PC-3 cells treated with the triterpenoids or Akt inhibitor VIII.

The mechanisms by which Akt protects cells from programmed cell death have been subject to many investigations. Akt has been shown to affect the apoptotic processes by phosphorylation of several components of the apoptotic machinery, including BAD and caspases in prostate cancer cells (Chinni and Sarkar, 2002). Several results correlate the antiapoptotic activity of Akt with a direct phosphorylation and inactivation of Bcl-2 proapoptotic members. BAD is a member of Bcl-2 family and its phosphorylation by Akt promotes cell survival (Hanada et al., 2004). BAD phosphorylation by Akt promotes its sequestration by 14-3-3 protein in the cytosol and prevents its binding to Bcl-xL, restoring the Bcl-xL antiapoptotic function (Li et al., 2005). Akt inhibition by triterpenoids resulted in dephosphorylation of BAD, caspase-3 activation and induction of apoptosis in LNCaP and PC-3 cells.

The results obtained in this study together with the published data confirm a central role of the PI3-K/Akt pathway in prostate cancer cell proliferation and survival, and they have validated the Akt pathway as a promising therapeutic target. Efforts have been made to develop small molecule kinase inhibitors targeting directly PI3K or Akt. Compounds, such as wortmannin and LY294002, which inhibit the p110 catalytic subunit of PI3-K, have been widely used in *in vitro* studies (Li et al., 2005). Recently molecular targeted anticancer therapies using either small-molecule tyrosine kinases inhibitors (TKI) or monoclonal antibodies have been introduced to treat patient with various cancers, including breast cancer, colorectal cancer, chronic myeloid leukaemia and gastrointestinal stromal tumors, but little progress has been observed in the treatment of advanced prostate cancer (Faivre et al., 2006). We have shown now that the isolated tetracyclic triterpenoids OTA, αATA, and βATA are potent inhibitors of Akt signaling in prostate cancer cells. Treatment of prostate cancer cells in *vitro* with the tetracyclic triterpenoids resulted in inhibition of several substrates of Akt, such as GSK-3β, β-catenin, androgen receptor, p65/NF-κB, c-myc, p53 and BAD, finally resulting in inhibition of proliferation and induction of apoptosis in prostate cancer cells.

The important antiproliferative and proapoptotic effects obtained in *in vitro* experiments with triterpenoids on prostate cancer cells were further verified by *in vivo* experiments. The experiments were performed in the Institute of the Pharmacology of Natural Products and Clinical Pharmacology using LNCaP and PC-3 cells xenotransplanted onto the chick chorioallantoic membranes of fertilized eggs (Syrovets et al., 2005). For the in *vivo* application of triterpenoids, water-soluble PVP microparticles were designed. The microparticles alone had no effect on growth and proliferation of LNCaP and PC-3 tumor xenografts. The PVP microparticles loaded with tetracyclic triterpenoids inhibited growth and proliferation of tumor xenografts. Immunohistochemical analysis was carried out with antigen Ki-67 as an indicator of tumor cell proliferation. Furthermore, TUNEL analysis was used to determine induction of apoptosis. These results confirmed that the isolated triterpenoids inhibit proliferation of prostate cancer cells and induce apoptosis in *vitro* as well as in *vivo*.

5. Summary

Akt are serine/threonine kinases controling cell metabolism, proliferation, and apoptosis. Akt activation is associated with aggressive behaviour of prostate cancer. Akt1 and Akt2 are the major isoforms expressed in LNCaP, PC-3 and DU 145 prostate cancer cells. Two structurally different synthetic Akt inhibitors were cytotoxic for prostate cancer cells indicating that the Akt pathway is indispensable for their viability. Oleogum resins from *Boswellia* species contain a complex mixture of triterpenoids with antitumor properties. In search for well-tolerated stable Akt inhibitors, several tetracyclic triterpenoids were isolated and purified to chemical homogeneity. 3-Oxo-tirucallic acid, 3-α-acetoxy-tirucallic acid and 3-β-acetoxy-tirucallic acid potently inhibited the activities of human recombinant Akt1 and Akt2 in *in vitro* kinase assays, and Akt immunoprecipitated from PC-3 cells, but not IκB kinase. The triterpenoids inhibited the phosphorylation of cellular Akt and glycogen synthase kinase-3β, whereas extracellular signal-regulated kinase 1/2 phosphorylation was increased. Further, the compounds inhibited nuclear accumulation of p65/relA, the androgen receptor, β-catenin, c-myc and the expression of cyclin D1, followed by hypophosphorylation of retinoblastoma protein. These events culminated in cell cycle arrest and induction of apoptosis. These results suggest that inhibition of Akt is sufficient to trigger apoptosis in prostate cancer cells. Tetracyclic triterpenoids represent a new class of Akt inhibitors with anticancer properties in human prostate cancer cells.

6. Publications

1. **Estrada A**, Syrovets T, Büchele B, Schimana-Pfeifer J, Schmidt T, Simmet Th. Inhibition of Akt by tetracyclic triterpenoids induces cell cycle arrest and apoptosis in prostate cancer cells (submitted)

2. Büchele B, Zugmaier W, **Estrada A**, Genze F, Syrovets T, Paetz C, Schneider B, Simmet T. Characterization of 3α-acetyl-11-keto-α-boswellic acid, a pentacyclic triterpenoid inducing apoptosis in vitro and in vivo. Planta Med. 2006;72:1285-9.

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Abstracts

1. **Estrada AC**, Syrovets T, Büchele B, Schmidt T, Schimana-Pfeifer J, Simmet Th (2007) Inhibition of the Akt by novel tetracyclic triterpenoids induces cell cycle arrest and triggers apoptosis in human prostate cancer cells. 14th European Cancer Conference, Barcelona, Spain, September 23-27. Eur J Cancer 5(4):315.

2. **Estrada AC**, Syrovets T, Büchele B, Schmidt T, Simmet Th (2007) Inhibition of the Akt pathway by tetracyclic triterpenoids induces cell cycle arrest and triggers apoptosis in human prostate cancer cells. 55th International Congress and Annual Meeting of the Society for Medicinal Plant Research, Graz, September 2-7. Planta Medica 73:P573.

3. **Estrada AC**, Syrovets T, Büchele B, Schmidt T, Simmet Th (2007) Down-regulation of the Akt pathway by novel tetracyclic triterpenoids induces cell cycle arrest and triggers apoptosis in human prostate cancer cells. 48th Spring Meeting of the Deutsche Gesellschaft für experimentelle und klinische Pharmakologie und Toxikologie, Mainz, March 13-15. Naunyn-Schmiedeberg's Arch Pharmacol 375 (Suppl 1):159.

4. Syrovets T, **Estrada AC**, Büchele B, Simmet T (2006) Inhibition of TCF-signaling by triterpenoids from *Boswellia Carterii* induces G_0/G_1 checkpoint arrest and apoptosis in human prostate cancer cells. Phytopharmaka und Phytotherapie 2006, Berlin, September 28-30. Z Phytotherapie; Kongressband 2006:S15.

5. Syrovets T, **Estrada AC**, Büchele B, Gschwend JE, Simmet Th (2006) Triterpenoids from tirucallic and boswellic acid families induce G_0/G_1 checkpoint arrest and apoptosis in prostate cancer cells. 97th Annual meeting of American Association for Cancer Research, Washington, DC, April 1-5. Proc Am Assoc Cancer Res 47:453, #1920.

6. **Estrada AC**, Syrovets T, Büchele B, Simmet Th (2006) Triterpenoids from the tirucallic and boswellic acids families induce G_0/G_1 checkpoint arrest and apoptosis in prostata cancers cells. 47th Spring Meeting of the Deutsche Gesellschaft für experimentelle und klinische Pharmakologie und Toxikologie, Mainz, April 4-6. Naunyn-Schmiedeberg's Arch Pharmacol 372 (Suppl 1):61.

7. Syrovets T, **Estrada AC**, Büchele B, Simmet T (2006) Inhibition of TCF-signalling by triterpenoids from *Boswellia carterii* induces G_0/G_1 checkpoint arrest and apoptosis in human prostate cancer cells. Phytopharmaka und Phytotherapie 2006, Berlin, September 28-30. Z Phytotherapie, Kongressband :S15.

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9. Annexes

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9.3 Curriculum Vitae

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