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LIST OF ORIGINAL PUBLICATIONS

The current thesis is based on the following original publications, which will be referred to by their Roman numerals in the text:

I. **Mäemets-Allas, K**., Viil, J., Jaks, V. (2015) A Novel Inhibitor of AKT1-PDPK1 Interaction Efficiently Suppresses the Activity of AKT Pathway and Restricts Tumor Growth *In Vivo*. *Mol Cancer Ther*. 14(11):2486–96

II. **Mäemets-Allas, K**., Belitškin, D., Jaks, V. (2016) The inhibition of AKT-PDPK1 interaction efficiently suppresses the growth of murine primary liver tumor cells. *Biochem Biophys Res Commun*. 474(1):118–125

III. Viil, J., Maasalu, K., **Mäemets-Allas, K**., Tamming, L., Lõhmussaar, K., Tooming, M., Ingerpuu, S., Märtson, A., Jaks, V. Laminin-rich blood vessels display activated growth factor signaling and act as the proliferation centers in Dupuytren's contracture. (2015) *Arthritis Res Ther*. 17(1):144–153

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My contribution to these articles:

REF I – designed and performed the experiments, analyzed the data and wrote the manuscript

REF II – designed and performed the experiments, analyzed the data and wrote the manuscript

REF III – designed and performed the gene expression analysis, analyzed the data, revised the manuscript

ABBREVIATIONS

ABC	ATP binding cassette
ACK1	activated CDC42 kinase 1
ACL	ATP citrate lyase
ALDH1	aldehyde dehydrogenase 1
ALT	alanine aminotransferase
APAF-1	apoptotic protease activating factor 1
AS160	AKT substrate of 160 kDa
AST	aspartate aminotransferase
ATM	ataxia telangiectasia mutant
ATRA	all-trans-retinoic-acid
BAD	Bcl-2-associated death promoter
BCL-2	B-cell lymphoma 2
Bcl-XL	B-cell lymphoma-extra large
bFGF	basic fibroblast growth factor
BH3	Bcl-2 homology domain 3
BIM	Bcl-2-like protein 11
CC3	cleaved caspase 3
CCND	cyclinD1 gene
CDK	cyclin dependent kinase
CK	cytokeratin
CK2	casein kinase 2
cMyc	cellular myelocytomatosis oncogen
CREB	cAMP response element-binding protein
CSC	cancer stem cell
CTGF	connective tissue growth factor
DC	Dupuytren contracture
DEN	diethylnitrosamine
Deptor	DEP domain-containing mTOR-interacting protein
DMSO	dimethyl sulfoxide
DNA-DSB	DNA double strand breaks
DNA-PK	DNA-dependent protein kinase
ECM	extracellular matrix
EGF	epidermal growth factor
EGFP	enhanced green fluorescent protein
ELISA	enzyme-linked immunosorbent assay
EMT	epithelial-mesenchymal transition
eNOS	endothelial nitric oxide synthase
ERK	extracellular signal–regulated kinase
EYFP	enhanced yellow fluorescent protein
FasL	Fas ligand
FOXO	Forkhead family subclass O
FP	fluorescence polarization

FRET	fluorescence energy transfer
GADD45	growth arrest and DNA damage inducible protein 45
GFP	green fluorescent protein
GLUT	glucose transporter
GPCR	G protein–coupled receptor
GSK3	glycogen synthase kinase 3
HCC	hepatocellular carcinoma
Hek293	human embryonic kidney 293 cells
HES	hairy and enhancer of split-1
HEY	hairy/enhancer-of-split related with YRPW motif protein
HF-SC	hair follicle stem cell
HGF	hepatocyte growth factor
HIF1	hypoxia-inducible factor 1
HM	hydrophobic motif
Hnf4α	hepatocyte nuclear factor alpha
ICC	intrahepatic cholangiocellular carcinoma
IGF	insulin-like growth factor
IκB	inhibitor of nuclear factor kappa-B
IKK	IκB kinase
JNK	c-Jun N-terminal kinases
LSC	leukemic stem cell
MAPK	mitogen-activated protein kinase
MDM2	mouse double minute 2
mLST8	mammalian lethal with SEC13 protein 8
MMP1	matrix metalloproteinase-1
MRCK	myotonic dystrophy-related Cdc42-binding kinases
MST	microscale thermophoresis
mTORC1/C2	mechanistic/mammalian target of rapamycin complex 1/2
NFκB	nuclear factor kappa-light-chain-enhancer of activated B cells
NO	nitric oxide
NPF	normal palmar fascia
PCA	protein complementation assay
PCNA	proliferating cell nuclear antigen
PDE	phosphodiesterase
PDGF	platelet-derived growth factor
PDPK1	phosphoinositide-dependent protein kinase 1
PGC1α	peroxisome proliferator-activated receptor γ coactivator 1
PH	pleckstrin homology
PHLPP	PH domain and leucine rich repeat protein phosphatases
PI3K	phosphatidylinositide 3-kinase
PIKfyve	FYVE-type zinc finger containing phosphoinositide kinase
PIKK	phosphatidylinositol 3-kinase-related kinases
PIP2	phosphatidylinositol (4,5)-bisphosphate
PIP3	phosphatidylinositol (3,4,5)-trisphosphate
PKA	protein kinase A

PKB	protein kinase B
PKC	protein kinase C
PKG	protein kinase G
PLA	proximity ligation assay
PLC	phospholipase C
PP2A	protein phosphatase 2A
PPI	protein-protein interaction
PRAS40	proline-rich AKT substrate of 40 kDa
PRR5	proline-rich protein 5
PTEN	phosphatase and tensin homolog
PTK6	protein tyrosine kinase 6
PUMA	p53 upregulated modulator of apoptosis
RAF	rapidly accelerated fibrosarcoma
Rheb	Ras homolog enriched in brain
Rluc	Renilla luciferase
ROCK1	Rho-associated protein kinase 1
RTK	receptor tyrosine kinase
SCF	Skp, Cullin, F-box containing complex
SGK	serum and glucocorticoid-regulated kinase
Skp2	S-phase kinase-associated protein 2
SMA	smooth muscle actin
SREBP	sterol regulatory element-binding protein
STXBP4	syntaxin binding protein 4
TGFβ	transforming growth factor beta
TM	turn motif
TSC1/2	tuberous sclerosis complex 1/2
TSP-1	thrombospondin-1
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labeling
VEGF	vascular endothelial growth factor
vWF	von Willebrand's factor
XIAP	X-linked inhibitor of apoptosis protein

INTRODUCTION

Cells are exposed to several extracellular stimuli, like hormones, cytokines and growth factors, which induce intracellular signaling pathways that ultimately regulate cell proliferation, apoptosis, differentiation, metabolism and angiogenesis. Strict balance between growth promoting and suppressing factors ensures the normal life cycle of the cell. With time the accumulating mutations and epigenetic changes can alter the signaling network, which drives the normal cell. Cancer may arise when normal cellular growth goes awry due to defects in critical signal transduction pathways. Most of the somatic mutations found in cancers, typically 40–90 per cell, are considered harmless. A smaller set of mutations occurring frequently in critical genes, like proto-oncogenes and tumor suppressors, are called "driver" mutations, which form the basis of oncogenesis. The identification of tumorigenic mutations and elucidating their impact on cell functioning has defined core signaling pathways that participate in multistep development of cancer. Protein-protein interactions (PPI) in these identified signaling cascades, which play crucial role in malignant transformation, are attractive targets for intervention with small molecular compounds. Despite of the challenges that the researchers face when designing the PPI inhibitors, the number of small molecules specifically interfering PPIs, which are crucial for the integrity of the pathway of interest, is constantly growing.

In last decades it has become apparent that PI3K/AKT signaling pathway, which has a key role in normal physiological processes, such as cell cycle progression, differentiation, survival, transcription, translation, endocytosis, motility, metabolism and autophagy, is also one of the most frequently dysregulated pathways in human tumors. Aberrantly activated AKT pathway has been commonly described in prostate, breast, liver, and colorectal carcinomas. Recently, active AKT pathway has also been linked to cancer stem cells. Moreover, the constitutive activation of the PI3K/AKT pathway confers resistance to many chemotherapeutic drugs and is a poor prognostic marker for a number of cancer types. Due to AKT involvement in critical steps of human tumor pathogenesis, targeting AKT pathway has become a promising strategy in anti-cancer therapy. Although a number of small molecule AKT kinase inhibitors have been developed, severe side effects have prevented their use in clinical trials.

The current thesis is focused on active AKT pathway in cancer cells and in Dupuytren contracture tissue. Screening, based on *Renilla* luciferase protein complementation assay (PCA), identified a small molecule compound NSC156529, that efficiently suppressed the signal transduction in PI3K/AKT pathway by interfering AKT1 and PDPK1 interaction. Since NSC156529 efficiently decreased the proliferation of different human cancer cells *in vitro* and the growth of prostate tumor xenografts *in vivo*, it could be a new promising compound for drug development to inhibit the growth of cancer cells with elevated AKT activity. We also found that the activated AKT signaling might play a role in the progression of benign hyperproliferative pathology – the Dupuytren's contracture.

LITERATURE OVERVIEW

1. AKT kinases – classification and structure

Protein kinase B or AKT (PKB/AKT) is a serine/threonine kinase, which belongs to AGC group of protein kinases (named after PK**A**, PK**G** and PK**C**), that share structural homology within their catalytic domain and have a similar mechanism of activation [1]. AKT/PKB was first described by three independent groups. Coffer *et al*. found that the catalytic domain of kinase has 65% homology with PKA and 75% homology with PKC, hence the name PKB. In addition, they demonstrated that PKB is a downstream effector of PI3K activation [2]. Jones *et al*. analyzed kinases in a similar way but named it RAC-PK (related to A and C protein kinases) [3]. Bellacosa *et al*. found PKB, or c-Akt, to be a cellular homologue of v-Akt, the gene product of AKT-8, an acute transforming retrovirus which was isolated from a rodent T cell lymphoma [4-6]. In mammals **AKT family comprises three highly homologous members** known as PKBα (AKT1), PKBβ (AKT2), and PKBγ (AKT3). AKT isoforms are encoded by different genes on chromosomes 14q32, 19q13, and 1q44, respectively, and their amino acid sequences share approximately 80% similarity [7]. Despite of a close homology, the three isoforms of AKT localize in distinct subcellular compartments and have different functions in normal cell physiology and development [8, 9]. AKT1/PKBα, which is localized in the cytoplasm, is required for whole body normal growth and mammary morphogenesis [10, 11]. AKT2 colocalizes with the mitochondria and is involved in glucose metabolism, adipogenesis and β-cell function [12]. AKT3, which is mainly found in the nucleus and nuclear membrane, is essential for the attainment of normal brain size [13]. All three AKT isoforms are expressed in a tissue-dependent manner. AKT1 is expressed ubiquitously at high levels with exception of the kidney, spleen and liver [3, 14, 15]. AKT2 expression is elevated in insulin-sensitive tissues such as fat cells, skeletal muscle and liver [16-19]. AKT3 is relatively highly expressed in the brain and testis, with low levels in skeletal muscle and liver [20].

To assess the **functions of individual AKT isoforms**, the gene expression of each of the Akt was disrupted in the mouse germ line via homologous recombination. Mice lacking individual Akt isoforms are mostly viable. In knockout mouse experiments Akt1–/– mice have placental hypotrophy, partial neonatal mortality and reduced animal size from the embryonic stages [11, 21, 22]. The exposure to genotoxic stress decreases lifespan of Akt1−/− mice [21]. Akt1 deficiency has also been related to angiogenesis and tumor development [11]. Interestingly, Akt1−/− mutants did not have a diabetic phenotype; the mice had either normal glucose tolerance and insulin-stimulated disposal of blood glucose, or an improved insulin sensitivity and enhanced energy consumption compared to wild type animals [11, 23, 24]. Akt2 –/– knockout mice have normal growth characteristics, but they have a mild to severe insulin resistance (impaired insulin action in skeletal muscle, fat and liver) [25, 26]. Akt3–/– mice

have a specific deficiency in postnatal development of the brain and 20–25% decrease in brain size, which is at least partially caused due to smaller and fewer cells [13, 27]. Akt1/Akt3 double knockout causes embryonic lethality at around embryonic days 11 and 12, with more severe developmental defects in the cardiovascular and nervous systems, apoptosis was also increased in the developing brain of double mutant embryos [28]. This indicates that Akt1 may have more important role in developing embryo than Akt3. Akt1/Akt2 double-knockout mice show severe growth deficiency and die shortly after birth. These mice display impaired skin development due to a proliferation defect, severe skeletal muscle atrophy because of a marked decrease in individual muscle cell size, and impaired bone development [29].

All AKT proteins have a similar conserved **protein structure** – an amino terminal pleckstrin homology (PH) domain, a central kinase domain and a carboxyl-terminal regulatory domain (Figure 1) [1].

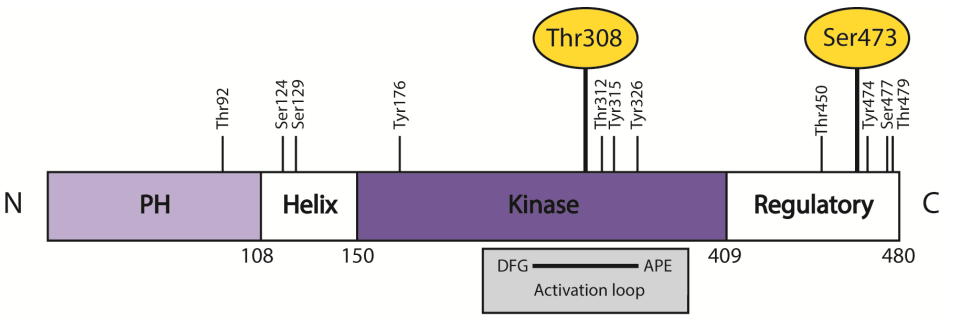

Figure 1. Schematic structure of human AKT1 protein. AKT1 protein (length 480 amino acids) contains a pleckstrin homology domain (PH), helical region (Helix), kinase domain (Kinase) and a regulatory motif (Regulatory). The two phosphorylation sites essential for complete activation (Thr308 and Ser473) and other post-translational modifications of AKT1 are indicated in the diagram. The activation loop is encompassed by DFG and APE motifs. C: carboxyl-terminal; N: amino-terminal.

AKT pleckstrin homology (PH) domain, which comprises approximately 100 amino acids in the N-terminal region of the protein, consists of seven β-strands forming two antiparallel β-sheets capped by a C-terminal α-helix. The β1–β2, β3–β4, and β6–β7 loops form a positively charged pocket that can adapt the PI3K-generated phospholipids in a complementary fashion through specific hydrogen-bonding interactions. The residues Lys14, Arg25, Tyr38, Arg48, and Arg86 form the bottom of the binding pocket and specifically interact with the 3- and 4-phophate groups of the phospholipids, while residues Thr21 and Arg23 are situated at the wall of the binding pocket and bind to the 1-phosphate group [30]. AKT PH domain interacts with membrane lipids, such as phosphatidylinositol (3,4,5)-trisphosphate (PIP3), the product of phosphatidylinositol 3-kinase (PI3-kinase, PI3K), and is believed to play significant role in recognition by upstream kinases as well as membrane translocation.

The kinase domain is located in the central region of the AKT molecule and it shares high similarity with other AGC group members, such as PKA, PKC, p70S6K and p90RSK [31]. The phosphorylation of the conserved threonine residue in the activation segment, which is required for enzymatic activation, is located in the C-lobe of the kinase domain between the DFG and APE motifs in the activation loop [31, 32].

Approximately 40 amino acid long carboxyl-terminal regulatory domain of AKT contains hydrophobic motif F-X-X-F/Y-S/T-Y/F (where X is any amino acid), which is characteristic to the AGC protein kinase family [31]. Phosphorylation of the Ser or Thr residue in this hydrophobic motif is necessary for full activation for all AGC family kinases. In mammalian AKT isoforms this motif is identical (FPQFSY) and very important, because deletion of it completely abolishes the enzymatic activity of the kinase [33]. It has been found that rat PKBγ/AKT3 and the human PKBγ-1 splice variant do not possess this motif, suggesting that these variants are activated by mechanisms independent of phosphorylation of the Ser/Thr residues in the hydrophobic motif [34, 35].

2. AKT activation and modification

2.1. AKT1 phosphorylation at Thr308 and Ser473

Full activation of AKT protein is a multistep process in which several proteins have been identified and characterized [36]. Stimulation of cells with growth factors, such as platelet derived growth factor (PDGF), insulin, epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), insulin-like growth factor I (IGF-I) and vascular endothelial growth factor (VEGF), leads to the recruitment of phosphoinositide 3-kinase (PI3K) to the plasma membrane where it phosphorylates phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P2, PIP2) to generate phosphatidylinositol (3,4,5)-trisphosphate (PtdIns(3,4,5)P3, PIP3) [37]. The major mechanism by which PIP3 exerts its physiological effect, is the interaction with proteins possessing pleckstrin homology (PH) domain [38]. AKT interaction with PIP3 via PH domain leads to a conformational change so that PH domain folds back and the phosphorylation sites become available. AKT interaction with PIP3 and recruitment to the membrane brings it to the close proximity to another PH domain containing serine/threonine kinase called phosphoinositide-dependent kinase 1 (PDK1 or PDPK1), which phosphorylates AKT1 at the site **Thr308** (Thr 309 and 305 in AKT2 and AKT3 respectively) in the activation loop of the kinase domain.

PDPK1 is one of the most conserved protein kinases found in eukaryotes, which belongs to AGC kinase family [39]. PDPK1 human gene product is a 556 amino acid long polypeptide. Folded as a globular protein, it constitutes of two domains: N-terminal serine–threonine kinase domain and a C-terminal PH domain [40]. The kinase domain can be subdivided in two lobes (a small N-terminal lobe and a large C-terminal lobe), which include two important

regulatory sites: the PIF-pocket (or PIF-binding pocket) and the activation loop (or T-loop). The phosphorylation of PDPK1 at Ser241 in T-loop is done *in trans* by another PDPK1 molecule and is essential for its kinase activity [41]. Another important structural element of the kinase domain is the αC-helix which connects the PIF-pocket with the activation loop and the bound ATP [42]. PDPK1 is mainly localized in the cytoplasm. Upon increased PI3K activity, PDPK1 moves to PIP3-enriched plasma membrane regions, which has been linked to its ability to regulate cell migration [43]. In specific situations it translocates into the nucleus with the mechanism that involves the inhibition of its Nuclear Export Sequence [44, 45].

AGC kinases, such as AKT, p90RSK, p70S6K, SGK and some PKC isoforms, carry two phosphorylation sites required for the regulation of their activity, one localized in the activation loop within the kinase domain, the other in the hydrophobic motif [46]. PDPK1 phosphorylates AGC kinases on their activation loop by two different mechanisms. By the first mechanism PDPK1 phosphorylates AKT at Thr308 in the activation loop, which is essential for its activation [40]. PDPK1 and AKT both bind to PIP3 or PIP2 produced by PI3K at the plasma membrane by their PH domain [40, 47], which co-localizes the two proteins at the plasma membrane. AKT binding to these phospholipids determines a conformational change that allows the phosphorylation of Thr308 on the activation loop by PDPK1 [48]. By the second mechanism PDPK1 phosphorylates and activates other AGC kinases, such as p70S6K [49], SGK [50], p90RSK [51] and atypical PKC isoforms [52]. In this case, PDPK1 binds the phosphorylated hydrophobic motif (HM) of these AGC kinases through its PIF-pocket, which leads to their phosphorylation on the activation loop and their full activation [53]. Moreover, PDPK1 is able to bind and activate in a kinase independent manner some proteins belonging to AGC kinase family, such as ROCK1 [54] and MRCKα [43], or proteins not belonging to this family, such as PLCγ1 [55] and β3 integrin [56, 57].

Since PDPK1 knock-out mice die during the embryonic development, this protein is considered to be strictly required for survival of all eukaryotes. The main role of PDPK1 is to be a signal transducer in signaling pathways activated by several growth factors and hormones. PDPK1 is involved in glucose metabolism by promoting glucose storage [58], development [59], blood vessel formation [60], and neuron differentiation [61]. Moreover, alterations of PDPK1 functions have also a relevant role in pathology such as Alzheimer's disease [62], diabetes [63] and cancer [64, 65].

For full activation, AKT needs to be phosphorylated at **Ser473** in the hydrophobic motif (Ser474 and Ser472 in AKT2 and AKT3 respectively) [66]. Phosphorylation of Ser473 is believed to be the key step in the activation of AKT because it stabilizes the active conformation state [67]. Molecular identity of the kinase phosphorylating AKT at Ser473 (referred also as „PDK2") has been under debate for many years. Proposed candidates include PDPK1 [68], integrin-linked kinase (ILK) [69], AKT itself [70], mitogen activated protein kinase activated protein kinase 2 (MAPKAPK2) [32], protein kinase C II

(PKCII) [71] and the members of the atypical PI 3-kinase related protein kinase (PIKK) family: DNA-dependent protein kinase (DNA-PK) [72] and ataxia telangiectasia mutant (ATM) [73]. Currently it is accepted that the main candidate for this action is the mechanistic/mammalian target of rapamycin complex 2 (mTORC2).

mTOR is a serine/threonine kinase that mediates signals from nutrients, stress and growth factors and thereby participates in cell metabolism, proliferation, autophagy and migration regulation [74-76]. mTOR is a member of a family of protein kinases termed the PIKKs (phosphatidylinositol-3-kinase-related kinases) and functions as serine/threonine protein kinase. As the name implies, mTOR is a specific target of the natural compound rapamycin. mTOR interacts with several proteins and forms two complexes named mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). The conserved components of mTORC2 are mTOR, Rictor (mAVO3), SIN1 and mLST8 (GβL). Other proteins found to be associated with mTORC2, such as PRR5/Protor, PRR5L and DEPTOR. Rictor and mSIN1 stabilize each other and are believed to create the basis for mTORC2 complex [77, 78]. mLST8 is essential to mTORC2 stability and activity [79]. Deptor is the negative regulator of mTORC2 activity [80].

mTORC2 phosphorylates AKT at Thr450 of the turn motif (TM) and Ser473 of the hydrophobic motif (HM) [66, 81]. Thr450 phosphorylation is a post-translational modification and occurs upon recruitment of AKT to the membrane via affinity of its PH domain to membrane lipids. Phosphorylation at Ser473 is induced by such stimuli as growth factors and hormones and it leads to increased activity of AKT. Ser473 phosphorylation may be related to substrate specificity, since AKT phosphorylation by mTORC2 at Ser473 has influence on Forkhead subclass O family of transcription factors (FOXO1/2) phosphorylation, but not on other substrates like GSK3 (glycogen synthase kinase 3) and TSC2 (tuberous sclerosis complex 2) [79]. Phosphorylation at Ser473 has been shown to be elevated in a number of cancers, indicating that the regulation of AKT by mTORC2 could play a significant role in cancer progression.

2.2. Alternative AKT modifications

Although, current evidence suggests that phosphorylation at Thr308 and Ser473 are required for full activation of AKT1 protein, there is information about other post-translational modifications, which influence AKT activity, subcellular localization, protein stability, protein folding etc. (Figure 1) [82].

Thr92 phosphorylation indicates to proper folding of AKT protein, whereas single mutation only marginally affects expression and activation of this kinase, but double mutation of Thr92 and Thr450 residues makes AKT unstable [83]. **Ser124** high phosphorylation level has been detected in different cell lines [32]. According to one hypothesis phosphorylation of AKT at Ser124 renders the protein responsive to growth factor-induced and PI3K-dependent activation [84], but so far, there is no evidence of identified regulatory stimulus or kinase

involved with this event. **Ser129** phosphorylation *in vitro* and *in vivo* by casein kinase (CK2) is associated with an increase in the catalytic activity of AKT, which is supported by the fact that down-regulation of CK2 catalytic subunit or a mutation of the CK2 target site in AKT correlates with decreased AKT activity [85]. **Thr312** is believed to have an inhibitory effect on AKT activation. GSK3α phosphorylates AKT within the substrate-binding site of the protein and this modification is considered to interfere with the interaction of AKT and its substrates preventing AKT kinase function [86]. Since mutation of **Thr450** only slightly inhibits the activation of AKT by growth factors [87], Thr450 is indicated as constitutively phosphorylated site, which controls AKT protein folding and maturation and has been proposed to be the first step in AKT activation [81, 88, 89]. In addition, constitutive phosphorylation of Thr450 inhibits co-translational ubiquitination and subsequent degradation of AKT protein [89]. Very recently it was found that AKT activity fluctuation during the cell cycle is in correlation with cyclinA2 expression pattern. Further investigation revealed cyclinA2 phosphorylation sites at the very end of the C-terminal domain of AKT protein – residues **Ser477** and **Thr479**. Phosphorylation of these two residues is proposed to trigger AKT1 activation either through enhancing its association with mTORC2 to promote phosphorylation of Ser473 or by functionally compensating the absence of pSer473 to lock Akt1 in its active conformation. In breast cancer samples as well as in breast-cancer-derived cell lines they found a positive correlation between the phosphorylation of AKT1 at Ser477/Thr479 and Ser473. These results indicate that a physiological role of cyclinA2 is to promote pro-survival and oncogenic properties of AKT1 protein [90]. Evolutionarily conserved **Tyr176** can be directly phosphorylated by non-receptor tyrosine kinase ACK1 (also known as ACK or TNK2), which causes PIP3-independent recruitment of AKT to the plasma membrane and subsequent phosphorylation of Thr308/Ser473 leading to AKT activation. In addition, activated ACK1 expression in the prostate of transgenic mice specifically induces phosphorylation of AKT at Tyr176, which promotes the development of murine prostatic intraepithelial neoplasia (mPINs). Positive correlation between activated ACK1 and Tyr176 phosphorylation in breast tumor samples was also found to be related with poor prognosis of breast cancer patient survival [91]. Chen *et al*. found that AKT is a direct substrate of Src both *in vitro* and *in vivo* and that Tyr176 phosphorylation of AKT is critical for its full activation by growth factors.

Two identified residues near the activation loop of AKT, **Tyr315** and **Tyr326,** are found to be indispensable for AKT activation. Replacing these residues with phenylalanine blocked AKT kinase activity upon growth factor stimulation [92]. Non-myristylated Src-related intracellular tyrosine kinase protein tyrosine kinase 6 (PTK6) is also capable of phosphorylating AKT at Tyr315 and Tyr326 [93]. Conus *et al*. showed that AKT activation by pervanadate or serum is associated with **Tyr474** phosphorylation on AKT. Tyr474 substitution with phenylalanine abolished its phosphorylation and resulted in up

to 55% inhibition of AKT activation, indicating that phosphorylation at Tyr474 is required for full activation of the kinase [94].

AKT inhibition. Among the many molecules that stimulate AKT are also these that inhibit AKT activity. Phosphatase and tensin homologue deleted chromosome 10 (**PTEN**) is a tumor suppressor, which has been frequently mutated in a variety of solid tumors [95]. This lipid phosphatase inhibits PI3K/AKT signaling indirectly by catalyzing the conversion of PIP3 into PIP2 (Figure 2) [96]. PTEN is also involved in DNA-DSB repair following genotoxic stress such as exposure to ionizing radiation [97]. Protein phosphatases such as **PHLPP**s (PH-domain leucine-rich repeat protein phosphatases) and **PP2A** (protein phosphatase 2A) have a direct effect on AKT activity. PHLPP dephosphorylates Ser473 and thereby inactivates AKT proteins. Two PHLPP isoforms have distinct impact on AKT proteins: PHLPP2 dephosphorylates AKT1 and PHLPP1 dephosphorylates AKT2 and AKT3 [98]. Indicated as tumor suppressor, PHLPP expression is commonly lost in human cancers [99]. PP2A preferentially dephosphorylates AKT at Thr308 [100].

3. AKT signaling pathway

PI3K/AKT pathway is initiated by various growth factors, hormones and cytokines, such as insulin, insulin like growth factor (IGF), epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF) and platelet derived growth factor (PDGF). These factors bind to receptor tyrosine kinases (RTKs), cytokine receptors or GPCRs and trigger the activation of the lipid kinase PI3K. Activated PI3K phosphorylates PIP2 to produce PIP3, which recruits AKT to the cell membrane where it is phosphorylated at Thr308 and Ser473. Once activated, AKT migrates to the cytosol, mitochondria and nucleus, phosphorylates numerous substrates throughout the cell and thereby regulates multiple cellular events and processes like cell growth, survival, differentiation and metabolism (Figure 2) [101, 102].

Up to now, more than 100 proteins have been identified as potential AKT targets, including caspase-9, $p27^{Kip1}$, $p21^{Cip1/WAF1}$, glycogen synthase kinase 3 (GSK3) cAMP response element-binding protein (CREB), murine double minute 2 (MDM2), BAD, proline-rich AKT substrate 40 (PRAS40), Forkhead family subclass O (FOXO) transcription factors etc. [54, 101, 102]. Most of the AKT substrates contain the minimal consensus sequence RxRxx(S/T), where x is any amino acid and S/T is the Ser/Thr phosphorylation site [103]. Many targets of the pathway modulated by AKT are also subjects for regulation by other pathways, further highlighting the extreme complexity of AKT signaling regulation [104–106].

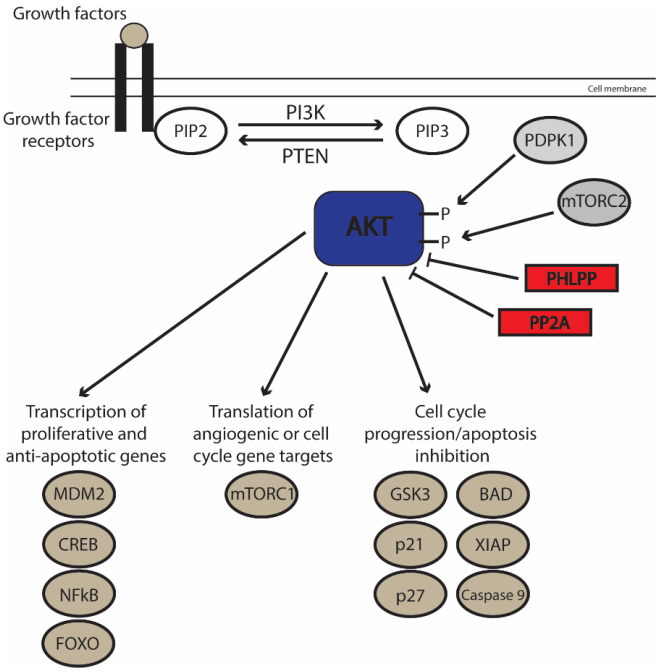

Figure 2. Schematic representation of the PI3K/AKT pathway. Growth factor receptor activated PI3K phosphorylates PIP2 and produces PIP3, which recruits AKT to the cell membrane where it is phosphorylated by PDPK1 and mTORC2. Phosphorylated AKT regulates a plethora of downstream cellular processes from cell survival, angiogenesis, proliferation, translation and metabolism. AKT is dephosphorylated by PHLPP and PP2A.

AKT signaling specificity. There is a large body of evidence about the diverse functions of AKT isoforms, but the mechanisms by which AKT isoform-specificity is conferred are largely unknown. Selectivity might at least in part be reached through tissue-specific expression, spatial segregation at distinct subcellular locations and phosphorylation of isoform-specific substrates [103]. Since the subcellular localization of AKT isoforms in cancer cells is different [104], the current model of AKT activation by PI3K/PDPK1/mTORC2 might only be applicable to AKT1.

3.1. AKT regulates cell survival and apoptosis

AKT promotes cell survival by inhibiting the functions of pro-apoptotic proteins and apoptosis related processes, or activates transcription factors, which regulate the expression of genes with anti-apoptotic activity (Figure 3).

AKT inhibits the functions or expression of several Bcl-2 homology domain 3 (BH3)-only proteins, which exert their proapoptotic effects by binding to and inactivating prosurvival Bcl-2 family members. AKT can directly phosphorylate

pro-apoptotic Bcl-2-antagonist of death (**BAD**), BAX and BIM [105-108]. Unphosphorylated BAD interacts with Bcl-XL, which leads to a release of cytochrome c from the mitochondrial compartment into the cytoplasm where it binds to Apaf1 thereby activating apoptotic proteases called caspases [109]. Phospho-serine residues Ser112 (phosphorylated by Raf1) and Ser136 (phosphorylated by AKT) on BAD form high-affinity binding sites for cytoplasmic 14-3-3 molecules, which prevent BAD binding with BclXL [110, 111]. Ser112 and Ser136 residues on BAD can also be phosphorylated by p21-activated kinase 1 (Pak) [112]. Pak activation is triggered by oncogenic Ras stimulation and subsequent AKT activation, which means that AKT can regulate BAD also indirectly [113].

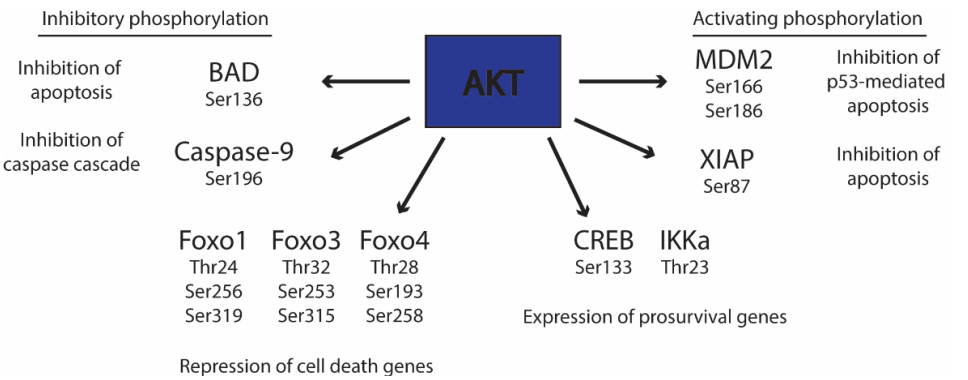

Figure 3. The substrates of AKT involved in blocking apoptosis and promoting cell survival.

AKT regulates positively some transcription factors to allow expression of pro-survival genes. Cell survival promoting cAMP response element binding protein (**CREB**) is phosphorylated by AKT at Ser133, which stimulates recruitment of CREB-binding protein (CBP) to the promoter of target genes, such as Bcl-2 [114]. AKT can phosphorylate and activate the IκB kinase IKKα at Thr23, causing degradation of IκB and nuclear translocation of **NF-κB** where it promotes expression of caspase inhibitors, c-Myb and Bcl-XL [115]. AKT also inhibits the expression of BH3-only proteins through effects on **FOXO** transcription factors [116]. AKT phosphorylates FOXO1 at Thr24, Ser256, Ser319 and FOXO3a and FOXO4 at three equivalent sites in the nucleus [117]. Phosphorylation of FOXO proteins by AKT triggers the rapid relocalization of FOXOs from the nucleus to the cytoplasm. Phosphorylated Thr24 and Ser256 are binding sites for 14-3-3 proteins, which masks FOXO nuclear localization signal (NLS) and causes FOXO transcription factor translocation to the cytoplasm [118]. By this mechanism AKT blocks FOXO-mediated transcription of target genes that promote apoptosis, cell-cycle arrest, and metabolic processes, such as BIM [119] and proapoptotic cytokine Fas ligand (FasL) [120]. Down-

stream of cytochrome c release AKT can phosphorylate **caspase 9** at Ser196 in human cells, which leads to an inhibition of caspase activity through an unknown mechanism [121]. AKT can interact and phosphorylate X-linked inhibitor of apoptosis protein (**XIAP**) at residue Ser87 *in vitro* and *in vivo*. Phosphorylation of XIAP by AKT prevents XIAP ubiquitination and degradation in response to cisplatin and also XIAP autoubiquitination [122]. By stabilizing XIAP, AKT promotes cell survival.

3.2. AKT regulates cell cycle progression and proliferation

AKT kinase modulates the activity, localization and functions of several substrates related to cell cycle progression at the G1/S and G2/M transitions, either by directly phosphorylating the target proteins or indirectly regulating their expression levels (Figure 4). Growth-factor-mediated AKT activation causes cells to exit G0 via directly increasing the transcription of proto-oncogene **c-Myc** (cellular myelocytomatosis oncogen), which executes its multiple activities mostly through transcriptional regulation of the target genes [123]. c-Myc induces the expression of D-type cyclins (cyclinD) and inhibits the expression of negative cell cycle regulators such as $p21^{Cip1/WAF1}$, $p27^{Kip1}$ and p15INK4b [124-126]. Indirectly AKT controls the stability of c-Myc and cyclinD1 via its downstream substrate GSK3β. c-Myc phosphorylation at Thr58 and cyclinD1 phosphorylation at Thr286 by GSK3β is required for ubiquitin-dependent proteolysis of these proteins [127, 128].

$p21^{Cip1/WAF1}$ is a critical regulator of cell cycle progression and cell survival. $p21^{Cip1/WAF1}$ inhibits the kinase activity of the cyclin-dependent kinases (CDKs), leading to cell growth arrest at specific stages in the cell cycle. $p21^{Cip1/WAF1}$ binding to proliferating cell nuclear antigen (**PCNA**) interferes PCNA-dependent DNA polymerase activity and thereby inhibits DNA replication and modulates various PCNA-dependent DNA repair processes [129]. Elevated $p21^{Cip1/WAF1}$ protein levels have been observed in various aggressive tumors and it has been linked to enhanced survival and chemoresistance [27]. $p21^{Cip1/WAF1}$ is reported to be a direct substrate of AKT [130]. AKT phosphorylation of $p21^{Cip1/WAF1}$ at Thr145 in the nuclear-localization signal (NLS) leads to the cytoplasmic localization and suppression of the inhibitory effect of $p21^{Cip1/WAF1}$ on cell cycle progression [130]. In addition, AKT-dependent phosphorylation of $p21^{Cip1/WAF1}$ at Thr145 prevents the formation of DNA replication inhibiting complex between $p21^{Cip1/WAF1}$ and PCNA, and decreases the binding of the cyclin-dependent kinases CDK2 and CDK4 to $p21^{Cip1/WAF1}$ [131]. Alternatively, it has been found that AKT phosphorylation of $p21^{Cip1/WAF1}$ at Ser146 significantly increases $p21^{Cip1/WAF1}$ protein stability and thereby modulates $p21^{Cip1/WAF1}$ protein level [132]. AKT induced $p21^{Cip1/WAF1}$ protein can promote the assembly and activation of cyclinD1-CDK4 complex, which controls G1 to S phase progression and enhances cell cycling [132]. These data suggest that $p21^{Cip1/WAF1}$

protein stabilization is an additional mechanism, by which AKT regulates tumor cell survival and/or proliferation.

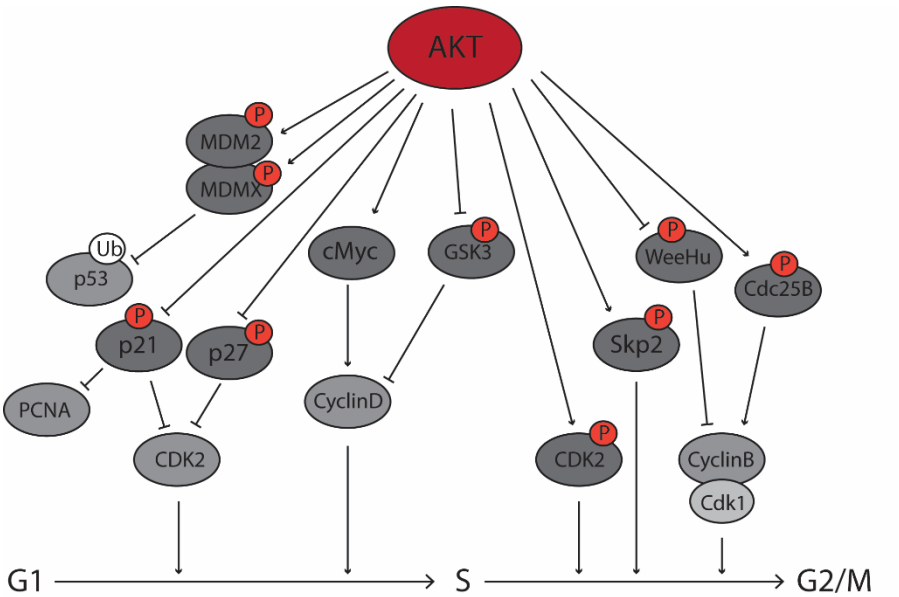

Figure 4. AKT role in cell cycle progression. Activated AKT kinase regulates the function of numerous proteins involved in cell cycle progression, either by direct phosphorylation of the target proteins or indirectly, by controlling protein expression levels.

AKT controls the expression, localization, and protein stability of another major cyclin/CDK inhibitor - **p27^{Kip1}**. AKT directly binds to and phosphorylates p27^{Kip1} at several residues, such as Ser10, Thr157, Thr187 and Thr198 [133-137]. Thr157 phosphorylation within the nuclear localization sequence (NLS) leads to cytoplasmic accumulation of p27^{Kip1}. In the cytoplasm p27^{Kip1} is not able to bind and inhibit nuclear cyclinE/CDK2 kinase activity and thus halting cell cycle progression. p27^{Kip1} phosphorylation by AKT at Thr198 promotes 14-3-3 binding and cytoplasmic localization [138]. Cytoplasmic localization of p27^{Kip1} has been observed in various human tumors [139, 140]. In up to 40% of primary human breast cancers, cytoplasmic p27^{Kip1} correlates with AKT activation, indicating that AKT-mediated cytoplasmic mislocalization of p27^{Kip1} may be critical in the development of human cancers. In addition, AKT can downregulate p27^{Kip1} transcription by phosphorylation-dependent inhibition of the FOXO transcription factors [141]. Protein stability of p27^{Kip1} is controlled by AKT-dependent phosphorylation of S-phase kinase-associated protein 2 (**Skp2**) [142, 143]. Skp2 is a key component of SCF (Skp1/Cul-1/F-box) E3 ubiquitin ligase, which binds to p27^{Kip1} and targets it for ubiquitination and degradation [144, 145].

The tumor suppressor p53 binds to DNA and up-regulates the expression of several genes involved in cell cycle control, DNA repair, senescence, angiogenesis, and apoptosis [146-148]. In normal cells p53 is maintained at low levels by **MDM2,** which targets p53 for ubiquitination, translocation from the nucleus to the cytoplasm and proteolysis [149, 150]. By growth factor stimulation activated AKT binds to and phosphorylates MDM2 at Ser166 and Ser186, which is necessary for the translocation of MDM2 from the cytoplasm to the nucleus, to enhance protein stability and facilitate the function of MDM2 to promote p53 ubiquitination [151, 152]. MDMX, which is a MDM2 homologue, has also been shown to be an AKT target. MDMX alone does not have E3 ubiquitin ligase activity, but it binds to and stabilizes MDM2 thereby promoting p53 ubiquitination [153]. AKT phosphorylation of MDMX at Ser367 generates a 14-3-3 binding site and leads to the stabilization of the MDM2-MDMX complex [154]. Through MDM2 phosphorylation and activation AKT has impact also on transcriptional targets of p53, like BH3-only proteins PUMA (p53 upregulated modulator of apoptosis) and Noxa, which appear to be essential for p53-induced apoptosis [155].

AKT regulates the phosphorylation of cyclin-dependent kinase 2 (**CDK2**), which is a key regulator of G1-S cell cycle progression [156]. AKT phosphorylation of CDK2 at Thr39 in the nucleus causes temporary localization of cyclinA/CDK2 complex into the cytoplasm, which is required for cell cycle progression from S to G2/M phase [157]. Alternatively, AKT-mediated phosphorylation and cytoplasmic translocation of CDK2 is also important for stress induced apoptosis [158]. Phosphatase Cdc25B has an essential role in control of cyclinB/CDK1 activity [159] The function of Cdc25B is tightly linked to its intracellular localization [160, 161]. AKT-mediated Ser353 phosphorylation causes the cytoplasmic accumulation and inactivation of Cdc25B by avoiding the access to its substrates [162]. WeeHu, a CDK1 inhibitor, is also an AKT target. AKT phosphorylates WeeHu at Ser642, which does not affect its kinase activity, however, it promotes the interaction of WeeHu with 14-3-3 protein and translocation into the cytoplasm [163].

3.3. AKT regulates cell growth and metabolism

In response to growth factors, AKT also regulates nutrient uptake and cell metabolism. One of the most important physiological functions of AKT is to regulate the glucose uptake in response to insulin. AKT activity increases the transcription of the glucose transporters **GLUT1** and the translocation of **GLUT4** to the plasma membrane in muscle and fat cells, which increases the uptake of glucose into cells [164, 165]. Other AKT substrates that may regulate cell metabolism by mediating the regulation of GLUT4 translocation include AKT substrate of 160 kDa (**AS160**), phosphoinositide kinase for five position containing a Fyve finger (**PIKfyve**) and syntaxin binding protein 4 (**STXBP4/synip**) [166-168]. After entering into the cell, glucose is converted

by hexokinase to glucose-6-phosphate. AKT stimulates the association of hexokinase with mitochondria, where it can access its glucose substrate more readily, but the direct target of AKT is currently unknown [169, 170]. Next, glucose 6-phosphate is stored by conversion to glycogen or catabolized to produce cellular energy through glycolysis. AKT regulates glycogen synthesis and suppression of glycogenolysis through the AKT-dependent phosphorylation and inactivation of **GSK3**. Nonphosphorylated GSK3 is catalytically active and inhibits the activity of glycogen synthase [171]. Activated AKT phosphorylates and inhibits GSK3 and prevents GSK3 from phosphorylating and inhibiting glycogen synthase, thereby stimulating glycogen synthesis. AKT can regulate glucose homeostasis also by phosphorylation and inhibition of **FOXO1**, as FOXO1 promotes hepatic glucose production and regulates the differentiation of cells involved in metabolic control [172]. In hepatocytes, AKT can inhibit gluconeogenesis and fatty acid oxidation through direct phosphorylation of peroxisome proliferator-activated receptor γ (PPARγ) coactivator 1 α (**PGC-1α**) at Ser570, which is a coactivator that can regulate genes with FOXO1 and other transcription factors [173]. AKT activation also increases the rate of glycolysis, which is common event seen in tumor cells [174]. AKT ability to enhance the rate of glycolysis is at least partly related to its ability to promote the expression of glycolytic enzymes through hypoxia-inducible factor 1-alpha (**HIF1α**) [175, 176]. AKT-dependent phosphorylation and inhibition of GSK3 also regulates lipid metabolism. Phosphorylation of substrates by GSK3 often targets them for proteasomal degradation. GSK3 has been shown to promote degradation of the sterol regulatory element-binding proteins (**SREBP**s), which are transcription factors that turn on the expression of genes involved in cholesterol and fatty acid biosynthesis. AKT-mediated inhibition of GSK3 promotes SREBP stability and enhances lipid production [177]. AKT can also phosphorylate Ser454 on **ATP citrate lyase** (ACL), indicating a potential role in regulating ACL activity and fatty acid synthesis [178]. In addition, AKT activates the 3B isoform of cyclic nucleotide phosphodiesterase (**PDE**) via phosphorylation at Ser273, resulting in reduced cyclic-AMP levels and inhibition of lipolysis [179].

Promoting the cell growth (including an increase in cell mass) is one of the conserved functions of AKT. The predominant mechanism of AKT-dependent cell growth involves **mTORC1** (mTOR complex 1 or the mTOR-raptor complex), which is activated by nutrients or growth factors. mTORC1 has a critical role in cell growth control, translation initiation and ribosome biogenesis [180]. mTORC1 is composed of mTOR, raptor (regulatory associated protein of mTOR), mLST8 (also called G-protein-subunit-like protein, GL), PRAS40 and deptor (death domain containing mTOR interacting protein) (Figure 5) [181]. mTORC1 regulates translation by phosphorylating its various downstream effectors from which the best characterized are p70 ribosomal protein S6 kinase 1 (p70S6K1) and eukaryotic translation initiation factor 4E-binding protein 1 (EIF4E-BP1). Ras homolog enriched in brain (Rheb) GTPase directly binds to the catalytic domain of mTOR and acts as a positive regulator of mTORC1 kinase activity [182]. TSC1 and TSC2 proteins form *in vivo* a complex, which

negatively regulates mTORC1 by acting as Rheb GAP (GTPase activation protein) that converts Rheb into an inactive GDP bound form [183]. In response to growth factors, AKT directly phosphorylates TSC2 on several distinct residues (Ser939 and Thr1462), which prevents the formation of TSC1-TSC2 complex, thus allowing GTPase Rheb to convert back into the GTP-bound active state leading to mTORC1 activation (Figure 5) [184].

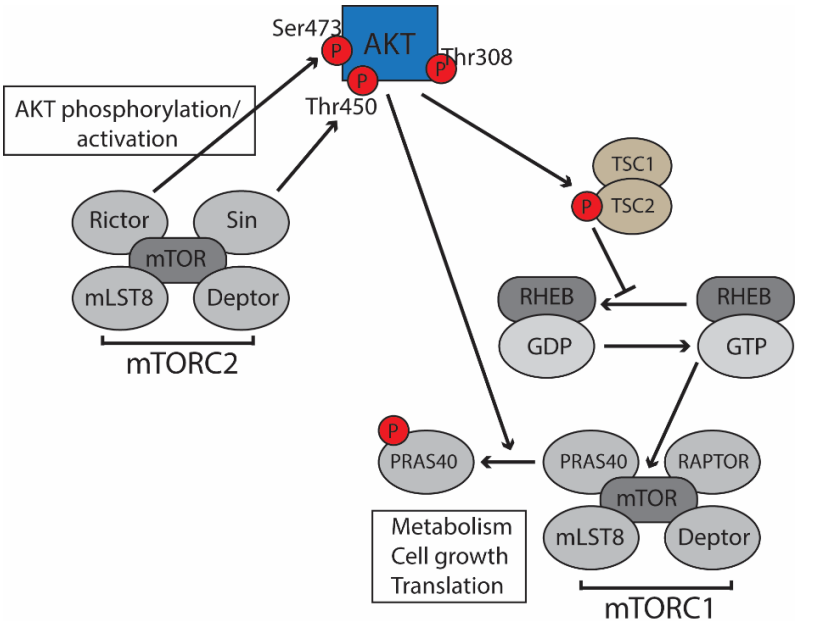

Figure 5. PI3K–AKT signaling activates mTOR complex 1 (mTORC1) through the phosphorylation of TSC2 at position Ser1462. This phosphorylation event results in the dissociation of the TSC1–TSC2 complex and thus prevents TSC2 from stimulating the intrinsic GTPase activity of the small G protein RHEB, resulting in the accumulation of GTP-bound RHEB, which is a positive regulator of mTORC1. AKT also phosphorylates 40 kDa proline-rich AKT1 substrate PRAS40 at position Thr246, resulting in its dissociation from mTOR and thus blocking PRAS40-mediated mTORC1 inhibition. mTOR complex 2 (mTORC2) phosphorylates AKT at Ser473 and Thr450.

Thus PI3K/AKT signaling pathway regulates mTORC1 by phosphorylation and inhibition of TSC2 which impairs its ability to inhibit Rheb, thereby resulting in the subsequent activation of Rheb and mTORC1. AKT activation by growth factors can stimulate mTORC1 also in a TSC1/2-independent manner through proline-rich AKT substrate 40 kDa (PRAS40). Unphosphorylated PRAS40 binds raptor and inhibits mTOR kinase activity. PRAS40 phosphorylation by AKT at Thr246 promotes the dissociation of PRAS40 from mTORC1 and subsequent mTORC1 activation [182, 185].

3.4. AKT regulates angiogenesis

Angiogenesis is essential in many normal physiological processes, including embryogenesis, wound healing, tissue repair and organ regeneration, but it also contributes to several pathological conditions, like rheumatoid arthritis, tumor progression and metastasis. AKT participates in both physiological and pathological angiogenesis through promoting angiogenic signal production and regulating the response of endothelial cells to these stimuli. Under physiological conditions, angiogenesis is tightly regulated through a local balance between angiogenic stimulators, such as growth factors VEGF, bFGF, TGFβ and PDGF, and angiogenic inhibitors like thrombospondin-1 (TSP-1), tissue inhibitor of matrix metalloproteinase-1 (MMP-1), angiostatin and endostatin. The formation of new blood vessels in pathological disorders is often due to either a decrease in levels of inhibitors, an increase in levels of stimulators or a combination of both [186].

In endothelial cells PI3K/AKT pathway is activated by vascular endothelial growth factor (VEGF), which thereby promotes the survival, growth and proliferation of these cells [187]. One of the major stimuli for increased VEGF production by tumor cells is hypoxia, which is characteristic to advanced solid tumors. Hypoxia leads to the induction of hypoxia-inducible factor 1 (HIF-1) [188]. HIF-1 is a heterodimeric transcriptional factor, which is composed of HIF-1α and HIF-1ß subunits. Under hypoxic conditions the ubiquitin-mediated degradation of HIF-1α is inhibited and stabilized α subunit binds to the β subunit, which is constitutively present. Composed heterodimer binds to numerous promoters containing hypoxia response elements (HREs), leading to the transcription of multiple target genes including VEGF [189]. In addition, HIF-1α production and VEGF expression can be increased by the activation of EGFR/PI3K/AKT/mTOR pathway and PTEN mutation [190]. HIF-1α activation in endothelial and other surrounding cells leads to elevated expression and secretion of VEGF, which therefore stimulates angiogenesis through autocrine and paracrine manner.

AKT regulates angiogenesis through the activation of endothelial nitric oxide synthase (eNOS), which catalyzes nitric oxide (NO) synthesis in endothelial cells. AKT phosphorylates eNOS at Ser1177, by leading to a persistent, calcium-independent eNOS activation [191]. The activity of eNOS is also regulated by subcellular localization and protein-protein interactions. eNOS can be localized in a specific domain of plasma membrane called caveolae, where it interacts with caveolin-1 through caveolin-1 scaffolding domain, which inhibits eNOS activity. Stimulation with VEGF or stress induces eNOS to interact with heat shock protein 90 (Hsp90), and this interaction enhances eNOS activity. Remarkably, on stimulation, AKT can also interact with Hsp90 and this interaction enhances AKT enzymatic activity, which suggests that Hsp90 may serve as a scaffold protein for the efficient phosphorylation of eNOS by AKT at caveolae [192].

Elevated levels of VEGF and subsequent eNOS activity can lead to vascular permeability, leaky vessels, slow blood flow, and elevated interstitial pressure.

Constitutive activation of the ERK/MAPK pathway could induce angiogenesis but not vascular permeability, while activation of PI3K/AKT was required for both angiogenesis and vascular permeability [193]. In animal studies over-expression of constitutively active AKT in the vascular endothelium increased resting diameter and blood flow, whereas transduction of dominant-negative AKT attenuated endothelium-dependent vasodilation induced by acetylcholine, suggesting that AKT regulates the vasomotor tone in vivo [194].

4. AKT and cancer

4.1. AKT signaling in cancer

Since AKT kinases control essential cellular processes, it is inevitable that the dysregulation of AKT signaling pathway leads to the loss of the strict balance between AKT-regulated tumor suppressor factor activities and proto-oncogenes, which yields severe consequences. Less active or defective AKT-dependent pathway does not provide an efficient protection from apoptotic signals, which may contribute to the pathogenesis and/or progression of several human disorders, such as type-2 diabetes, neurodegenerative diseases or illnesses of the cardiovascular system [195-200]. The overexpression and/or constitutively increased activity of the AKT-regulated pathway has been observed in a wide variety of human cancers, including carcinomas, *glioblastoma multiforme* and various hematological malignancies [201, 202]. Elevated AKT activity is usually not a result of any single event, but rather a consequence of multiple causes, which lead to AKT-dependent suppression of apoptosis, differentiation and to uncontrolled cell cycle progression. The AKT activity may be caused of increased amounts of extracellular factors that stimulate AKT signaling, altered number of receptors, which mediate these signals, constitutively activating mutations in these receptors as well as deregulation of intracellular AKT-activating mediators, like Ras or PI3K and their regulators [203]. The control mechanisms that regulate AKT activity can be downregulated or deleted; for example, tumor suppressor protein PTEN has been found mutated or deleted in many advanced cancers [204, 205]. The elevated expression of AKT isoforms has been described in some cancer types, like pancreatic, prostate and mammary cancer [20, 206-208]. Amplification of AKT1 is rather rare, but amplification of AKT2, found in several cancer types, is more common [206, 209, 210]. AKT3 amplification has recently been reported to promote DNA repair and glioma progression [211]. Carpten *et al*. described a transforming point-mutation E17K (lysine substituted with glutamic acid at position 17) within the PH domain of AKT1 in human breast, ovarian and colorectal cancer, which increases the plasma membrane recruitment and confers to the constitutively active state of the protein [212]. Analogous point-mutation in the AKT3 has been found in human melanoma [213]. AKT hyperactivation has frequently been found in less differentiated tumors, which are more invasive, grow faster and do not respond

to treatment. In addition, hyperactivated AKT in primary tumors is considered as poor prognostic marker for disease outcome [214].

Considering the central role in cell signaling and frequent aberrant regulation in human cancers, AKT has become an attractive target in anti-cancer drug design. A better understanding of upstream regulators and downstream targets is necessary to control and regulate activity of AKT-related pathway in human malignancies. However, it has to be remembered that AKT signaling pathway is not only multifaceted and complicated but it is a part of a more complex and integrated network. To date, direct or indirect communication of AKT pathway with RAF-MEK1/2-ERK1/2, NFkB, JNK, p38, Wnt, p53 and NOTCH pathways has been described, which means that activation or inhibition of one of these pathways may have a strong impact on other signaling pathways as well [152, 215-219].

4.2. AKT and cancer stem cells

Since PI3K/AKT/mTOR pathway is implicated in resistance mechanisms to antineoplastic therapies, it is rapidly emerging as a signaling network important for cancer stem cell (CSC) survival. CSC have suggested to be immortal tumor-initiating cells that have self-renewal and pluripotent capacity [220]. CSCs have been identified in multiple malignancies, including leukemia and various solid cancers, such as lung cancer, colon cancer, prostate cancer, ovarian cancer, brain cancer and melanoma. CSCs are thought to be responsible for tumor initiation, development, metastasis and recurrence, and to be resistant to conventional anti-tumor treatment, like polychemotherapy, which only acts on more mature cells. The reason could be the different regulation of gene expression and some signaling pathways compared to other tumor cells, like the elevated expression of ATP-binding cassette (ABC) family membrane transporters [221], or the fact that CSCs have low proliferation rate [222, 223]. Cell surface proteins CD133, CD24 and CD44 are considered as putative markers for cancer stem cell populations, associated with aggressive cancer types and poor prognosis. However, distribution of these markers differs between patients and cell lines [224]. In contrast, tumor initiating cells from head-and-neck and breast cancer have been shown to be CD24-negative [225].

Probably due to different experimental models and methods, the published findings regarding the role of AKT in promoting cancer stem-cells are controversial. AKT co-expression with CD133 has been shown to provide the cells resistance to chemotherapeutics [226]. Whereas AKT activation has been found both negatively as well as positively correlated with CD44 expression [227, 228]. Radiation itself can increase the expression of AKT, CD133, and reduce the expression of CD44 in colorectal cancer cells [229]. The study with colon cancer cell lines showed that cells highly expressing CD133/CD44 have elevated expression of AKT and these cells are more resistant to radiation. In addition, AKT isoforms had different influence on CD44 and CD133 expres-

sion. Knocking down AKT1 but not AKT2 in colon cancer cell line, reduced CD133 expression. CD44 expression was increased in both knockdown variants [230]. Gargini *et al*. demonstrated a close relationship between maintenance of the CSC-like phenotype and the survival of tumor initiating cells [231]. Inhibiting PI3K activity or AKT1 activity was not only related to increased apoptosis but also reduced significantly stem cell/mesenchymal phenotype and recovery of epithelial-like markers. They also revealed that maintenance of stem cell-like phenotype and survival of these cells is linked to AKT-FOXO-Bim pathway [231]. Studies with hair follicle stem cells (HF-SCs) showed that the expression of permanently active form of AKT leads to HF-SC activation and increases the response to proliferative stimuli, at the same time maintaining their stem-cell identity and functionality. In addition, active AKT signaling increased tumor development and malignancy [232]. Recently it was found that cells overexpressing Twist, a key transcriptional factor of epithelial mesenchymal transition (EMT), had cancer stem cell-like properties, such as tumorsphere formation and significantly elevated expression of ALDH1 (aldehyde dehydrogenase 1) and CD44. In addition, activation of β-catenin and AKT pathways correlated with the expression of CD44 in these cells and knockdown of β-catenin expression and inhibition of the AKT pathway substantially suppressed the expression of CD44 [233]. Recent study claims that the EMT and stem cell renewal programs are influenced by imbalance between AKT1 and AKT2, which dysregulates the microRNAs that control these processes, rather than overall activity of AKT [234]. It has been noted that inactivation or deletion of tumor suppressor PTEN, the negative regulator of PI3K/AKT/mTOR signaling cascade, is important in conferring CSC properties in several types of solid tumors, like glioblastoma, hepatocellular carcinoma, prostate carcinoma, lung adenocarcinoma and breast carcinoma [235-240]. In glioblastomas, AKT activation correlated with stemness, invasivity, and increased tumorigenicity, whereas ERK activation correlated with tumor cell proliferation [241]. PTEN gene down-regulation was also detected when leukemic stem cells (LSCs) from chronic myelogenous leukemia (CML) patients were analyzed and compared with healthy hematopoietic stem cells (HSCs) [242].

Although the present information about AKT kinase relationship with CSCs is controversial and needs further research, it is still obvious that AKT signaling can have an important role in preserving CSC characteristics, like tumor initiation, self-renewal and pluripotency.

5. Protein-protein interaction inhibitors

Protein-protein interactions (PPI) have a central role in cellular biological processes, like extracellular signaling, protein translation, DNA-synthesis, protein degradation, programmed cell death etc. There are roughly 30 000 protein sequences in human genome, an estimated number of PPIs in human body reaches up to ≈400 000, and therefore PPIs are a large and important class of

highly promising targets for human therapeutics [243]. In cancer cells, PPIs are responsible for transmitting signals along molecular networks, by promoting tumorigenesis, invasion and metastasis. Large number of PPIs have a role in signaling networks that allow the acquisition and maintenance of cancer hallmarks. PPIs promote oncogenic signals by cell proliferation stimulation, for example by aberrantly activated epidermal growth factor receptor (EGFR) and its downstream target Ras protein [244]. Cell survival signaling is activated by proteins like insulin-like growth factor 1 (IGF-1) and PI3K, which enables cells to resist apoptosis through several different ways. Transcription-dependent mechanism uses AKT-FOXO3a-14-3-3 axis and transcription-independent antiapoptotic mechanism AKT-BAD-14-3-3 pathway [105, 116]. In addition, by regulating mTOR complex, AKT promotes translation of growth promoting genes, like c-Myc [245]. Disruption of PPIs, which are critical for cancer progression and that cancer cells rely on for survival, offers a potential effective, although challenging strategy for development of anticancer treatment. The challenges to discover compounds that could inhibit PPIs are for example the lack of good starting points for drug design and the difficulty to distinguish the real interaction from false/artefactual binding. The concerning properties of PPIs include large PPI interface areas, lack of deep pockets, where small molecule can dock, lack of noncontiguous binding sites and general lack of natural ligands. Protein-protein complexes are typically hydrophobic and have different amino acid residue composition than small-molecule binding sites [246]. The presence of hot spots (small subsets of amino acid residues that contribute the most of the free binding energy), post-translational modifications and natural ligands simplifies defining of targeting suitable interfaces.

Current approaches to design PPI modulators include structure based design and different small-molecule screening methods [246]. Structural analysis helps to identify specific amino acid or peptide fragments, which are critical for protein-protein interactions. These methods include computational design of peptides and peptide engineering [247], design of small molecules based on α-helix and β-sheet scaffolds [248], protein design strategies to stabilize α-helical motif [249]. Small-molecule screening allows the identification of PPI modulators when structural information is lacking or limited. Most widely used high throughput screening techniques in such cases include fluorescence energy transfer (FRET) and fluorescence polarization (FP) method [250], ELISA, flow cytometry, surface plasma resonance, label free methods [251], cell-based reporter assays, which are also able to identify cell-permeable compounds [252] and fragment based screening [253]. The screening method and structure based approach used in combination helps to find modulators with better pharmacological and physicochemical properties.

Natural compounds like taxanes for tubulin and rapamycin for mTOR, which were discovered in the late 90s as potent stabilizers of PPIs, show the potential of PPI modulation as therapeutic target. Despite of challenges in PPI modulator design, more than 15 years after the mentioned discoveries novel small molecules targeting specific PPIs have entered clinical trials, and in some cases

already resulted in new therapeutics or optimized treatments. Probably one of the most studied PPI in cell-cycle pathway is MDM2-p53 interaction. MDM2 interacts with a hydrophobic surface groove with three key hydrophobic residues Phe19, Trp23 and Leu26 on p53. These residues make up the "hot spot" which was targeted to identify molecules that can interrupt this specific interaction [254]. For now, seven MDM2-p53 inhibitors have progressed to clinical trials with promising results [243]. The inhibitors of apoptosis proteins (IAPs) are overexpressed or constitutively activated in tumor cells, resulting in evasion of programmed cell death. The XIAP (X-linked IAP) is the most potent caspase inhibitor among the IAP protein family, with the natural inhibitor SMAC [255]. After discovery of the SMAC protein in 2000, a number of SMAC mimetics have been designed, from which seven have reached clinical trials and five molecules are in clinical development [256]. The antiapoptotic Bcl2 members of the protein family, which protect the cells against apoptosis, by inhibiting the actions of the proapoptotic members, are in some cancer types overexpressed. Bcl-2 specific inhibitor ABT-199 (RG7601) is in phase 1 trials for chronic lymphocytic lymphoma or small lymphocytic lymphoma. Another Bcl2 inhibitor Obatoclax (GX015-070) is currently assessed in multiple phase 2 clinical trials offers the opportunity to treat many forms of cancer both as a single agent and in combination with current treatments [243]. The inhibition of Hsp90 chaperone protein, which regulates the activity and stability of numerous targets, is able to shut down multiple oncogenic pathways [257]. Hsp90 inhibitors not only elicit potent anti-cancer effects, but also render tumor cells susceptible to chemotherapy and/or radiation therapy [258].

Novel small molecules targeting PPIs, which have entered to clinical trials and already resulted in optimized treatments or new therapeutics, clearly demonstrate the potential of PPIs in drug development.

6. AKT inhibitors

Although AKT has been targeted by several small-molecule inhibitors, the development of an AKT-specific inhibitor is challenging due to its high sequence and structural homology to other kinases and proteins containing PH domains. Despite of that, a plethora of AKT inhibitors is under pre-clinical and clinical trials.

AKT inhibitors can be divided into six major classes based on their mechanisms of action [259]. The first class contains ATP competitive inhibitors, which bind to enzyme active site and display ATP-competitive behavior, like AKT2 inhibitor CCT128930 [260], AKT1 inhibitor GDC-0068 [261], which has entered to Phase II trials, the pan-AKT kinase inhibitors such as GSK2110183 (afuresertib) [262], which has entered Phase II trials, and AT7867 [263], which is still in pre-clinical phase. The second class contains phospholipid-like molecules, which prevent the generation of PIP3 by PI3K, thereby avoiding AKT translocation to the cell membrane and its subsequent activation.

This mechanism is utilized by phosphatidylinositol analogs such as Calbiochem AKT Inhibitors I, II and III (category no. 124005, 124008, 124009 respectively) [264] or other PI3K inhibitors such as PX-866 [265]. This category also includes compounds such as Perifosine that entered phase III trials for colorectal cancer and multiple myeloma, but failed in 2012 [266]. The third class contains a group of compounds called pseudosubstrate and substrate-mimetic inhibitors, which include compounds like AKTide-2 T and FOXO3 hybrid [267]. These peptides are potent selective inhibitors of AKT, that bind to substrate binding site and inhibit AKT1 enzyme activity. Unfortunately, their size makes them poor leads for small molecule inhibitor development. The fourth class consists of allosteric inhibitors of AKT kinase domain, like MK-2206. MK-2206 is an orally bioavailable allosteric inhibitor of AKT, which binds to and inhibits the activity of AKT in a non-ATP competitive manner by inducing a conformational change of AKT to the closed cytoplasmic conformation [268]. MK-2206 is currently widely examined in several clinical trials in phase II status in combination with other targeted drugs and chemotherapy. The fifth class consists of antibodies and include entities such as GST-anti-AKT1-MTS [269]. The last class comprises of compounds that interact with the PH domain of AKT, and include compounds such as Triciribine and PX-316 [270]. Triciribine is a cell-permeable tricyclic nucleoside that inhibits the phosphorylation, activation, and signaling of all three family members of AKT [271]. RX-0201 (Archexin) is an antisense oligonucleotide directed toward AKT1 mRNA, which significantly downregulated the expression of AKT1 at both mRNA and protein level [272].

Although, various drug trials have been initiated for AKT inhibitors, only miltefosine has been recently approved by FDA for treatment of leishmaniasis, which is a disease caused by an intracellular protozoan parasite (*genus Leishmania*) transmitted by the bite of a female phlebotomine sandfly.

RESULTS
Objectives of the study

The main objectives of present work were as follows:

- to identify AKT pathway inhibitors with principally novel mechanism of action, by targeting the crucial step in the AKT pathway – the interaction of PDPK1 and AKT1 proteins, which leads to AKT phosphorylation and subsequent activation of AKT-related pathways (**REF I**).
- to use DEN-phenobarbital induced mouse liver tumor tissue derived primary mouse liver tumor cell line K07074 as a model system for testing the effect of chemotherapeutic agents that could target the pathways deregulated in liver tumors (**REF II**).
- to study the specific areas in Dupuytren´s contracture (DC), which sustain active cell proliferation, to describe the potential molecular factors that could promote the DC pathogenesis (**REF III**).

1. A Novel Inhibitor of AKT1–PDPK1 Interaction Efficiently Suppresses the Activity of AKT Pathway and Restricts Tumor Growth *In Vivo* (REF I)

Ser/Thr kinase AKT has a central role in regulating survival and proliferation of normal and cancerous cells [201, 273]. Since inadequately regulated AKT pathway has been found in several human malignancies [274], it has become an attractive target for treatment of different types of cancer. Although a number of small molecule AKT kinase inhibitors have been developed, only a few are suitable for clinical trials due to severe side effects, which have prevented their further use. To find inhibitors of AKT1 signaling with principally novel mechanism of action, we carried out a live cell-based screen for small molecule inhibitors of physical interaction between AKT1 and its primary activator PDPK1.

1.1. Screening for the inhibitors of AKT1 and PDPK1 interaction

Small-chemical library screen was carried out in live cells by using the NCI Diversity Set I. The screening method, protein complementation assay (PCA), is based on *Renilla reniformis* luciferase (Rluc) fragments (F1 and F2) that are fused to the proteins of interest [275]. When the proteins of interest interact, the *Renilla* fragments are brought in close proximity, which results in reconstitution of the enzyme activity (**Figure 1 in REF I**), and vice versa, the inhibition of protein-protein interaction unfolds the functional enzyme and destroys the Rluc activity. To assess the quality of the screen setup, Z-factor was measured using the constructs encoding p53-F1 and HDM2-F2 fusion proteins and the inhibitor of p53-HDM2 interaction – Nutlin-3. The value of the Z-factor for this assay

was 0.5, demonstrating its suitability for detecting the inhibition of the protein-protein interactions.

For screening experiments, H1299 cells, which harbor an active AKT signaling pathway and are well-suited for DNA transfection experiments, were used [276] (**Figure 1B in REF I**). To exclude unspecific readouts of luciferase modulation, a number of controls were included in the screen setup (**Table S4 in REF I**). Well-to-well variations were eliminated by normalizing the luciferase read values to cell viability counts. The viability counts were also used to identify and exclude toxic compounds. Baseline values were set according to DMSO (vehicle) treated cells. As controls, each experiment assay included cells that were transfected with p53-F1 and HDM2-F2 fusions and treated with Nutlin-3 or vehicle. At least 2-fold reduction in luciferase activity upon Nutlin-3 addition was considered as successful experiment. To eliminate the chemicals, which directly inhibited the Rluc activity, cells transfected with plasmids encoding full-length Rluc were treated with the chemical library in parallel. Taken together the selection criteria for compounds were set as follows: 1) at least 2-fold reduction of normalized Rluc activity representing the disruption of AKT1-PDPK1 interaction; 2) 25% or less reduction in cell viability counts when compared to the average of untreated controls; 3) less than 10% reduction in the normalized luciferase signal in cells transfected with full-length Rluc when compared to the average reading of untreated cells (**Figure 1C in REF I**).

The screen was performed in two major steps. First, 74 chemicals out of 2,000 were identified, as inhibitors of AKT1-F1–PDPK1-F2 interaction that reduced the luciferase signal at least to 50% or less. In the second step, the selected 74 chemicals were retested for AKT1–PDPK1 PCA inhibition and the inhibition of full-length Rluc and cell toxicity were evaluated. From 74 chemicals that inhibited the AKT1–PDPK1 PCA, 29 chemicals also inhibited the activity of full-length Rluc, and 13 chemicals were toxic for cells. Nine more chemicals from remaining 45 were excluded from further analysis due to toxicity as these reduced the viability counts more than 25%. Consequently, 36 chemicals were selected for the next evaluation step.

1.2. NSC156529 reduces AKT phosphorylation level and inhibits AKT1-PDPK1 endogenous interaction

Since the hallmark of AKT1 activation by PDPK1 is the phosphorylation of AKT1 at Thr308 [32, 33, 84, 277], we evaluated the ability of the chosen chemicals to inhibit the phosphorylation of AKT protein at Thr308. H1299 cells were treated with 36 chemicals selected in the previous step (**Figure 2A in REF I**). GSK2334470, a direct inhibitor of PDPK1, was used as a positive control. By using Western blot method and pAKT(T308) specific antibody, we identified 12 compounds from 36, which inhibited AKT1 protein phosphorylation to a various degree. The cell cultures treated with these compounds were carefully examined for signs of toxicity and 4 chemicals of 12, which did not cause

notable changes in cell density and morphology were chosen for further analysis (**Figure 2A, 2B, Figure S1A-S1D in REF I**).

To find out, whether the selected chemicals were able to inhibit the interaction of endogenous AKT1 and PDPK1 proteins, we used in situ proximity ligation assay (*in situ* PLA), which allows specific and sensitive detection of protein-protein interactions in their native conditions. PLA is based on the immunodetection of the interaction partners with specific primary and oligonucleotide-tagged secondary antibodies. When the proteins of interest interact, the *in situ* PCR generates specific DNA sequence, which is detected by the hybridization with fluorescently-labelled DNA oligonucleotides [278]. PLA experiment was performed using PC-3 prostate cancer cells, which harbor an activated AKT signaling pathway [279]. PC-3 cells were seeded to glass slides and incubated with the 4 chemicals selected in the previous step. The number of AKT1-PDPK1 interaction sites detected in the cells treated with the NSC156529 was significantly decreased when compared to PC-3 cells treated with DMSO (**Figure 2C in REF I**). The number of AKT1-PDPK1 interaction sites in cells treated with other three chemicals (**Figure S2A-S2G in REF I**) remained at the same level as in control cells. Remarkably, NSC156529 had no significant effect on AKT2-PDPK1 and AKT3-PDPK1 interactions, indicating to the specific effect of NSC156529 to AKT1-PDPK1 interaction.

1.3. AKT1-PDPK1 interaction inhibitor NSC156529 suppresses the activity of AKT downstream targets and cancer cell proliferation *in vitro*

To promote cell-cycle progression and proliferation, active AKT1 protein phosphorylates and thereby inactivates a large number of downstream targets [102]. Therefore, we next sought to find out, whether the disruption of AKT1-PDPK1 interaction and reduction of AKT phosphorylation by NSC156529 influences the phosphorylation of AKT1 downstream targets, such as GSK3β, FOXO3a, BAD and procaspase 9 [121, 280-283]. We found that in PC-3 cells incubated with NSC156529 the phosphorylation of the studied AKT1 target proteins was significantly decreased when compared to control cells incubated with DMSO (**Figure 3B in REF I**). PDPK1 inhibitor was used as a positive control. The basal level of these proteins did not change, with the exception of FOXO3a, the overall protein level of which was also reduced. These results indicated that NSC156529 treatment inhibited the key biochemical activities of the AKT signaling pathway.

The main goal of anticancer drugs is to suppress the growth of cancerous cells. Therefore, we incubated a selection of immortalized and tumor cell lines (Hek293, PC-3, H1299, K07074, and Hep3B) with NSC156529 to verify whether this compound is able to inhibit the proliferation of these cells. As a result, 96-hour treatment with NSC156529 strongly inhibited the growth of selected cell lines, in addition, NSC156529 was more efficient compared to

PDPK1 inhibitor which was used as an assay control (**Figure S4A-S4D in REF I**). Importantly, NSC156529 inhibited the growth of PC-3 cells to a larger extent than that of primary human fibroblasts, and osteoblasts (**Figure 3C in REF I**).

1.4. AKT1-PDPK1 interaction inhibitor NSC156529 suppresses the cell proliferation and induces expression of differentiation markers *in vivo*

To examine whether NSC156529 has an effect on tumor growth *in vivo*, we used a tumor xenograft model (**Figure 4A in REF I**). Nude mice were injected subcutaneously with PC-3 prostate cancer cells, which constitutively expressed EGFP.

The mice bearing a tumor with the median size of 29–32 mm^3 were injected subcutaneously 3 times a week with NSC156529 at concentrations 1 mg/kg, 5 mg/kg, 10 mg/kg, or with vehicle only. Tumor size was measured externally by using a caliper, and additionally the number of tumor cells was monitored by using an *in vivo* imaging device. The reason for using fluorescent imaging was to measure the pure amount of human GFP-expressing xenografts. As the human GFP-expressing xenografts are gradually infiltrated by mouse stromal cells the caliper measurements might have yielded incorrect results. Tumor growth was monitored during the 28-day treatment period. Measurements obtained with external caliper were consistent with GFP measurements and showed clearly that all used NSC156529 concentrations reduced the tumor growth *in vivo* (**Figure 4B and 4C in REF I**). Since no adverse side effects, such as weight loss, ulcerations, or decline in general health conditions of the animals, were observed, and the ALT and AST levels, the biomarkers of liver health, stayed within the normal values, it was concluded that in our experimental system NSC156529 did not exert hepatotoxicity (**Figure S5 in REF I**).

NSC156529 inhibited AKT pathway in tumor xenografts, as the number of cells positive for the pAKT(T308) and the phosphorylated form of BAD protein were notably reduced in NSC156529-treated PC-3-GFP xenografts compared to control xenografts (**Figure 5A and 5B in REF I**). The mitotic activity, which was detected by the number of pH3-positive cell nuclei, was decreased in NSC156529-treated xenografts, indicating that at least in part the inhibition of tumor growth was caused by the inhibition of cell proliferation (**Figure 5C and 5D in REF I**). The presence of cells, which spontaneously undergo apoptosis, is a characteristic feature of malignant neoplasms. To study the role of apoptosis in NSC156529-induced tumor growth suppression, we used terminal deoxynucleotidyl transferase nick end labelling (TUNEL) assay, and measured the amount of apoptosis markers – fragmented DNA and cleaved caspase-3 (CC3) – in tumor xenograft cryosections. The treatment with NSC156529 did not significantly increase the number of cells positive for TUNEL nor CC3, which indicates that apoptosis did not have a significant role in the growth reduction of

mouse tumor xenografts induced by NSC156529 (**Figure S6A and S6B in REF I**). Interestingly, we found that the expression of cytokeratins 15 and 17 (CK15/17) (**Figure 6A, C, D in REF I**) and cytokeratins 8 and 18 (CK8/18) (**Figure 6B, E, F in REF I**) was increased in NSC156529-treated PC-3-GFP xenografts. CK15/CK17 label the human prostate basal epithelial cells and CK8/CK18 expression characterizes the differentiated luminal cells [284]. Since PC-3 cells display properties of poorly differentiated prostate cancer [285], increased levels of CK15/CK17 and CK8/CK18 indicate that NSC156529 could limit the tumor growth at least in part by directing the cancer cells to differentiate.

1.5. NSC156529 interacts preferentially with PDPK1

Interaction inhibition between AKT1 and PDPK1 might be caused by direct interaction of NSC156529 either with AKT1 or PDPK1. To study the potential interaction between NSC156529 and EYFP-tagged AKT1 or PDPK1 in cell lysates we used microscale thermophoresis method (MST). For MST analysis, H1299 cells were transfected with either EYFP-tagged AKT1 or PDPK1 (AKT1–EYFP; PDPK1–EYFP) expressing vectors. Cell lysates containing fluorescently labeled proteins were incubated with serially diluted unlabeled NSC156529 and analyzed using a dedicated MST instrument. While NSC156529 stable binding to AKT1–EYFP could not be detected (**Figure S3A in REF I**), the binding event with the Kd of 981±131 nmol/L was determined for the NSC156529 and PDPK1– EYFP interaction (**Figure S3B in REF I**). These data suggested that NSC156529 binds directly to PDPK1. The PDPK1 protein has two well-defined docking sites, which are required for the phosphorylation of selected PDPK1 targets: ATP pocket and PIF pocket [286]. The potential of NSC156529 to bind to these sites was evaluated by docking calculations using Glide (Glide, v6.2, Schrodinger, LLC, 2014) and with standard settings performed by the previously published PDPK1 crystal structure (PDB ID–1H1W, [287]). Since the results of the calculations demonstrated no reliable binding of NSC156529 to these two sites, NSC156529 most likely interacts with PDPK1 at other less structured locations (U. Maran and A.T. García-Sosa; personal communication).

2. The inhibition of AKT-PDPK1 interaction efficiently suppresses the growth of murine primary liver tumor cells (REF II)

Most frequently upregulated pathways in liver cancer are related to hyper-activated growth-factor signaling, such as epidermal growth factor (EGF), platelet-derived growth factor (PDGF), hepatocyte growth factor (HGF), and vascular endothelial growth factor (VEGF), which lead to signal transduction

involving cytoplasmic tyrosine kinases like RAS/MAPK, AKT/PI3K/mTOR and ERK1/2 pathways [288, 289]. AKT signaling in liver cancer progression and the level of AKT activation has been found to correlate with increased hepatocellular carcinoma (HCC) recurrence risk [290]. By using a primary mouse liver tumor cell line K07074 as a model system, we tested the effect of novel chemotherapeutic agents on pathways deregulated in liver tumors.

2.1. Increased activity of liver tumor-associated pathways in K07074

K07074 is a primary cancer cell line derived from DEN-phenobarbital induced mouse liver tumor tissue. The origin of K07074 cells is not clear. Expression of CK19 is characteristic to cholangiocytes, though, the polygonal shape and low expression of Hnf4α in K07074 cells might also indicate to hepatocyte origin (**Figure 1A and 1B in REF II**).

Based on previously published data about active signaling cascades in HCC and intrahepatic cholangiocellular carcinoma (ICC) [291, 292], we evaluated the gene and protein expression of Wnt, Hedgehog, p53, Notch and AKT1 targets. Significantly higher gene expression level of Notch pathway target genes *Hes1*, *Hey1*, *Notch3*, *Trib1*, *Ccnd1* and the Hedgehog pathway target genes *Gli1* and *Gli2* in K07074 cells compared to cultivated hepatocytes (**Figure 3A-G in REF II**) indicates to elevated activity of these pathways. Although the protein level of Axin2, a Wnt signaling pathway target gene, was higher in K07074 cells (**Figure 3H and 3I in REF II**), the expression of *Axin2*, was not significantly different from that in the control cells (**Figure S1 in REF II**), suggesting that stability of this protein is regulated post-transcriptionally. Immunofluorescence analysis showed that in hepatocytes β-catenin localized primarily near cell membrane and in cytosol. In the K07074 cells perimemebraneous β-catenin content was decreased and the signal was mainly detected in the nucleus (**Figure 3J in REF II**), which indicated to activated Wnt pathway in K07074 cells. The p53 protein level (**Figure 3H and 3I in REF II**) and the expression of p53 target genes *p21, CyclinG1* and *Gadd45* (**Figure S1 in REF II**) was moderately increased in K07074 cells, though, the expression of p53 target and its negative regulator *Mdm2* and the proapoptotic gene *Puma* were not significantly different from those of control cells. Since sequencing of the *trp53* gene in K07074 cells revealed no p53 mutations, it is assumed that p53 transcriptional activity is partially inactivated in K07074 cells.

Flow cytometry analysis of AKT1 protein expression and phosphorylation revealed an increased AKT phosphorylation at its activation sites Thr308 and Ser473 in K07074 cells when compared to normal hepatocytes (**Figure 3H and 3I in REF II**). In K07074 cells treated with AKT1-PDPK1 interaction inhibitor NSC156529 and PDPK1 inhibitor GSK2334470 the phosphorylation of AKT at Thr308 but not at Ser473 was decreased (**Figure 4A in REF II**).

Next, the phosphorylation status of AKT target proteins was analyzed by Flow cytometry to examine, whether inhibition of Thr308 phosphorylation is sufficient to downregulate the AKT pathway activity (**Figure 4B in REF II**). Although Bad protein expression and the level of Bad phosphorylation at Ser136 were downregulated in NSC156529 treated cells, the change in phosphorylated Bad signal was proportional to the change in Bad protein level. Decreased phosphorylation of Gskβ at Ser9 and Foxo at Thr32 indicated to downregulated AKT pathway activity in K07074 cells after incubation with NSC156529 and GSK2334470. Since studied phosphorylation sites are also modified by other Ser/Thr kinases, like serum- and glucocorticoid-inducible kinases (SGKs) [217, 293], the treatment with AKT pathway inhibitor NSC156529 did not completely abolish the phosphorylation of these sites.

2.2. AKT and Notch inhibitors efficiently suppress the growth of K07074 cells

Previous results indicated to increased activity of several liver tumor associated pathways in K07074 cells, therefore, these cells were treated with Nutlin-3 (Trp53-Mdm2 interaction inhibitor) [294], Gant61 (Hedgehog pathway inhibitor at the level of Gli transcription factors) [26], PNU74654 (Wnt inhibitor) [295], GSK2334470 (PDPK1 inhibitor) [296], LY3039478 (Notch inhibitor) [297] and NSC156529 (AKT1-PDPK1 interaction inhibitor) [298] to study the potential effect of these modulators on K07074 cell growth. PNU74654 and Gant61 had no significant impact on K07074 cell growth, and Nutlin-3 and GSK2334470 had only a modest impact on cell viability of these cells. The moderate effect of Nutlin3 on K07074 growth may be explained by the fact that the expression of a subset of p53 target genes, including Mdm2, was not activated. On the contrary, Notch inhibitor LY3039478 and AKT1-PDPK1 interaction inhibitor NSC156529 incubation decreased the number of K07074 cells notably during 96-hour treatment (**Figure 4C in REF II**).

Since the antitumor efficacies of current therapies are limited, most likely because of the high degree of cancer clonal heterogeneity, intratumor genetic heterogeneity and cell signal complexity, a shutdown of a single target does not necessarily eradicate the cancer and it has been proposed that combined therapies should be used. Therefore, we tested the combined treatment of K07074 cells with NSC156529 and Hedgehog, Notch or Wnt pathway inhibitors. While co-treatment of the cells with a reduced amount (5μM) of NSC156529 and working concentrations of Gli and Wnt and Notch inhibitors had no significant additive effect (**Figure 4D in REF II**), the lower NSC156529 concentration (3μM) in conjunction with LY3039478 reduced the cell number more effectively than either inhibitor alone (**Figure 4E in REF II**). Conclusively, AKT pathway inhibitor NSC156529 is potentially powerful antitumor agent in liver cancer treatment and a combination therapy with Notch inhibitors may be considered when using suboptimal doses of NSC156529.

3. Activated growth factor signaling and increased phosphorylation of AKT in small blood vessels provide supportive environment for Dupuytren's contracture pathogenesis (REF III)

Dupuytren's contracture (DC) is a chronic and progressive transformation of connective tissue beneath the palm skin. When the disease progresses, palmar fascia within the hand becomes abnormally thick, which severely impairs the hand functioning. DC is non-malignant, but it invades locally and has progressive and irreversible nature [299, 300]. Fibrotic lesions in DC of the palmar fascia include two distinct structures, the nodule and the cord. Histologically the Dupuytren's nodule is a vascular tissue which comprises numerous myofibroblasts (alpha smooth muscle actin expressing fibroblasts). The Dupuytren's cord has a rich connective tissue matrix containing a low density of elongated spindle-shaped fibroblasts. Although widely studied, exact pathogenic process of the disease is largely unknown. The aim of the study was to shed light into the mechanisms and potential molecular targets related to DC pathogenesis.

3.1. Blood vessels in the DC contain proliferating cells and endothelial cells with activated phenotype

It is widely accepted that uncontrolled proliferation of atypical fibroblasts and their differentiation into myofibroblasts forms the cellular basis of DC [301]. The distribution of proliferative cells in the DC tissue and in normal palmar fascia (NPF) samples was visualized by antibodies recognizing proliferation marker Ki67 and the myofibroblast marker smooth muscle actin (SMA). While the NPF samples were Ki-67-negative (**Figure S1A in Additional file 2 in REF III**), numerous Ki67-positive proliferating cells were concentrated in the surrounding area and inside of the SMA-expressing blood vessels in the DC tissue (**Figure 1A in REF III**). Co-staining of DC samples with the SMA and endothelial marker von Willebrand's factor (vWF) verified SMA-positive structures as blood vessels (**Figure S1B in Additional file 2 in REF III**). Additional co-staining with antibodies recognizing vWF, pericyte marker desmin and Ki67 revealed that vast majority of proliferating cells were located in SMA-positive muscular layer of the blood vessels (**Figure 1B and 1D in REF III**), small part of the proliferative cells was located in vWF-expressing endothelial compartment (**Figure 1C in REF III**) and no proliferative desmin-positive pericytes could be identified (**Figure 1E in REF III**). Increased number of proliferating cells near the blood vessels in the DC tissue may indicate to activated growth factor signaling in the DC-associated blood vessels. The expression of CD90 (Thy-1) and CD105 (endoglin) has been associated with the activated state of the endothelial cells in response to growth factor signaling [302, 303]. Tissue sample staining revealed that rare small blood vessels found in NPF did not contain CD105-expressing cells (**Figure S1g in Additional file**

2 in REF III), however, a number of CD105/CD90 double-positive cell clusters were present in DC tissue (**Figure 2A, Figure S1E in Additional file 2 in REF III**). As CD105 expression coincided with the vWF-positive endothelial cells (**Figure 2B, Figure S1f in Additional file 2 in REF III**) and SMA-expressing CD90 negative myofibroblasts surrounded the CD90-expressing endothelial compartment (**Figure 2E, Figure S1H in Additional file 2 in REF III**) we concluded that endothelium displays an activated phenotype in DC.

3.2. The blood vessels in DC tissue contain cells with activated AKT signaling

Previous studies, which described protein expression profile in the diseased palmar fasciae of DC patients indicated the presence of activated AKT pathway [304]. While AKT has an essential role in cell proliferation regulation we hypothesized that AKT may contribute to the cell division in vascular/perivascular compartment of DC. Since phosphorylated AKT (pAKT) protein is the hallmark of activated AKT pathway, DC samples were stained with pAKT(Thr308) recognizing antibody to evaluate activation status of AKT protein. pAKT was present in all layers of SMA-positive blood vessels and their surrounding structures (**Figure 3A in REF III**). High level of pAKT was also detected in the acini and ducts of sweat glands (**Figure 3B in REF III**), which were surrounded by small blood vessels. AKT kinase is activated by growth factors like CTGF, bFGF and IGF-2, which have also been linked to DC pathogenesis [305-307]. Analysis of CTGF, bFGF and IGF-2 mRNA expression in NPF and DC samples revealed that the level of all three growth factors was increased in the DC samples compared to NPF (**Figure 3D-F in REF III**). DC tissue section staining with anti-CTGF antibody detected a strong signal in the K15-positive acini of the sweat glands (**Figure 3G in REF III**), while in other parts of the DC tissue, including K15-negative sweat gland ducts and blood vessels, CTGF was not expressed (**Figure 3H in REF III**). Although activated AKT was found in the acini and ducts, the cells secreting CTGF in acini were not proliferatively active (**Figure 3I in REF III**). IGF-2 expression was distributed all over the DC tissue without any preference in respect of small blood vessels (**Figure 3J-L in REF III**). Only the expression of bFGF was confined to the endothelium of the small blood vessels (**Figure 3M and 3N in REF III**) correlating with the increased number of proliferative cells in and near the blood vessels.

Since the composition of the ECM plays a critical role in the regulation of cell fate and regenerative potential [32], the presence of specific components of the ECM in the areas of DC tissue with increased proliferation was studied. Laminin subunits α4, α5, β1, β2, and γ1, which correspond to laminins 411/421 and 511/521, were enriched in the DC tissue by localizing in the myoepithelial compartment of the blood vessel wall (**Figure S2A-E in Additional file 3, Figure 4 in REF III**). These laminins are normal components of the endothelial basement membrane [22] and the high laminin content of the DC nodule was likely caused by the increased vascularization.

DISCUSSION

Extensive biological and clinical studies during the last decades have identified signaling cascades responsible for cancer progression, maintenance and metastasis. AKT signaling pathway, which has important role in cell growth, survival and proliferation, has been found to be hyperactivated in many human cancers. In addition, activated AKT is an indicator of poor prognosis and resistance to cancer therapy. Since protein-protein interactions are responsible for transmitting oncogenic signals in pro-oncogenic pathways they have become promising therapeutic targets in cancer drug design. Thereof, specific small molecule compounds that could block protein-protein interactions in tumors possessing aberrantly activated AKT signaling pathway, would be promising tools for cancer treatment.

The purpose of the first study (**REF I**) was to discover small-molecule compounds with principally novel mechanism of action that would target the critical step in the AKT cascade – the interaction between AKT1 and PDPK1. We took advantage of the reversible protein complementation assay, which utilized two *Renilla* luciferase fragments and enabled to detect the interaction between two proteins in live cells [275]. The screening and following experiments identified one chemical – NSC156529 – which reduced the interaction of ectopically expressed and endogenous AKT1 and PDPK1 proteins, decreased AKT1 phosphorylation and inhibited tumor cell growth *in vitro* and *in vivo* in a tumor xenograft model. The detailed molecular mechanism of inactivation by NSC156529 is unknown, but based on our data NSC156529 interferes AKT1–PDPK1 interaction by the direct binding of the compound to PDPK1. NSC156529-treated xenografts expressed increased levels of differentiation markers, suggesting that inhibiting activity of AKT pathway at the level of AKT1–PDPK1 interaction, may possibly open up an alternative way for tumor treatment via stimulation of cell differentiation. Since poor differentiation is an important hallmark of cancer cells, novel substances that could promote tumor cells to differentiate rather than induce cell death is a promising way for cancer treatment. To date, the only successful clinical application of differentiation-based tumor therapy is used in the acute promyelocytic leukemia (APML) treatment scheme. All-trans-retinoic acid (ATRA)-based therapy induces granulocytic differentiation of APML leukemic blasts, which dramatically improves patient survival [308]. Since limited therapeutic effectiveness of conventional chemotherapy is often associated with the development of drug resistance and systemic toxicity [309], the combined therapy scheme, which consists in addition a tumor cell differentiation inducing component, could offer a possibility to reduce a drug dosage, limit the occurrence of side effects, and decrease the incidence of drug resistance [310, 311]. *In vitro* cell growth analysis confirmed that NSC156529 inhibits the proliferation of the cell lines of variable origin. Since hyperactivated AKT pathway has been found in many human tumor types [201], the potential use of this chemical should not be limited with one cancer type only. In fact, tumor xenograft assays conducted by NCI within the frame-

work of Developmental Therapeutics Program confirm, that NSC156529 inhibits also the growth of B16 melanoma cells and leukemia cell lines L1210 and P388 [312]. Although NSC156529 inhibited also the growth of normal human fibroblasts and osteoblasts *in vitro*, the cancer cell proliferation seems to be more sensitive to NSC156529 inhibition. *In vivo* subcutaneous administration of the compound was well-tolerated by the animals, since severe symptoms of irritation at the injection sites of the skin were not seen. Intraperitoneal administration of the compound caused severe irritation indicating to potential local toxicity on mucous membranes and endothelium. Because systemic administration of this compound to the blood stream was not tested, the relatively low general toxicity observed during our studies might be caused by a reduced exposure of NSC156259 to the host organism. Further toxicity studies are required to assess and resolve drug administration issues.

The potential anti-tumor effects of NSC156529 were further elaborated in **REF II,** where primary mouse liver tumor cell line K07074, which was derived from DEN-phenobarbital induced mouse liver tumor tissue, was used as a model system to test the tumor-suppressive effects of NSC156529 and other novel antitumor agents that target cancer-related pathways. Analysis of gene and protein expression demonstrated an increased activity of Notch, Hedgehog and AKT pathways in these cells. Activation of AKT and Notch pathways, has been shown to convert the hepatocytes to cholangiocyte-like cells that act as precursors of rapidly progressing ICC [313, 314]. The nuclear localization of β-catenin found in K07074 cells, commonly refers to the Wnt pathway activation. Nevertheless, we were unable to detect an increase in *Axin2* mRNA and found only a modest increase in Axin2 protein level. Therefore in our model system β-catenin nuclear localization may appear as a consequence of constitutive AKT signaling activity, which phosphorylates and deactivates GSKβ, the kinase responsible for β-catenin phosphorylation, cytoplasmic localization and degradation [315]. This could also be the explanation why Wnt pathway inhibitor did not have any effect on the growth of K07074 cells. p53 tumor suppressor gene inactivation or mutation is a frequent event in tumorigenesis. Accumulation of a stable mutant p53 protein is regarded as a hallmark of cancer cells [316]. In K07074 cells trp53 gene was not mutated, but the expression of a subset of p53 target genes including Mdm2 was not activated. This could be the reason why in our experiments the inhibitor of p53-Mdm2 interaction – Nutlin3 – was not efficient on K07074 cell growth inhibition.

While the gene and protein expression analysis showed an increased activity of several pathways, only AKT and Notch inhibitors efficiently decreased the growth of K07074 cells. At higher concentrations, AKT-PDPK inhibitor NSC156529 alone was sufficient to inhibit the cancer cell growth, but at suboptimal concentrations LY3039478 and NSC156529 co-treatment was more effective than either compound alone. These results confirm the effective antitumor properties of NSC156529 as demonstrated previously by its ability to suppress the growth of human tumor cell-line xenografts in nude mice (**REF I**).

Taken together, the small molecular compound NSC156529 is a potent inhibitor of AKT1 pathway in tumor cells. NSC156529 reduces AKT activity, inhibits the activation of AKT target proteins that are involved in regulating cell survival, proliferation, and metabolism, and inhibits the growth of different tumor cells *in vitro* and *in vivo*. Since treatment with NSC156529 increased the differentiation status of cancer cells in *in vivo* xenograft treatment, this compound presents a promising lead for the development of a novel class of tumor therapeutics.

AKT signaling is not only involved in the appearance of malignancies but it may also play a role in formation of benign pathologies. Dupuytren's contracture (DC) is a chronic, progressive fibroproliferative disease of the hand, which is non-malignant but invades locally and has progressive and irreversible nature [317]. Although, uncontrolled proliferation and accumulation of contractile myofibroblasts is believed to have the central role in DC pathogenesis [301], the exact molecular mechanism of the disease is still unclear. The analysis of proliferative cell distribution in the DC tissue (**REF III**) revealed that these were concentrated in the small blood vessels or in their surrounding area, primarily in the myofibroblast layer of the blood vessel walls. Previously it has been shown that small blood vessels can act as stem cell niches in normal and malignant brain tissue [318], therefore, small blood vessels in DC might provide a favorable microenvironment for sustaining myofibroblast proliferation. In addition, proteomic studies have implicated the proliferation promoting AKT pathway in DC pathogenesis [304]. In correlation with these studies, elevated levels of phosphorylated AKT, a hallmark of activated AKT signaling, was detected in the blood vessels and sweat glands of the DC tissue. The potential AKT activating growth factors, including basic fibroblast growth factor (bFGF) in the endothelium of the blood vessels, insulin-like growth factor 2 (IGF-2) uniformly throughout the nodular tissue and connective tissue growth factor (CTGF) in sweat glands near the nodule, were also highly upregulated in DC tissue. These observations are supported by previous studies, which suggest that IGF-2 has a role in regulating cellular contractility and proliferation in DC [307] and that CTGF, a TGF-β target gene and a potent activator of the AKT signaling pathway is implicated in the pathogenesis of fibrotic diseases including DC [304]. Li *et al*. reported that in response to FGF signaling, constitutively active AKT induces the transcription of laminin β1 and collagen IV α1 isotypes and causes their translocation to the basement membrane [319]. Since in our study we found active AKT and laminin subunits α4, α5, β1, β2, and γ1 expressed in the DC tissue, it provides additional support to the hypothesis of AKT role in DC pathogenesis. Conclusively, we proposed that small blood vessels, which contain increased expression of growth factors and favorable extracellular matrix components, form a microenvironment that supports the activation of AKT pathway and sustains the persistent myofibroblast proliferation promoting thereby the progression of DC.

SUMMARY

Extensive biological and clinical studies during the last decades have identified signaling cascades responsible for cancer progression, maintenance and metastasis. AKT-related signaling pathway, which has important role in cell growth, survival and proliferation, has been found hyperactivated in many human cancers. In addition, activated AKT has been linked also to poor prognosis and resistance to cancer therapy. Since protein-protein interactions in tumorigenesis promoting pathways are responsible for transmitting oncogenic signals, they have become promising therapeutic targets in cancer drug design. Thereof, specific small molecule compounds that could target protein-protein interactions in tumors possessing aberrantly activated signaling pathways, would be promising tools in cancer treatment.

Small molecule library screen utilizing a *Renilla* luciferase protein complementation assay (PCA)-based approach, which was aimed to discover AKT1 and PDPK1 interaction inhibitors, identified one compound – NSC156529 – that interacted preferentially with PDPK1, inhibited AKT1 phosphorylation and suppressed AKT-mediated signal transduction to several AKT1 substrates. The discovered compound efficiently decreased the proliferation of human cancer cells *in vitro* and inhibited tumor growth in a prostate tumor xenograft model *in vivo*. In addition to the reduction in the mitotic activity in NSC156529-treated tumors, the tumor cells exhibited a significant increase in the expression of prostate differentiation markers, suggesting that the inhibition of tumor growth was achieved at least in part through enhanced differentiation of tumor cells toward prostate epithelium. In the study with primary mouse liver tumor cell line, the AKT-PDPK interaction inhibitor NSC156529 and the Notch pathway inhibitor LY3039478, significantly reduced the growth of K07074 cells. Although gene and protein expression analysis suggested the presence of activated Hedgehog, Wnt and p53-related pathways, the inhibitors targeting these pathways had little or no effect on K07074 cell growth. In addition, no additive effect to NSC156529 could be seen when the cells were co-treated with the pathway inhibitors suggesting that the disruption of AKT-PDPK interaction is sufficient to inhibit the growth of primary liver tumor cells.

Dupuytren contracture sample analysis showed that proliferative cells in DC nodules localized in the vicinity of small blood vessels, predominantly in the myofibroblast layer. The small blood vessels also contained increased levels of phosphorylated AKT, which is a hallmark of activated growth factor signaling. Respectively, expression of potential activators of AKT signaling, such as bFGF, IGF and CTGF, was detected in DC samples. bFGF was expressed in the endothelium of the small blood vessels, IGF-2 was present uniformly in the DC tissue, and CTGF expressed in the DC-associated sweat gland acini. The blood vessels in DC nodules contained also increased amounts of laminins 511 and 521, which have been previously shown to promote the proliferation and stem cell properties of different cell types. Conclusively, DC-associated small blood

vessels, with the presence of growth factors and subsequent AKT activation in combination with extracellular matrix components, provide a supportive environment for myofibroblast proliferation and play an important role in DC pathogenesis.

Taken together, the results presented in current thesis demonstrate that targeting AKT related signaling pathway in cancer cells is effective way to suppress the growth of cancerous cells. The small molecular compound NSC156529 is a potent inhibitor of AKT1 signaling pathway, which reduces AKT activity, inhibits the activation of AKT target proteins that are involved in regulating cell survival, proliferation, and metabolism, and inhibits the growth of tumor cells *in vitro* and *in vivo*. NSC156529 is powerful antitumor agent in monotherapy, which can also be used at lower doses in combined therapy. Since the treatment with NSC156529 increases the differentiation status of cancer cells, this compound has an advantage for the development of a novel class of tumor therapeutics. In addition, we found that the activated AKT signaling might promote the progression of benign hyperproliferative pathology – the Dupuytren's contracture.

SUMMARY IN ESTONIAN

Rakkude paljunemist soodustav AKT signaalirada kui potentsiaalne kasvajavastase ravi sihtmärk

Rakud võtavad väliskeskkonnast vastu mitmesuguseid signaale, näiteks hormoonide, kasvufaktorite ja tsütokiinide vahendusel, mis antakse edasi mööda signaaliülekande radasid, mõjutades seeläbi nende rakkude paljunemist, eluiga ja metabolismiga seotud protsesse ning diferentseerumist. Rakkude jagunemine ja suremine on rangelt kontrollitud sündmused, millega tagatakse organismi normaalne funktsioneerimine ja kahjustatud rakkude asendamine uutega. Aja jooksul, rakkude vananedes või kokkupuutel kahjulike ühenditega võivad raku DNAsse tekkida mutatsioonid, mis omakorda põhjustavad muutusi raku normaalset elutsüklit toetavates olulistes signaaliradades. Kasvajarakkude piiramatu kasv on väga sageli seotud mutatsioonidega just onkogeenides, mis tekitab nende liigset aktiivsust, ja tuumor-supressor valkudes, mis tingib nende geenide väljalülitumise. Vähkkasvaja areng on mitmeastmeline protsess, milles põhiroll on valk-valk interaktsioonidel kindlates signaaliradades. Seetõttu on kriitilise tähtsusega signaaliülekande kaskaadides olevad valk-valk interaktsioonid perspektiivikaks sihtmärgiks potentsiaalsete kasvajavastaste terapeutikumide väljatöötamisel.

AKT kinaas on seriin/treoniinkinaas, mida kirjeldati esmakordselt umbes 25 aastat tagasi. Praeguseks on selge, et AKT kinaasiga seotud signaalirajal on keskne roll normaalsete rakkude füsioloogilistes protsessides nagu näiteks rakkude elulemus, rakutsükli edenemine, metabolism, transkriptsioon, valgu süntees, rakkude liikuvus jne. Samal ajal on kontrollimatult aktiivset PI3K/AKT signaalirada kirjeldatud väga paljudes inimese tuumorites nagu näiteks maksa-, aju-, rinna-, soole ja eesnäärmekasvajates. Lisaks sellele on leitud, et AKT signaalirada on seotud ka kemoteraapia käigus tekkivate resistentsusmehhanismidega ja üldiselt peetakse AKT aktiivsust mõnede kasvajate puhul halva prognoosi markeriks. AKT kinaasi keskse rolli tõttu olulistes raku elutsükli protsessides ja tema liigse aktiivsuse tõttu kasvajates peetakse AKT valguga seotud signaalirada heaks märklauaks kasvajavastaste terapeutikumide väljatöötamisel. Praeguseks on avastatud ja kliinilistesse katsetustesse võetud juba mitmeid AKT signaaliraja inhibiitoreid, kuid paraku on tugevad mittespetsiifilised kõrvaltoimed ja toksilisus takistanud nende edasise kasutamise raviskeemides.

Käesoleva doktoritöö esimese osa põhieesmärk oli leida AKT signaaliraja aktiivsust pärssivaid inhibiitoreid, mida võiks kasutada potentsiaalsete kasvajavastaste ravimitena. Otsitavate inhibiitorite täpsem märklaud oli AKT1 ja PDPK1 valkude omavaheline interaktsioon, mille käigus PDPK1 fosforüleerib AKT1 valgu – esimene etapp, mis on vajalik AKT1 aktiviseerimiseks. Väikesemolekulaarsete ainete skriinimiseks kasutati *Renilla* lutsiferaasi valgu komplementaarsusel põhinevat lähenemisviisi. Skriinimine ning sellele järgnenud erinevatest meetoditest koosnenud valideerimisprotsess tuvastas ühe kemikaali – NSC156529 – mis pidurdas erinevat päritolu kasvajarakkude paljunemist nii koekultuuri

tingimustes kui *in vivo* hiire kasvajamudelis. Tuumorirakkude töötlemine NSC156529 kemikaaliga mõjutas lisaks AKT kinaasi enda aktiivsusele ka AKT signaalirajas allpool asuvate märklaudvalkude aktiivsust, viidates sellele, et antud kemikaal mõjutab AKT toimet laiemalt. *In vivo* kasvajamudelitest eraldatud tuumorite analüüsimisel tuvastati, et NSC156529 kemikaaliga töödeldud kasvajates oli suurenenud rakkude diferentseerumisele viitavate markerite (tsütokeratiin 8 ja 18) ekspressioon, millest järeldub, et lisaks suudab NSC156529 tuumorirakkude paljunemist pärssida neid diferentseeruma suunates. Käesoleva töö teises osas testiti NSC156529 ühendit koos teiste potentsiaalsete kasvajaspetsiifilisi signaale pärssivate kemikaalidega hiire primaarse maksakasvaja rakuliinis K07074. See mudelsüsteem peegeldab paremini kui püsirakuliinid maksakasvajates esinevaid signaaliradade aktiivsuse muutusi. Kuigi geeni- ja valguekspressiooni analüüs viitas ka selliste signaaliradade nagu Notch, Wnt, Hedgehog ja p53 aktiivsusele, leiti et NSC156529 on üksinda piisavalt efektiivne kasvajarakkude proliferatsiooni inhibeerimisel. Ainult tingimustes, kus NSC156529 inhibiitorit kasutati madalates kontsentratsioonides, andis Notch inhibiitori ja NSC156529 koos kasutamine rakkude kasvu pidurdamisele tugevama efekti.

Dupuytreni kontraktuur (DK) on pihukilekõõluse (palmaaraponeuroosi) paksenemisega kaasnev sõrme liikuvuse piiratus. Koe tasandil iseloomustab seda haigust atüüpiliste müofibroblastide kontrollimatu proliferatsioon. Kuna praeguseni on DK täpne molekulaarne mehhanism ebaselge, siis oli käesoleva töö kolmanda osa eesmärk kirjeldada täpsemalt paljunevate rakkude asukohta DK koes ja selgitada võimalikke selle haiguse tekkimise mehhanisme. DK koe analüüsimisel leiti, et aktiivselt jagunevad rakud on koondunud veresoonte lähedusse, peaasjalikult neid ümbritsevasse silelihaskihti. Samal ajal oli võrreldes normaalse koega DK koe veresoontes ja higinäärmetes suurenenud fosforüleeritud AKT valgu hulk, mis võis olla tingitud sellest, et DK koes oli suurenenud ka potentsiaalsete AKT signaalirada aktiveerivate kasvufaktorite nagu bFGF, IGF ja CTGF tase. Lisaks sisaldasid DK koe veresooned suuremal hulgal laminiine 511 ja 521, mida on varasemalt seostatud rakkude tõusnud proliferatsiooni ja tüvirakuliste omadustega. Varasemalt on näidatud, et vastuseks FGF stimulatsioonile indutseerib aktiivne AKT kinaas laminiin β1 ja kollageen IV α1 isotüüpide transkriptsiooni. Seega viitavad antud töö tulemused sellele, et DK väikesed veresooned koos suurenenud kasvufaktorite ekspressiooni ja aktiivse AKT valgu tasemega loovad soodsa keskkonna müofibroblastide paljunemiseks ning DK haiguse edenemiseks.

Kokkuvõtvalt, antud töös esitatud tulemused kinnitavad, et AKT kinaasiga seotud signaalirada on oluline märklaud kasvajavastaste ravimite väljatöötamisel. Kuna leitud AKT1-PDPK1 interaktsiooni inhibiitor NSC156529 on efektiivne kasvajarakkude kasvu pärssimisel nii *in vitro* koekultuuri tingimustes kui *in vivo* hiire tuumori mudelis ning lisaks on NSC156529 kemikaalil rakkude diferentseerumist soodustav toime, siis võiks antud ühendit kasutada potentsiaalse vähivastase ravimi väljatöötamisel. Lisaks näitasime, et aktiveeritud AKT signaalirada osaleb healoomulise hüperproliferatiivse patoloogia – Dupuytreni kontraktuuri – arengus.

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PUBLICATIONS

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List of Publications

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Teaduspublikatsioonid

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