Åbo Akademi University

Knotwood extract from Norway Spruce (*Picea abies*) as treatment for androgen-independent prostate cancer

In vitro study of insulin signaling and proliferation in PC-3 cells

Toveann Ahlnäs
38818
toveann.ahlnas@abo.fi
213995.0 Master's thesis in biochemistry
Faculty of Science and Engineering
Åbo Akademi University and Turku Bioscience Centre
Supervisor: Prof. John Eriksson
2020

ÅBO AKADEMI UNIVERSITY

Biochemistry, Faculty of Science and Engineering

Toveann Ahlnäs

Knotwood extract from Norway Spruce (*Picea abies*) as treatment for androgen-independent prostate cancer: *in vitro* study of insulin signaling and proliferation in PC-3 cells

Master's Thesis (74 pages, 1 appendix)

April 2020

Keywords: *Picea abies*, Lignan, Hydroxymatairesinol, Prostate cancer, PC-3, Insulin signaling, Akt, Growth factor-depletion, Proliferation

ABSTRACT

Despite advancements in the therapeutic field during the past decades, prostate cancer (PCa) remains one of the most common cancers in men, with a relatively high mortality rate when the disease progresses, and available treatment methods fail. Androgen-independent PCa is an advanced form of the disease due to the cells not relying on androgens anymore for proliferation and survival in comparison to the androgen-dependent prostate cancer cells, which are more responsive to therapy. Instead, androgen-independent PCa cells heavily exploit other growth factors such as insulin and insulin-like growth factor (IGF) for sustained growth. With an increasing incidence worldwide as well as insufficient and costly medication, novel treatment methods are in great demand.

Lignans are polyphenols found throughout the plant kingdom mainly in fiber-rich vegetables and seeds and are known for their many health-promoting properties such as being potent antioxidants, exerting anti-inflammatory features, as well as reducing the risk of cardiovascular diseases. Importantly, lignans show selective toxicity to a variety of cancers including PCa without damaging healthy cells. In this thesis, a knotwood extract from the Norway spruce tree (*Picea abies*) was studied for its effects on the androgen-independent cell line PC-3 with the main focus on the insulin signaling pathway. Previous studies have investigated the antineoplastic properties of individual lignans, whereas extracts containing a mixture of these compounds have rarely been studied.

Initial analysis of the extract by gas chromatography-mass spectrometry revealed the lignan content to be 31.3%, of which hydroxymatairesinol was the

dominant lignan. Live-cell imaging of 48h treated PC-3 cells in serum-rich medium showed a distinct concentration-dependent suppression of proliferation. Further, the cells arrested in the G0/G1-phase of the cell cycle without apparent cell death as determined by flow cytometry. To identify if the extract targeted insulin-mediated signaling, the cells were treated in serum-free conditions with added insulin for up to 60 min. An evident inhibition of Akt phosphorylation as well as its downstream target Gsk-3β were observed in a concentration-dependent manner. Expression of p21 was additionally upregulated during a longer treatment time. Intriguingly, however, treatment in growth factor-depleted conditions revealed PC-3 cells to be highly sensitized to the extract, initiating rapid necroptosis-like cell death.

The results in this thesis demonstrate the antineoplastic effects of the *P. abies* knotwood extract on PC-3 cells *in vitro*. Mechanism of action is tied to altered PI_3K/Akt and MAPK signaling, where the amount of available growth factors as well as the cell's energy metabolism influence the lethal effects, possibly revealing how lignans exert their selectivity to cancer cells in general.

Table of Contents

LIST OF ABBREVIATIONS..... I

1. REVIEW OF LITERATURE 1

1.1. Hallmarks of cancer 1

1.2. Prostate Cancer..... 3

1.2.1. Disease progression..... 5

1.2.2. Treatment methods for prostate cancer 6

1.3. Intracellular signaling in regulating cancer cell proliferation and survival . 7

1.3.1. Insulin signaling in cell homeostasis..... 7

1.3.2. Cell cycle progression and regulation 12

1.3.3. Intrinsic and extrinsic signaling pathways in apoptosis 13

1.4. Lignans in human health and disease..... 15

1.4.1. Introduction to lignans and their biosynthesis 15

1.4.2. Dietary lignans, metabolism and benefits 19

1.4.3. Lignans as anticancer agents..... 20

2. Aims of the study 21

3. Experimental procedures 22

3.1. Gas chromatography – Mass spectrometry 22

3.2. Live-cell imaging of PC-3 cell proliferation..... 23

3.3. Flow cytometry analysis of cell cycle using propidium iodide staining.... 24

3.4. Western blot analysis of insulin signaling 24

3.5. Cytotoxicity assay of *P. abies* knotwood extract on serum-starved PC-3 cells 26

3.6. Statistical analysis 27

4. Results 28

4.1. Gas chromatography – Mass spectrometry analysis of *Picea abies* knotwood extract reveals HMR as dominant active lignan 28

4.2. *P. abies* knotwood extract suppresses PC-3 cell proliferation in a concentration-dependent manner 31

4.3. *P. abies* knotwood extract initiates cell cycle arrest in G0/G1-phase in PC-3 cells 34

4.4. Insulin-induced Akt and ERK signaling are affected by *P. abies* knotwood extract in a concentration-dependent manner 36

4.5. Growth factor-depletion sensitizes PC-3 cells to *P. abies* knotwood extract resulting in rapid cell death 40

5. Discussion and Conclusions 42

6. Summary in Swedish – Kvistnötsextrakt från gran (*Picea abies*) som behandling mot androgenoberoende prostatacancer: *in vitro*-studie om insulinsignalering och tillväxt hos PC-3-celler 49

7. Acknowledgements 53

8. References 54

9. Supplementary information 67

LIST OF ABBREVIATIONS

α-CONI	α-Conidendrin
ADT	Androgen deprivation therapy
Akt, PKB	Protein kinase B
AR	Androgen receptor
Bad	Bcl-2-associated agonist of cell death
Bak	Bcl-2 homologous antagonist killer
Bax	Bcl-2-associated X protein
Bcl	B-cell lymphoma
Bid	BH3 interacting-domain death agonist
CDK	Cyclin dependent kinase
DHT	Dihydrotestosterone
DISC	Death-inducing signaling complex
EGF	Epidermal growth factor
END	Enterodiol
ENL	Enterolactone
ERK	Extracellular-signal regulated kinase
GC	Gas chromatography
GDP	Guanosine diphosphate
GFR	Growth factor receptor
GLUT	Glucose transporter
GPER	G-protein-coupled estrogen receptor
Gsk-3β	Glycogen synthase kinase-3β
GTP	Guanosine triphosphate
HMR	Hydroxymatairesinol
HRP	Horseradish peroxidase
IGF-1	Insulin-like growth factor-1
IGF-1R	Insulin-like growth factor-1 receptor
IR	Insulin receptor
IS	Internal standard
MAPK	Mitogen-activated protein kinase
MAT	Matairesinol
MLKL	Mixed lineage kinase domain-like pseudokinase

MS	Mass spectrometry
mTOR	Mammalian target of rapamycin
PARP	Poly (ADP-ribose) polymerase
PBS	Phosphate buffered saline
PCa	Prostate cancer
PDK	Phosphoinositide-dependent protein kinase
PE	Pure extract
PI_3K	Phosphatidylinositol 3 -kinase
PINO	Pinoresinol
PTEN	Phosphatase and tensin homologue
PI	Propidium iodide
RC	Risk calculator
RIPK	Receptor-interacting serine/threonine-protein kinase
SDG	Secoisolariciresinol diglucoside
SECO	Secoisolariciresinol
S3M	Stockholm3 model
TRAIL	Tumor necrosis factor-related apoptosis-inducing ligand

1. REVIEW OF LITERATURE

1.1. Hallmarks of cancer

Maintaining balance, or so-called homeostasis, in the body is a remarkably intricate process where constant adjustments in physical and chemical conditions on the cellular level aim to keep the organism alive. External factors such as radiation, toxic chemicals, microorganisms, and dietary components all challenge the cells of the organism. Small errors occur constantly when cells grow and divide, ultimately governing the fate of the individual. Luckily, these faulty cells can be recognized and eradicated either by the cell itself or by the immune system. This process called regulated cell death or apoptosis is a vital part of both embryonal development and maintenance of homeostasis in all living organisms. However, some cells are able to avoid cell death, instead in many ways controlling their own fate. These cells often turn malignant and are capable of growing uncontrollably, acquiring certain properties through oncogenes to eventually create tumors in the organism.

The present understanding of tumorigenesis of cancer cells can be classified into six main cellular events known as the hallmarks of cancer (Fig. 1). These hallmarks such as resisting cell death and sustaining proliferation develop in a multistep manner during the life cycle of a cancer cell and are usually a consequence of genomic instability. Further, excess production of reactive oxygen species (ROS) which is detected in almost all cancers, promotes genomic instability through oxidative DNA damage (Liou & Storz, 2010). During the past decade, progress in the field has emerged with additional novel hallmarks, expanding our comprehension of tumorigenesis (Hanahan & Weinberg, 2011). These include the reprogramming of energy metabolism in the cell as well as the ability to avoid immune cell mediated apoptosis. Moreover, studying the tumor microenvironment has broadened our understanding of how cancer cells communicate with surrounding cells and the extracellular matrix, for instance, to migrate and form metastasis.

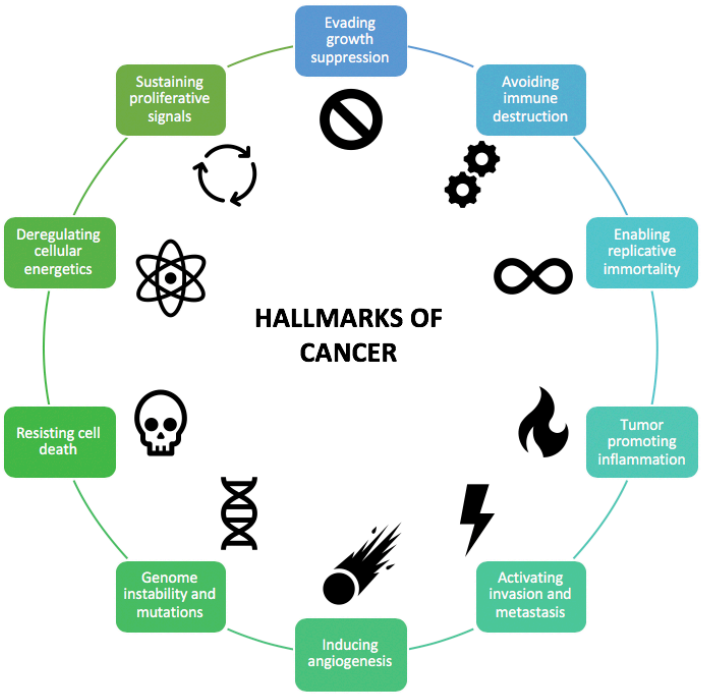

Figure 1 - Hallmarks of cancer. The classical characteristics of a cancer cell include sustained proliferative signaling, avoidance of growth suppression, resistance of cell death, replicative immortality, and activation of angiogenesis, invasion, and metastasis. Additional hallmarks of cancer have emerged during the past decade and are now generally accepted as part of the typical behavior of a cancer cell. These involve altered cellular energetics, promotion of inflammation, acquiring mutations through genomic instability, as well as avoiding immune cell detection. Original image modified from Hanahan & Weinberg, 2011.

Rapid cell division is costly, and hence rewiring the metabolism is an essential feature in malignant cells. One of the main hallmarks of an altered metabolism in these cells is the Warburg effect, which is defined as utilizing glycolysis instead of oxidative phosphorylation to produce energy regardless of the availability of oxygen (Liberti & Locasale, 2016). In normal cells, hypoxic conditions would initiate glycolysis, whereas cancer cells predominantly prefer glycolysis by constitutively taking up nutrients such as glucose to produce lactate through fermentation in order to meet the metabolic demands of proliferation. This occurs even though the energetic yield of ATP is much lower through glycolysis compared to oxidative phosphorylation. Many of the signaling pathways regulating these metabolic processes are frequently altered in cancers, inducing anabolic growth by enhanced protein and fatty acid synthesis (Lunt & Vander Heiden, 2011). The metabolic flexibility of cancer cells thus generally allows them to survive regardless of changes in nutrient availability in the varying

microenvironment. Intervention of these altered metabolic pathways has been of interest in drug development research since the modified energy production clearly partitions neoplastic cells from healthy ones. Yet, advancements in the field have been relatively slow and many challenges still remain (Martinez-Outschoorn, Peiris-Pagés, Pestell, Sotgia, & Lisanti, 2017).

1.2. Prostate Cancer

Prostate cancer (PCa) is the most common type of cancer in men in Finland, where approximately 5400 new cases are diagnosed annually. Worldwide, the incidence of PCa has been increasing during the past decades. According to the global cancer statistics of 2018, PCa has the third highest incidence and cancer-related death rate of all cancers, only topped by lung and breast cancer when combining the statistics from both sexes (Bray et al., 2018). Globally ca 350 000 deaths are resulted by PCa each year. In 2017 the corresponding mortality rate in Finland was 912 as reported by the Finnish Cancer Registry (Finnish Cancer Registry, 2019). The disease has been found to be less common in Asian countries compared to Europe and the Americas, which is likely due to dietary and lifestyle-related factors, such as large consumption of leafy green vegetables and soy products being part of the everyday diet in Eastern countries. Despite the high prevalence of PCa, not much is known about the cause of the disease. The incidence is however largely age-related, with some lifestyle factors such as low physical activity, smoking, as well as consumption of a high-fat diet greatly increasing the risk of developing the disease. Genetic predispositions have also been linked to an increased occurrence, yet only a small fraction of diagnosed prostate cancers are linked to hereditary factors (Wang, Zhao, Spring, & DePinho, 2018).

PCa progression is relatively slow, where the mean age of diagnosis is around 70 years. According to statistics gathered between 1999 and 2007, the 5-year survival rate of PCa in Finland is approximately 90%, having one of the best prognosis rates of all cancers overall in Europe (De Angelis et al., 2014). High-risk and metastasized PCa pose nonetheless a much greater mortality due to ineffective treatment methods, causing the 5-year survival rate to drastically drop to ca 30%. It is, however, important to note that some of the statistics of PCa incidence and prognosis during the past decades have been additionally influenced by the greatly developed diagnostics and

increased screening practices promoted for men. Screening for prostate-specific antigen (PSA) is one of the most commonly used efficient methods to detect early-stage PCa, since the patients have usually not developed clear physical symptoms yet at this point. PSA is a glycoprotein enzyme produced almost exclusively by the epithelial cells in the prostate gland, where its main physiological function is to liquefy semen for the sperm to flow more freely. Low levels of PSA are present in blood serum, but secretion is stimulated upon testosterone binding to cell surface receptors of prostate cells. Elevated serum PSA levels are used as a biomarker for prostate cancer since prostate tissue is increased due to tumor growth, however, high PSA status may also indicate nonmalignant conditions such as prostatitis (inflammation of prostate tissue) or benign prostate hyperplasia (prostate enlargement). Additionally, PCa can exist without increased PSA levels, such as the case in many aggressive or metastasized variants of PCa. Since PSA levels cannot accurately distinguish between benign and malignant prostate diseases, screening practices are found to be somewhat controversial due to potential false-positive results that can lead to painful biopsies and overtreatments for the patient (Lilja, Vickers, & Ulmert, 2008). For these reasons, research for novel biomarkers and secondary screening options are on the frontiers of PCa diagnostics. For instance, a variety of risk calculators (RCs) combined with patient PSA data and clinical parameters have been developed in order to maximize cost-effectiveness and predict biopsy outcome. Using this method of RCs in diagnostics alone is tempting due to the low cost, however, lack of accuracy is still a controversy (Osses, Roobol, & Schoots, 2019). The Stockholm3 model (S3M) is an example of a novel diagnostic tool for aggressive PCa that is based on blood tests that analyze plasma biomarkers in combination with genetic polymorphisms and clinical parameters. The S3M has been found to perform significantly better than PSA alone in disease prediction (Möller et al., 2019), however, the model is at the moment only in trial use in some Scandinavian countries and is more expensive compared to RCs. Multivariable risk assessment where data from clinical findings and RCs are compared to biomarker analysis would yield in highest diagnostic accuracy and patient benefit, however, the availability and high cost of these multivariable tests remains an issue. In addition, larger comparative cohort studies are still needed to fully assess the potential downsides and advantages of these new diagnostic methods.

1.2.1. Disease progression

Uncontrolled proliferation of abnormal prostate tissue cells leads to an enlarged prostate, causing symptoms such as trouble urinating, discomfort in the pelvic area, as well as potential blood in urine and erectile dysfunction. Patients have a tendency to seek medical help only when physical symptoms arise, at which point the cancer has usually already developed further from its initial stages and possibly even metastasized. Common areas for initial metastasis are bones and lymph nodes, from which the cancer can rapidly spread to other tissues and organs through the lymphatic system (Yoneda, 1998).

PCa is generally divided into two major phenotypes depending on the need for androgens for malignant cells to grow and survive. These are the androgen-dependent and androgen-independent cell types. *In vitro* and animal model studies use patient-derived cell lines such as LNCaP and PC-3 for modeling androgen-dependent and androgen-independent PCa, respectively. The PC-3 cell line, which was used in this study, originates from bone metastasis of human prostate adenocarcinoma (Kaighn, Narayan, Ohnuki, Lechner, & Jones, 1979). Balance of androgens such as testosterone and dihydrotestosterone (DHT) is an essential part of PCa development since they can regulate the ratio of proliferating and apoptotic prostate cells by either stimulating or inhibiting the processes on the molecular level through androgen receptor (AR) binding. It is currently not fully understood what the specific modifications are that drive prostate cancer cells to progress from an androgen-dependent to an independent state. Some possibilities for this development are the overexpression of AR or modifications caused by mutations that broaden the receptor's sensitivity to androgens and non-androgenic molecules present in the circulation, such as corticosteroids (Watson, Arora, & Sawyers, 2015). Importantly, AR can additionally be a target of growth factors such as insulin-like growth factor-1 (IGF-1), either through direct binding or downstream signaling via receptor tyrosine kinases (Wu et al., 2006). Overexpression of these growth factor receptors is often seen in prostate cancers, suggesting potential novel therapeutic targets outside of the AR (Heidegger, Massoner, Sampson, & Klocker, 2015; Krueckl et al., 2004). Progression from an androgen-dependent to an independent state seems to nonetheless be a multistep process, where an accumulation of mutations and modifications increases resistance to therapy and the overall survival of the cancer cells.

1.2.2. Treatment methods for prostate cancer

Current therapies for early-onset prostate cancer are chemo- and radiation therapy as well as castration. Androgen deprivation therapy (ADT) is regularly considered as a first-hand treatment option for patients with androgen-sensitive PCa, which aims to reduce the blood androgen levels and thereby suppress the spread of the disease. Even though ADT is commonly used, some controversy has been brought up during the past decades of its efficacy due to patients often relapsing with a more aggressive form of PCa. Deprivation of androgens through castration or ADT comes with additional adverse effects such as an increased risk for cardiovascular morbidity, loss of bone density, as well as psychological aspects including mood swings and lowered libido which can be compensated to an extent with other drugs but overall reduce the quality of life of the patient (Cary, Singla, Cowan, Carroll, & Cooperberg, 2014; Freedland, Eastham, & Shore, 2009; Saigal et al., 2007). Once the cancer develops into an androgen-insensitive stage, treatment methods become scarce and the risk for metastasis increases significantly. Unfortunately, no effective treatments for advanced prostate cancer are on the market at the moment, leading to a high mortality rate. Many of the late-stage PCa drugs such as docetaxel can initially be effective, however, cells frequently become resistant to the drugs during prolonged treatment and relapse commonly occur (Galletti, Leach, Lam, & Tagawa, 2017). Additionally, strong chemotherapeutics are harsh on the already compromised body, causing major side-effects and severely affecting the quality of life of the patients. Many doctors therefore focus also on easing the patient's discomfort and pain throughout the treatment process, often including drugs such as prescription opioids that bring yet another set of potential adverse effects to consider. For these reasons, there is an increasing need for novel potent drugs with minimal side-effects especially for metastasized PCa forms but also for the overall understanding of PCa epidemiology. Additionally, studying preventative methods for minimizing the incidence of PCa is especially important for men who are in risk groups of developing cancer, and generally for cutting down global treatment expenses.

Advancements in the therapeutic field of PCa has nonetheless been made during the past years. For instance, the widely used antidiabetic drug metformin has recently been shown to have great potential as a cytotoxic agent against PCa (Zaidi, Gandhi, Joshi, Smith, & Khan, 2019). This unexpected connection was initially noted since men with type II diabetes treated with metformin were found to have a

significantly reduced risk of developing prostate cancer. Similar results have since then been observed both *in vitro* and *in vivo*. The mechanism of action of metformin as an anticancer agent is still somewhat unknown, however, it is recognized that it targets the glucose and fatty acid metabolism as well as alters the androgen and IGF-1 signaling pathways (Loubière et al., 2015; Zaidi et al., 2019).

1.3. Intracellular signaling in regulating cancer cell proliferation and survival

The intracellular signaling network is the keystone for how cells regulate their survival, growth, metabolism, gene expression and other similar actions in regard to physical and chemical queues. Cell proliferation and growth are mainly initiated through the binding of extracellular growth factors (GFs) termed mitogens to their cell surface membrane receptors. These so-called growth factor receptors (GFR) are found in a vast variety of subtypes, which all consist of an extracellular ligand-binding domain, a transmembrane domain, and an intracellular catalytic domain which initiates the intracellular signaling cascades. Signaling through GFRs can regulate pathways controlling proliferation, survival, migration, metabolism, differentiation, and growth.

1.3.1. Insulin signaling in cell homeostasis

Insulin is a major mitogen that regulates multiple critical cellular processes, such as glucose and lipid metabolism, proliferation and survival (Fig. 2). The insulin signaling pathway is tightly interconnected with that of insulin-like growth factor-I and -II (IGF) since both mitogens have the ability to bind to the insulin receptor (IR), insulin-like growth factor receptor (IGFR), as well as hybrids of the two receptors (Antonino Belfiore, 2007). Typically, insulin-mediated signaling is more focused on metabolic processes, whereas IGF promotes mitogenic aspects. This distinction is however simplified to a high degree since the outcome seems not to be determined by receptor activation alone but rather a broad range of intracellular adaptor molecules and substrates (Siddle, 2012). Signal specificity and outcome distinguishing IR and IGF

activation and regulation are still however not thoroughly understood and require further research. For the sake of clarity, this thesis will focus on insulin signaling through IR, which nevertheless shares largely the same signaling pathways as IGFR.

IR belongs to a class of receptor tyrosine kinases, which consist of two subunits (termed α- and β-subunits) bound together by disulfide bonds that form either homo- or heterodimers together to create the complete receptor. At the time of ligand binding, changes in receptor configuration leads to autophosphorylation of tyrosine residues within the intracellular domains. This phosphorylation facilitates available binding sites for numerous signaling molecules that in turn can function as adaptors to carry the signal forward. The insulin receptor substrate (IRS) is a type of adaptor for IR signaling, which initiates downstream signal transduction through binding to and hence activating phosphoinositide 3-kinase (PI_3K). PI_3Ks are enzymes that phosphorylate the hydroxyl group on position 3 of the inositol ring on phosphatidylinositol molecules (PtdIns). PtdIns, in turn, are phospholipids residing at the cytosolic side of the plasma membrane. In this context, a phosphate group is added to phosphatidylinositol 4,5-bisphosphate (PIP_2) by PI_3K to create phosphatidylinositol 3,4,5-trisphosphate (PIP_3). The added phosphate group causes PIP_3 to bind and activate phosphoinositide-dependent protein kinase (PDK) 1 and -2.

The central target of PDK1/2 is the major kinase Akt (also termed protein kinase B or PKB). Akt is phosphorylated at sites Thr308 and Ser473 by PDK1 and PDK2, respectively (Nicholson & Anderson, 2002). This activation of Akt induces a variety of downstream signaling cascades, which regulate cellular processes such as survival and energy metabolism in response to insulin or IGF-I and -II (Fig. 2). Akt can additionally be activated via other signaling pathways. Some of the major targets

of Akt are for instance glycogen synthase kinase 3β (Gsk-3β), mammalian target of rapamycin complex 1 (mTORC1), and Bcl-2-associated agonist of cell death (Bad).

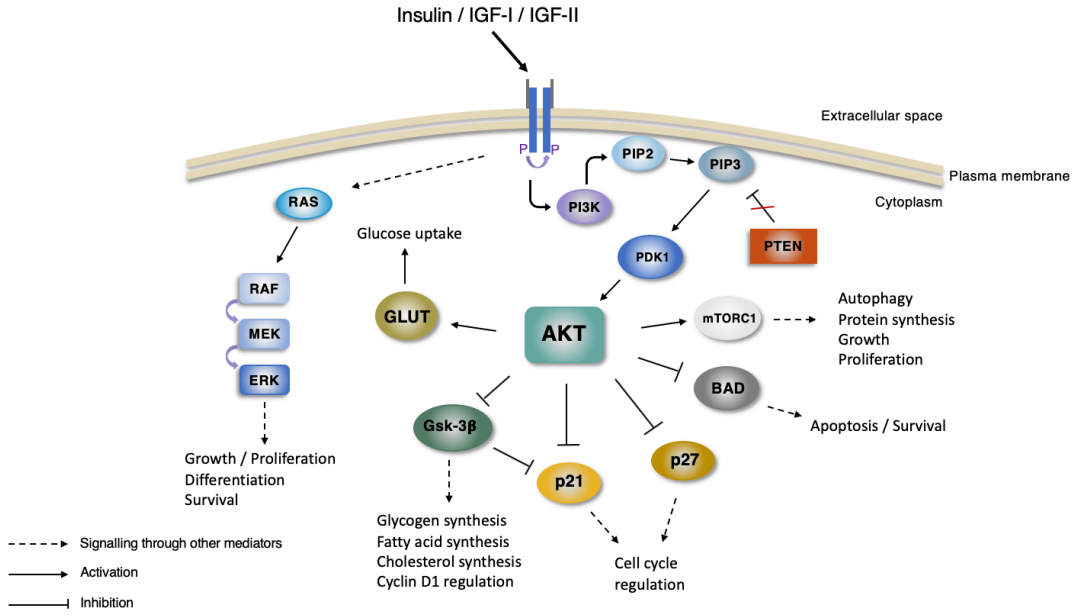

Figure 2 – Insulin signaling affects major PI_3K/Akt and Ras/MAPK-pathways. Receptor activation by ligand binding triggers downstream Ras and Akt mediated signaling, leading to regulation of cell survival, growth and proliferation, autophagy, glucose uptake through GLUTs, as well as alterations in the energy metabolism of the cell. Cancer cells commonly embody alterations in the insulin signaling pathway through mechanisms such as enhanced membrane receptor expression or lack of negative regulators like that of PTEN, leading to hyperactivity of kinases and consequently uncontrolled cell survival and division.

Gsk-3 is a highly conserved serine/threonine kinase that unlike many other kinases is in its active state when dephosphorylated. The kinase exists in two isoforms known as Gsk-3α and Gsk-3β, which together affect over 100 different substrates in the cell (Beurel, Grieco, & Jope, 2015). Gsk-3β was identified to act as a negative regulator of its major target glycogen synthase (GS), thereby inhibiting glycogen synthesis in resting cells (Embi, Rylatt, & Cohen, 1980). Phosphorylation of Gsk-3β at Ser9 by pAkt inhibits the kinase activity, which in turn stimulates glucose metabolism by phosphorylating and inhibiting GS, thereby initiating glycogen synthesis (Andjelkovich, Cohen, Alessi, Hemmings, & Cross, 1995). In addition to glycogen synthesis, Gsk-3β can regulate for example fatty acid and cholesterol synthesis. Furthermore, it is able to affect cell cycle progression by regulating cyclin D1 and cyclin-dependent kinase inhibitor 1A (p21) levels, which are important agents

in allowing the cell to proceed through the cell cycle. Phosphorylation of cyclin D1 by Gsk-3β initiates its proteasomal degradation, thereby halting cell cycle progression. In turn, inhibition of Gsk-3β by Akt phosphorylation induced through mitogenic signaling stabilizes cyclin D1 activity and allows the cell cycle to continue (Diehl, Cheng, Roussel, & Sherr, 1998). Similarly, phosphorylation of p21 by Gsk-3β triggers its degradation, whereas inhibition of Gsk-3β by stimulated PI_3K/Akt abolished Gsk-3β-mediated p21 degradation (Lothar Rössig, Cornel Badorff, Yvonne Holzmann, Andreas M. Zeiher, & Stefanie Dimmeler, 2002). The stability of p21 is nonetheless also affected by a variety of other substrates including Akt. Paradoxically, increased p21 activity is usually associated with cell cycle arrest, however, depending on the circumstances it can act as a tumor suppressor or an oncogene regarding cancer cells where p21 is often deregulated (Abbas & Dutta, 2009). Collectively, nonetheless, previous studies indicate that increased p21 activity upon treatment in metastatic PC-3 prostate cancers which normally express low levels of p21 leads to cell cycle arrest (Bott, Arya, Kirby, & Williamson, 2005; Gao, Zhang, Jiang, & Shi, 2003).

The PI_3K/Akt pathway is additionally able to initiate glucose uptake in muscle and adipose tissue via translocation of glucose transporter 4 (GLUT4) to the cell membrane, thereby increasing the energy production of the cell and balancing blood glucose levels (Chang, Chiang, & Saltiel, 2004). As previously reviewed, continuous uptake of glucose is necessary for glycolysis in cancer cells to meet their high energy demands, and consequently, a shutdown of glucose transporters halts this process. Insulin-mediated Akt signaling of glucose uptake is however only one of many regulatory mechanisms for this complex system.

Autophagy and cell growth are regulated mainly through the activity of the serine/threonine kinase mTOR, which exists in two complexes known as mTORC1 and mTORC2 (Yoon, 2017). Especially at times when available nutrients and growth factors are scarce, Akt regulates downstream mediators that lead to mTORC1 formation, thereby promoting autophagy (Saxton & Sabatini, 2017). Stimulated autophagy increases protein and lipid turnover by degrading cellular material in lysosomes. This breakdown of less vital material in times of e.g. starvation allows the building blocks to be used by the cell for synthesis of other more necessary proteins, lipids, and nucleotides.

Negative regulation of PI_3K/Akt is mainly based on dephosphorylation. PI_3K activity can be attenuated at the level of PIP_3, where the phosphatase and tensin

homologue (PTEN) inactivates the phospholipid PIP_3 by dephosphorylation, thus keeping PI_3K and consequently Akt in their inactive states (Maehama & Dixon, 1999). For this reason, PTEN is often referred to as a tumor suppressor since it inhibits Akt activity and therefore also its downstream targets, subsequently halting proliferation and growth (Fig. 2). However, PTEN is often mutated in many cancers (such as the case in prostate cancer), causing the phosphatase to dysfunction, leading to hyperactivity of Akt (McMenamin et al., 1999). Overall, PI_3K/Akt signaling is increased in PCa, especially in the androgen-independent type, and is in fact correlated with poor patient prognosis (Shazli N. Malik et al., 2002). One of the reasons why late-stage PCa tends to metastasize to the bone is the abundance of IGFs in the tissue. This signifies the importance of the IGF/insulin system on advanced PCa development and hence proposes an intriguing therapeutic target (Sachdev & Yee, 2007; Taichi Kimura et al., 2010). Furthermore, IGF-1 has also been found to stimulate integrin $\alpha v\beta 3$ expression in both DU-145 and PC-3 androgen-independent cells via the PI_3K/Akt pathway, thereby promoting migration and cell motility (Montagnani Marelli, Moretti, Procacci, Motta, & Limonta, 2006).

In addition to the PI_3K/Akt-pathway, IR and IGFR activation can stimulate the major Ras/MAPK-pathway (Fig. 2) (Boucher, Kleinridders, & Kahn, 2014). Once the tyrosine kinase receptor is autophosphorylated, adaptor molecules are recruited to the site which propagates downstream signaling, leading to activation of Ras. Ras belongs to a family of small GTPase enzymes, which are able to bind and hydrolyze guanosine triphosphate (GTP) to form guanosine diphosphate (GDP). By binding GTP, Ras is activated and able to transmit the signal forward to its downstream targets, these being a variety of cytoplasmic protein kinases. Subsequently, the *Ras*-gene is one of the most common oncogenes in human cancers and is also found to be mutated in a variety of prostate adenocarcinomas albeit at relatively low frequencies, where it is associated with increased proliferation and growth caused by enhanced signaling (Bakin, Gioeli, Sikes, Bissonette, & Weber, 2003; Cho et al., 2006). GTP-binding allows Ras to interact with the mitogen-activated protein kinase kinase kinase (MAPKKK), also known as Raf, thereby promoting its activation. Raf, in turn, phosphorylates its downstream target mitogen-activated protein kinase kinase (MAPKK) MEK, which completes the MAPK signal transduction pathway by adding a phosphoryl group to its final target mitogen-activated protein kinase (MAPK) called ERK. Once phosphorylated, ERK is able to regulate a variety of cellular processes mainly focusing on proliferation and cell growth, but also differentiation and survival. This is

accomplished by controlling transcriptional elements but also by interconnecting with other signal transduction pathways such as the discussed PI_3K/Akt (Aksamitiene, Kiyatkin, & Kholodenko, 2012).

1.3.2. Cell cycle progression and regulation

Suitable external and internal circumstances combined with mitogenic stimuli signals cells to grow and proliferate. During the time of preparation for cell division, cells undergo a variety of tightly regulated phases in the cell cycle (G1-, S-, G2-, and M-phase) which are separated by checkpoints, aimed to control for possible mistakes and unrestricted proliferation. Prior to dividing the cell into identical daughter cells during mitosis in the M-phase, the cell grows in size and multiplies its organelles in the G1-phase, replicating its DNA during the S-phase, and finally initiates mitosis in the G2-phase. When a cell is not actively dividing or receiving mitogenic signals, it resides in a G0-phase in quiescence, which is the state of most cells in the body that do not require constant renewal such as nerve or heart muscle cells in contrast to e.g. gut epithelia.

One of the main regulators of cell cycle initiation is the restriction point during the G0/G1-phase, where the cell commits to flow through from the G1- to the S-phase without further requirement of extracellular mitogens, thereby entering a state of no return. The major enzymes regulating cell cycle progression are cyclin-dependent kinases (CDKs), which in turn are controlled by oscillatory expression levels of cyclins D, E, A, and B as well as CDK inhibitors (Bai, Li, & Zhang, 2017). Basal low levels of cyclin D1 can be found in resting cells, however, at the time of mitogenic stimulation and progression through G1-phase, members in the cyclin D family (D1, D2, and D3) accumulate and are stabilized. Upon assembling with its catalytic CDK partners (CDK4 and CDK6) in the nucleus, cyclin D1 activates transcription of regulatory proteins and transcription factors involved in nucleotide metabolism and DNA synthesis, which subsequently allow the cell to commit to completing the remaining phases of the cell cycle. Due to the relatively fast turnover of cyclin D1, accumulation of the kinase is dependent on growth factor signaling which inhibits its ubiquitination and proteasomal degradation (Gwak, Kim, An, Dhanasekaran, & Song, 2017). Key factors keeping D-type cyclins active are involved in particularly the

PI_3K/Akt/Gsk-3β -pathway and the Ras/MAPK-pathway, which inhibit the phosphorylation of the cyclin during mitogenic signaling (Diehl et al., 1998; Lavoie, L'Allemain, Brunet, Müller, & Pouysségur, 1996). Numerous feedback loops and negative regulators of cyclin and CDK activity are however present, signifying that induction of cyclin D1 alone is not sufficient for the cell to transition from G1- into S-phase. Some of the main CDK inhibitors regulating cell cycle transitions are p21 and p27, as well as p15, p16 and p18. As mentioned previously, especially p21 is closely related to Akt signaling, and is able to promote cell cycle arrest when required.

Cyclin D1 is commonly overexpressed in malignant cells due to their upstream oncogenic signaling, thereby further promoting proliferation and tumorigenesis. Targeting cyclin D1 through suppression of its CDK partners by small molecules has shown promising results in cancer drug discovery and primary clinical trials, since this successfully blocks the cyclin D1 mediated cell cycle progression (Bai et al., 2017; Qie & Diehl, 2016). Yet, resistance to these types of drugs may occur and therefore treatment efficacy would benefit from combinational therapy instead of targeting only one specific molecule.

1.3.3. Intrinsic and extrinsic signaling pathways in apoptosis

Apoptosis, or controlled cell death, occurs when the cell no longer can sustain itself due to various intracellular or environmental factors. Generally, initiation of apoptosis is categorized into two signal transduction pathways: the intrinsic and the extrinsic pathway. In short, apoptosis via the intrinsic pathway is caused by intracellular factors such as DNA damage and oxidative stress. This disturbance influences members of the B-cell lymphoma (Bcl) family of proteins, which act either as pro- or anti-apoptotic agents (Singh, Letai, & Sarosiek, 2019). Bad is a member of the pro-apoptotic Bcl-2 protein subgroup, which in an unphosphorylated state can bind other Bcl proteins that reside at the mitochondrial membrane, such as homo- or heterodimers of Bcl-2-associated X protein (Bax) and Bcl-2 homologous antagonist killer (Bak). Once initiated, the intrinsic apoptosis pathway activates members of the pro-apoptotic Bcl-family proteins, causing a depolarization of the mitochondrial membrane and leading to an efflux of cytochrome c to the cytoplasm. Cytochrome c, in turn, recruits and activates caspases as well as forms the apoptosomes which further initiate and result

in cell death. Growth factor-stimulated Akt can regulate Bad activity through phosphorylation, which inhibits dissociation of Bax and Bak from the mitochondrial membrane and cytochrome c release (Fig. 2). Akt activity can, thus, to an extent control the balance between pro- and anti-apoptotic proteins and influence cell survival (Jeong et al., 2007). Hyperactive Akt, which is commonly observed in cancers, is therefore an intriguing drug target due to the ability of the kinase to promote survival signaling.

As the name implies, the extrinsic apoptosis pathway is initiated by external factors outside of the cell. These belong to the tumor necrosis factor (TNF) family of proteins, such as TNF-α, tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), and Fas ligand. Receptor binding at the cell membrane triggers the extrinsic apoptotic pathway, causing the formation of the death-inducing signaling complex (DISC) at the cytoplasmic side of the receptor. DISC formation serves as a platform for recruitment of caspases, which further prompt proteolysis and apoptosis (Falschlehner, Emmerich, Gerlach, & Walczak, 2007). Due to the nature of the extrinsic apoptosis pathway, it serves as a vital mechanism of the body's immune surveillance against malignant cells as well as pathogens by eliminating the potentially dangerous cells from the healthy ones. Importantly, however, many cancer cells tend to develop an immunity against death receptor-induced apoptosis. Prostate cancer cells generally express high levels of TRAIL receptors, but due to an altered pro-survival signaling are often insensitive to TRAIL-induced apoptosis (Chen, X. et al., 2001). Selective targeting of TRAIL receptors in cancerous cells is nonetheless an intriguing therapeutic area since the receptor stimulation seems to have minimal effect on healthy tissue. Yet, targeting TRAIL receptors in a variety of cancers appears not to be sufficient enough to kill the cells due to resistance, and combinational therapies with sensitizing agents are therefore explored, such as the use of common cytostatic drugs, natural compounds, or immunotherapeutic agents (von Karstedt, Montinaro, & Walczak, 2017).

Another type of cell death is necroptosis, which is a programmed form of necrotic cell death that is characterized by formation of the necrosome, or also known as the ripoptosome, through specific signaling patterns initiated via e.g. the TNF receptor or through cellular stress such as hypoxia, DNA damage, or reactive oxygen species (Galluzzi, Kroemer, Vandenabeele, & Vanden Berghe, 2010). In short, receptor activation at the plasma membrane leads to its association with adaptor proteins and the receptor-interacting serine/threonine-protein kinase 1 (RIPK1), which

in turn recruits RIPK3 to form the necrosome. Once the necrosome forms, it initiates oligomerization of the mixed lineage kinase domain-like pseudokinase (MLKL) through phosphorylation of the enzyme, which in turn causes MLKL to permeabilize organelles and to lyse the plasma membrane, subsequently leading to a type of cell death which is morphologically vastly different compared to apoptosis (Vanden Berghe, Linkermann, Jouan-Lanhouet, Walczak, & Vandenabeele, 2014). Necroptosis occurs hence via a caspase independent machinery, in contrast to intrinsic or extrinsic apoptosis, since in the presence of caspase 8 the necrosome cannot form due to RIPK1 cleavage by the caspase (Tummers & Green, 2017). The interconnectedness of apoptosis and necroptosis is, however, prominent considering that TNF-α can also induce e.g. NF-κB signaling to promote apoptosis. Exactly why necroptotic cell death occurs instead of apoptosis in certain conditions remains nevertheless largely uncovered. Previous studies have indicated that the metabolic state of the cell significantly affects this fate, for instance through ATP availability as well as necrosome proteins interacting with the autophagy machinery to promote necroptosis in certain conditions (Goodall et al., 2016; Kriel & Loos, 2019; Radogna, Dicato, & Diederich, 2015). Since necroptosis is primarily associated with an inflammatory response due to intracellular content leaking to the extracellular matrix as well as *de novo* production of pro-inflammatory mediators, selectively inducing necroptosis in cancer cells might promote immune cells to recognize malignant cells and thus improve antitumor immunity (Snyder et al., 2019). Targeting the necroptotic cell death machinery is therefore an intriguing approach for cancer treatment particularly to boost tumor immunotherapy, yet, it remains to be further scrutinized.

1.4. Lignans in human health and disease

1.4.1. Introduction to lignans and their biosynthesis

Lignans are a large group of polyphenolic compounds found throughout the plant kingdom where they are synthesized as secondary metabolites and have similar chemical and pharmacological profiles as other polyphenols such as flavonoids and stilbenes. Their main structure consists of two phenylpropanes linked in a β-β’ (8-8’)

fashion. Neolignans in turn are polyphenols where the coupling pattern of the dimers differ from β-β' linkage. Based on how oxygen is incorporated into the carbon skeleton and on the cyclization pattern of the molecule, lignans can be divided into eight main subgroups: dibenzylbutane, dibenzylbutyrolactone, furofuran, furan, aryltetralin, arylnaphthalene, dibenzocyclooctadiene, and dibenzylbutyrolactol (Umezawa, 2003). Additionally, the oxidation levels of the aromatic rings and the propyl side chains differ considerably within each subgroup and, therefore, lignans can be further categorized according to the oxidation state of the C9(C9') positions. These are lignans without 9(9')-oxygen, lignans with 9(9')-oxygen, and dicarboxylic acid lignans. For this reason, the structural diversity of lignans is enormous. Lignans with 9(9')-oxygen are the most studied in their biosynthesis pathway and chemical properties. Studies so far strongly suggest that the general biosynthesis of lignans in plant species emerges from enantioselective dimerization of two coniferyl alcohol units that form pinoresinol (furofuran) (Suzuki & Umezawa, 2007). This reaction occurs with the aid of a dirigent protein that enriches the yield of pinoresinol by dictating the stereochemistry of the compound synthesized. Depending on the plant and the subtype of dirigent protein expressed, the enantioselective dimerization leads to either (+)- or (-)-pinoresinol. Pinoresinol can then be further converted by e.g. several reduction or oxidation reactions to other lignans such as lariciresinol (furan), secoisolariciresinol (dibenzylbutane), and matairesinol (dibenzylbutyrolactone) (Markulin et al., 2019; Suzuki & Umezawa, 2007). Besides being the main monomeric precursor for lignan biosynthesis, coniferyl alcohol is also used by plants to synthesize lignins, which are complex cross-linked phenolic polymer structures that are essential e.g. for contributing to the mechanical strength and rigidity of cell walls by filling the spaces between cellulose, hemicellulose and pectin (Liu, Luo, & Zheng, 2018). Lignins are also crucial in the vascular tissue of plants due to their hydrophobic properties that help to conduct water more efficiently.

Lignans are found in a large variety in diverse plants and the quantity depends on the part of the plant, meaning the roots, stem, seeds, flowers, leaves, and fruits. Out of the hundreds of identified lignans, 7-hydroyxmatairesinol (HMR), matairesinol (MAT), secoisolariciresinol (SECO), pinoresinol (PINO), and secoisolariciresinol diglucoside (SDG) are examples of the most commonly found lignans in fiber-rich foods and plants (Fig. 3) (Smeds et al., 2007). The isolation of many lignans from nature has been challenging since many exist in glycosidic conjugates associated with fiber components of the plants. Coniferous trees, however, have been found to contain

many lignans in their unconjugated form, which makes the isolation and identification process less complex. Flaxseed is considered one of the richest dietary sources of lignans (mainly SDG and SECO), while other food sources such as legumes, rye, sesame seeds, and a large number of berries and vegetables also contain their own profile of varying lignans yet in much smaller quantities (Peñalvo, Haajanen, Botting, & Adlercreutz, 2005). The concentration of lignans in especially coniferous trees compared to foods such as flaxseeds is nevertheless enormous (Holmbom et al., 2003; Peñalvo et al., 2005; Smeds et al., 2007). Norway spruce (*Picea abies*) is known to contain extraordinary quantities of lignans especially in the knotwood of the tree (Holmbom et al., 2003). The amount of lignans fluctuate to some extent depending on the age of the tree, the climate it grows in, and possible fertilizers used (Piispanen, Willför, Saranpää, & Holmbom, 2008). For example, trees that grow in colder climates tend to have higher concentrations of lignans in their knotwood compared to those that grow in warmer climates (e.g. northern vs. southern Finland), which might be due to the heavy snow load that the branches must endure during winter. The lignan profile can additionally differ slightly depending on if the extraction process is done using dead or live knotwood. Generally, however, knotwood of *P. abies* contains 6-24% (w/w) lignans, of which 65-85% comprises of HMR (Ferreira et al., 2017; Willför, Hemming, Reunanen, Eckerman, & Holmbom, 2003). Additionally, knotwood also contain 2-6% (w/w) of various oligolignans, which are complex high-molar mass compounds resembling lignans but include several phenyl propane units (Smeds, Eklund, & Willför, 2016).

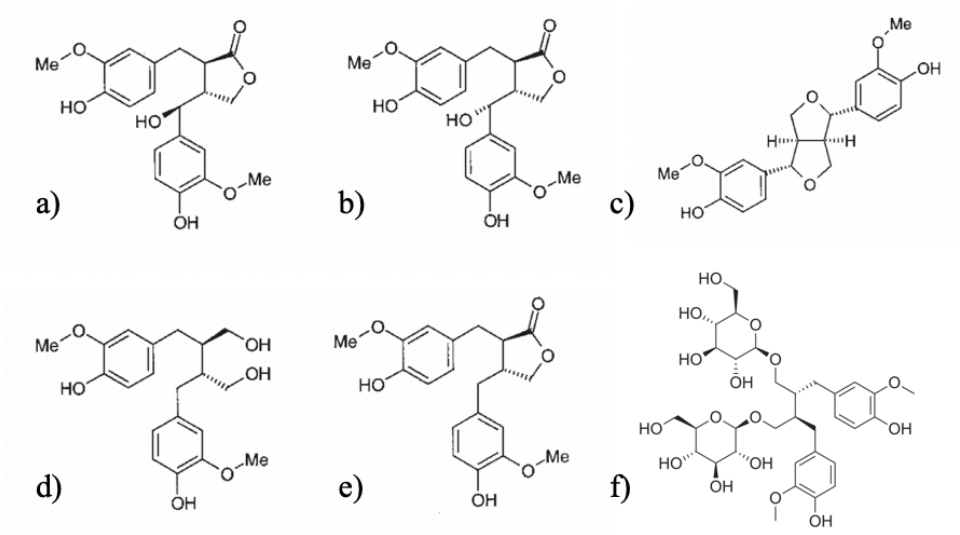

Figure 3 – Structures of commonly found lignans in the plant kingdom. a) 7-Hydroxymatairesinol (7-HMR 1); b) 7-Hydroxymatairesinol (7-HMR 2); c) Pinoresinol (PINO); d) Secoisolariciresinol (SECO); e) Matairesinol (MAT); f) Secoisolariciresinol diglucoside (SDG).

Due to the composition of the tree knots, they are thought as waste material at saw and pulp mills since they are not optimal starting components for paper production. Extraction of lignans from these knots is however a relatively simple process, which is why large-scale production of highly concentrated lignan-rich suspensions are obtainable also at a relatively low cost. It is estimated that hundreds of tons of lignan-rich extracts from knotwood could be produced per year from the leftover material from pulp and paper mills (Holmbom et al., 2003). Additionally, the individual lignans from the extract can be used as starting material for synthesis of other lignans. The extract can be used on its own or in combination with other active ingredients to increase the efficacy for therapeutic purposes. Since the lignan profile varies between tree species, combining extracts from e.g. Scots pine (*Pinus sylvestris*) with that of the Norway spruce opens up possibilities for fine tuning the lignan composition for personalized care. Furthermore, using a mixture of lignans instead of an individual compound may improve e.g. the overall toxicity against pathogens or malignant cells, in a similar fashion as seen in nature where plants with pathogen invasion trigger an increased production of various lignans and phenols to combat the microorganism through synergistic action (Harmatha & Dinan, 2003; Li et al., 2017; Sánchez-Elordi et al., 2020).

1.4.2. Dietary lignans, metabolism and benefits

In plants, lignans are thought to act as part of the immune system by actively reducing oxidization through free radical scavenging, as well as exerting antibacterial, antifungal and antiviral properties to combat potential pathogens. A variety of dietary lignans have been found to show similar health-promoting properties in humans for both disease prevention and treatment purposes (Adlercreutz, 2007). For instance, the potent antioxidant properties of lignans, which highly depend on the structure of the lignan molecule, reduce oxidative stress in cells by acting either as primary or secondary antioxidants, thereby reducing free radicals and hence preventing accumulation of damaging mutations and ultimately promoting longevity and health (Eklund et al., 2005; Hu, Yuan, & Kitts, 2007).

Dietary polyphenols in general are poorly absorbed by the small intestine due to being conjugated to e.g. glycosides or esters. For this reason, improved absorption is achieved with the help of gut microbes in the large intestine that convert the polyphenols to other metabolites in order to enhance the bioavailability in the body. Many of the lignans obtained from food sources are metabolized by the gut microbiome to so called mammalian lignans or enterolignans; enterolactone (ENL) and enterodiol (END) (Borriello, Stechell, Axelson, & Lawson, 1985). Conversion of lignans such as SDG to END and ENL can be achieved through intermediates produced by bacterial strains belonging e.g. to the *Peptostreptococcus* genus or *Eubacterium* genus (Li-Quan, Meselhy, Yan, Guo-Wei, & Masao, 2000), yet, the complex mechanisms governing the transformation of lignans to their mammalian metabolites in the gut is still largely unknown. Interestingly nonetheless, lignan intake as well as a high-fiber diet have been found to modify the microbiome and increase its diversity (Hullar et al., 2015). The gut microbiome in general is however influenced by many factors such as obesity, diet, environmental factors, as well as use of antibiotics, making it difficult to study the impact of lignans alone on the human gut microbiome in controlled settings. Antibiotic use especially has been linked to lower circulating enterolignan levels, which indicates a lower absorption capacity of the beneficial agents when the gut microbiome is affected. Pharmacokinetic analysis of HMR and its metabolite ENL in healthy postmenopausal women demonstrated that plasma levels of HMR peaked rapidly already at 1h after supplement ingestion, whereas ENL reached its maximum concentration at 24h during a 72h follow-up period (Udani, Brown, Tan, & Hardy, 2013). Similarly, a study analyzing SDG

absorption showed that serum levels of its converted metabolite SECO peaked at 5-7h following ingestion, after which ENL peaked around 24-36h (Setchell et al., 2014). This indicates clear differences in the bioavailability of lignans and that especially HMR can quickly be absorbed and used in the body before it is metabolized to enterolignans.

High dietary lignan intake has been associated with a protective role in many common diseases and conditions, such as diabetes, cardiovascular diseases and a variety of cancers, and therefore carry great potential therapeutic value (Adlercreutz, 2007; Basu & Maier, 2018; Biasiotto et al., 2018; Peterson et al., 2010). Interestingly, some lignans have also been found to pass the blood-brain barrier and have hence been studied for their function in prevention and care of brain tumors and neurodegenerative diseases, yet the mechanism of action of lignans on the molecular level against these types of diseases remains undefined (Das & Devi, 2019; Giuliano et al., 2020; Reddy et al., 2020).

1.4.3. Lignans as anticancer agents

Lignans are classified as phytoestrogens since they resemble the endogenous 17-β estradiol and are able to function as either agonists or antagonists to the estrogen receptor (ER). Due to the estrogenic activity, lignans have been found to exhibit protective and therapeutic features especially against hormone-related cancers such as breast and prostate cancer. Some of the epidemiological studies, however, show some controversies in the significance of reducing the cancer risk within a population due to the complexity of these types of studies as well as a lack of reliable analysis methods and biomarkers (Buck, Zaineddin, Vrieling, Linseisen, & Chang-Claude, 2010; Grosso et al., 2017). Nonetheless, the antineoplastic effects of lignans on a variety of cancers is prominent (Adlercreutz, 2007).

The antioxidant properties which prevent DNA damage and ROS production may be one of the main mechanisms in which lignans exert their anticarcinogenic activity and delay or prevent disease onset. Various lignans and their mammalian metabolites have, however, also been found to suppress also many of the vital signaling pathways related to growth, proliferation, and apoptosis in cancer cells. For instance, lignans has been associated with inhibiting the Wnt/β-catenin pathway in

colon cancer cells, suppressing MAPK activity as well as reducing expression of IGF-1R and EGFR in hormone-responsive breast cancer xenografts, modulating ERK/NF-κB/Snail signaling and downregulating mesenchymal markers vimentin and N-cadherin in triple-negative breast cancer, as well as altering FAK-Src signaling in lung cancer cells to reduce migration (Chikara, Lindsey, Borowicz, Christofidou-Solomidou, & Reindl, 2017; Mali, Joshi, Hegde, & Kadam, 2018; Saggar, Chen, Corey, & Thompson, 2010; Yoo et al., 2010). Furthermore, TNF-α-induced MAPK/NF-κB signaling in inflammation was observed to be reduced upon HMR treatment in *in vitro* conditions, indicating that HMR can possibly decrease low-grade inflammation and thereby prevent further stress in the body (Spilioti, Holmbom, Papavassiliou, & Moutsatsou, 2014; Yang et al., 2017). Targeting IGF/insulin transduction pathways in PCa has been of interest due to their increased activity during tumorigenesis. Lignans have in fact been shown to e.g. reduce IGF-1-mediated PI_3K/Akt activity, as well as sensitize PCa cells to TRAIL-induced apoptosis via inhibiting Akt phosphorylation and thereby modulating apoptotic signaling (Chen, L. et al., 2009; Peuhu et al., 2010). Other membrane receptor targets of lignans seem to be the G-protein coupled estrogen receptor (GPER), which is related to the estrogen receptor. Upon ENL binding, GPER was observed to cause sustained ERK phosphorylation and leading to suppressed proliferation in benign prostate hyperplasia (Chen, W. et al., 2016). Despite advancements in the field, many of the underlying mechanisms of action of lignans still remain somewhat unknown.

Most studies focus on determining the effects of individual compounds like SDG or ENL on cancer cells instead of using whole extracts or combinations of lignans. For this reason, it is of interest to determine if treatment efficacy for PCa can be increased by using multiple active compounds simultaneously to reach a synergistic effect, similar to other combinational therapies used at the moment in prostate cancer treatment and care.

2. Aims of the study

This master's thesis study aimed to identify and demonstrate the antineoplastic properties of a *Picea abies* knotwood extract on androgen-independent prostate

carcinoma cells (PC-3) *in vitro*. The main active agents in the extract were known to be lignans; polyphenolic compounds with phytoestrogenic activity. The precise composition of the extract was to be determined in order to identify the dominant lignans in the extract. In order to study the effects of the extract on prostate cancer cells, a cell viability assay, live-cell imaging as well as phase-contrast microscopy were to be performed. Another central objective of the thesis was to identify the intracellular signaling mechanisms governing the observed effects, with the focus on insulin receptor-mediated signaling. This was to be carried out by immunoblotting for key signaling proteins along with analyzing cell cycle progression using flow cytometry.

3. Experimental procedures

3.1. Gas chromatography – Mass spectrometry

Gas chromatography – mass spectrometry (GC-MS) is a commonly applied analytical method for identifying compounds from sample mixtures. The method is based on the principle that a sample is first run through gas chromatography, which separates the molecules from each other in the sample according to their chemical and physical properties in a long capillary column, after which the molecules are ionized and identified by mass spectrometry according to their mass-to-charge ratio.

Since the ready-made diluted stock solution of *Picea abies* knotwood extract (obtained from Oy Granula Ab Ltd.) was diluted in 1,3-propanediol, it was not optimal for GC-MS analysis due to propanediol interfering with the readout. For this reason, undiluted thick *P. abies* pure extract (PE) from the same lot (Oy Granula Ab Ltd.) was used instead for determining and identifying of the lignans and their concentrations in the diluted extract.

Sample preparation was conducted by weighing 6.46 mg of PE and placing it in an oven under vacuum (40 °C) overnight for drying. The dry weight of the extract was measured the following day to be 82%. A stock solution for GC-MS reaching a concentration of 3.34 mg/ml was made by dissolving 13.35 mg dry extract (equivalent

of 16.28 mg of pure extract) in 4 ml acetone. 200 µL of the stock solution (equivalent of 0.668 mg dry extract) was prepared for the analysis by incorporating 2 ml of internal standard solution (containing four pure standards of 40 µg each) to the sample and evaporating the solvent using nitrogen gas. Silylation was conducted by adding 20 µL dry pyridine, 80 µL BSTFA (N,O-Bis(trimethylsilyl)trifluoroacetamide), and 20 µL TMCS (trimethylchlorosilane) to the sample, and incubating it in an oven (70 °C) for 30 min. Silylation was done in order for the components in the sample to become more volatile for GC. The prepared sample was moved to an appropriate GC-vial and 1 µL of the sample was analyzed initially by short column GC-FID, followed by long column GC-FID, and subsequently using GC-MS in order to identify the peaks in the chromatograms. The concentration of the total and individual amount of lignans in the PE as well as in the diluted stock solution used in the *in vitro* experiments could thereby be calculated.

3.2. Live-cell imaging of PC-3 cell proliferation

Live-cell imaging using IncuCyte S3 system is a microscopy method in which real-time images of live cells can be obtained during long periods from hours up to days in sterile conditions. For the experiments in this study, PC-3 cells were cultured in RPMI-1640 medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. One day prior to the treatment, the cells were plated (20 000 cells/well) on glass coverslips on 24-well plates (Falcon) and incubated overnight at +37 °C (5% CO_2) in order for the cells to attach to the coverslips. The following day, the cells were washed with Dulbecco's phosphate-buffered saline (PBS) and treated with 0-60 µM (HMR) *P. abies* knotwood extract (Oy Granula Ab Ltd.) or solvent control (1,3-propanediol) in the corresponding concentrations. Additionally, a control using only growth medium was used. All samples were tested in triplicates. The cells were imaged for 48h with 2h intervals (9 images per well) using IncuCyte S3 Live-Cell Analysis System (Essen BioScience) to measure the proliferation of the cells. The experiments were repeated at least three independent times prior to performing statistical analysis of the proliferation data.

3.3. Flow cytometry analysis of cell cycle using propidium iodide staining

Flow cytometry is a technique widely used in research due to its diverse application options and its ability to quickly analyze large cell samples. The main function of a flow cytometer is to detect and measure different chemical and physical features of cells or particles within a fluid sample by individually sorting cells. Most often cells are labeled with specific fluorescent markers which are then detected by the machine and processed accordingly. In this case, propidium iodide was used as a stain to separate treated cells according to their DNA content, which corresponds to the cell cycle phase in which they reside.

In practice, PC-3 cells were seeded on 6-well plates (Falcon) with 200 000 cells/well in RPMI-1640 medium (Sigma Aldrich) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin and incubated in +37 °C, 5% CO_2 overnight. The following day, the cells were treated with the *P. abies* knotwood extract in concentrations of 0, 24 and 60 µM (HMR) and incubated for an additional for 24h. Cells were harvested the next day by trypsinization and centrifuged down (1200 rpm for 5 min in +4 °C), after which the pellets were resuspended in PBS (Biowest). The samples were then fixed for 15 min at RT using Cytofix/Cytoperm™ (BD Bioscience). After fixation, the cells were pelleted and washed with 1x BD Perm/Wash™ buffer (diluted in distilled water). The pellets were resuspended gently in FxCycle PI/RNase Staining Solution (ThermoFisher Scientific) and incubated for 30 min at RT in the dark in order to stain the cells with propidium iodide. Once stained, the samples were spun down and the pellets were resuspended in FACS buffer (2% FCS + 0.4% 0.5 M EDTA in PBS) prior to analyzing them using flow cytometry (BD LSR Fortessa analyzer). The experiment was repeated three independent times, where at least 20 000 events per sample were collected each time.

3.4. Western blot analysis of insulin signaling

In order to determine if the extract affects insulin receptor signaling and its downstream targets, western blot analysis was performed. The method is widely used

to detect amounts of a protein in a sample, its expression level, or evaluate the size of the protein of interest. Initially, the sample denatured and mixed with reducing agents to unfold the proteins from their 3D structure. Gel electrophoresis is next used to separate the proteins in the sample on a gel according to their molecular size. SDS-PAGE (sodium dodecyl sulfate - polyacrylamide gel electrophoresis) is most commonly used for this, where the SDS coats the proteins with a negative charge. The proteins from the gel are then transferred to a nitrocellulose or PVDF membrane, after which the membrane is incubated in a blocking solution to minimize unspecific binding and background signal. To detect the protein of interest, the membrane is incubated with a specific primary antibody that binds the protein. Lastly, any unbound antibody is washed away, and the membrane is coated with a secondary antibody that recognizes only the primary antibody. Secondary antibodies are usually conjugated to compounds such as horseradish peroxidase (HRP) that allow detection and visualization of the bands on the membrane.

For western blot experiments, PC-3 cells were plated on 6-well plates (120 000 cells/well) in complete RPMI-1640 medium and let attach to the plate for ca 6h as previously described. The medium was then changed to serum-free medium (SFM) prior to incubating the cells in +37° C (5% CO_2) overnight. The next day, the cells were treated with 0, 12 μM, 24 μM and 60 μM (HMR) extract for 15 min, 30 min and 60 min at +37 °C. Once the time points were reached, insulin was added (0.1 μM) and the cells were incubated for an additional 15 min for receptor activation (Fig. 4). Cells were harvested by removing the old media and washing them with cold PBS (Biowest). 1x Laemmli buffer (containing 3x Laemmli buffer + MQ-H_2O + β-mercaptoethanol) was thereafter added in order to lyse the cells and to disrupt any disulfide bonds in the proteins. The samples were collected by scraping, after which the whole cell lysate samples were boiled at +98 °C for 5 min for protein denaturation. SDS-PAGE was performed on 10-12.5% resolving gels in order to separate the proteins from each other. The proteins were transferred to polyvinylidene difluoride (PDVF) membranes (Thermo Fisher) and blocked for unspecific binding using 5% non-fat milk in 0.3% TBST. For detection of total and phosphorylated levels of proteins, antibodies against Akt, phospho-Akt (Ser473 and Thr308), phospho-Gsk-3β (Ser9), Erk1/2 (p44/42 MAPK) and phospho-Erk1/2 (p-p44/42 MAPK) with the corresponding HRP-conjugated secondary antibodies were used by first incubating the membranes for 1h in primary antibody, followed by washing with 0.3% TBST and 1h

incubation in secondary antibody. Primary antibody for p21 was additionally used to study downstream signaling of Akt. For these experiments, PC-3 cells were treated with the extract for 24h at +37° C followed by 30 min incubation with 0.1 µM insulin, instead of a 15-60 + 15 min treatment period. Enhanced chemiluminescence (ECL) was used to detect the protein bands with ChemiDoc MP Imaging System (Bio-Rad). Hsc70 was used to control for sample loading in each blot. PageRuler^{TM} Plus Prestained Protein Ladder (Thermo Fisher) was used as a marker for the molecular weight of proteins in all experiments. Each experiment was repeated at least three independent times. All primary antibodies were diluted according to the manufacturer's protocol in 1% BSA + 0.02% NaN_3 in PBS (Supplementary information). Secondary antibodies were diluted 1:10 000 in blocking buffer (5% non-fat milk in 1x TBS + 0.3% Tween 20).

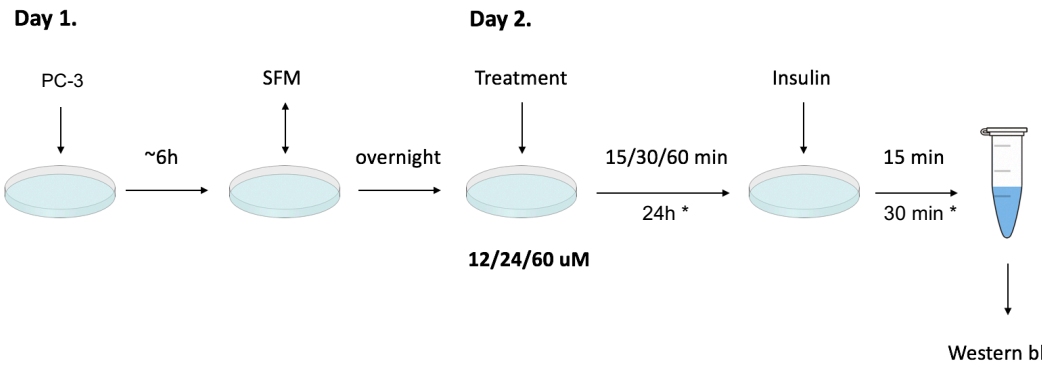

Figure 4 – General experimental setup for studying the insulin signaling pathway in *P. abies* extract treated PC-3 cells using western blot. Treatment periods varied from 15 min up to 24h followed by 15- or 30-min incubation with insulin depending on the protein analyzed. * indicates treatment times for studying p21 expression levels. SFM = serum-free medium.

3.5. Cytotoxicity assay of *P. abies* knotwood extract on serum-starved PC-3 cells

A cytotoxicity assay is generally used to measure the toxic effect of a compound on cells and to determine the optimal concentration to use. In this study, a reagent containing highly sensitive water-soluble tetrazolium salt (WST-8) was used to

evaluate the toxicity of the *P. abies* knotwood extract on PC-3 cells. The reagent enters cells and is reduced by dehydrogenase activity during cell respiration to produce a yellow-color formazan dye, which can then be evaluated and quantified by measuring the absorbance. The amount of the formazan dye is directly proportional to the number of living cells in the sample, meaning that the lower the absorbance, the lower the amount of living cells are in the sample.

In practice, PC-3 cells were seeded with 10 000 cells/well on a 96-well plate (Falcon) in RPMI-1640 medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. The cells were let attach for 6h prior to changing the medium to serum-free RPMI-1640 and incubating them overnight in +37 °C, 5% CO_2. After overnight serum starvation, cells were treated with *P. abies* knotwood extract in concentrations of 0-70 µM (HMR) in serum-free medium in replicate samples and incubated for 24h. The old medium was thereafter discarded and substituted with 100 µL 10% Cell Counting Kit-8 reagent (Dojindo Molecular Technologies) diluted in serum-free RPMI-1640 medium. The samples were incubated for an additional 1h prior to analyzing cell viability by measuring the absorbance at 450 nm using a microplate reader (Hidex Sense) according to the manufacturer's protocol. Non-treated cells were used as a negative control. The experiment was repeated at least three independent times for accurate cytotoxicity analysis. The IC_{50}-value was calculated using GraphPad Prism 6 software.

3.6. Statistical analysis

All statistical analyses shown in this thesis were carried out using the GraphPad Prism 6 software. For measuring inhibition of cell proliferation (described in 3.2 and 4.2), statistical analysis was calculated with one-way ANOVA followed by Dunnett's multiple comparison test, where ** represented a significance value of $P < 0.01$. In a similar fashion, cell cycle data (described in 3.3 and 4.3) was analyzed by one-way ANOVA followed by Tukey's multiple comparison test. Here, *** and **** represented significance values where $P < 0.001$ and $P < 0.0001$, respectively. Due to large variances within datasets in western blot experiments (described in 3.4 and 4.4), no significant statistical values were achievable using multiple t-test.

4. Results

4.1. Gas chromatography – Mass spectrometry analysis of *Picea abies* knotwood extract reveals HMR as dominant active lignan

The peaks in the chromatograms were identified from the GC-MS analysis by comparing the peaks to an existing database (Fig. 5). Concentrations of individual substances in the pure extract (PE) were calculated by dividing the individual peak areas with the peak areas of the internal standards (IS) and multiplying the value with the amount of added IS (40 µg). Lastly, the calculated value was divided by the used weight of the original dry extract (0.668 mg). Betulinol and terephtalic acid 21:0 were used as the IS for the lignan concentrations, where the final calculated value was additionally multiplied by 1.2 to correct for the detector response.

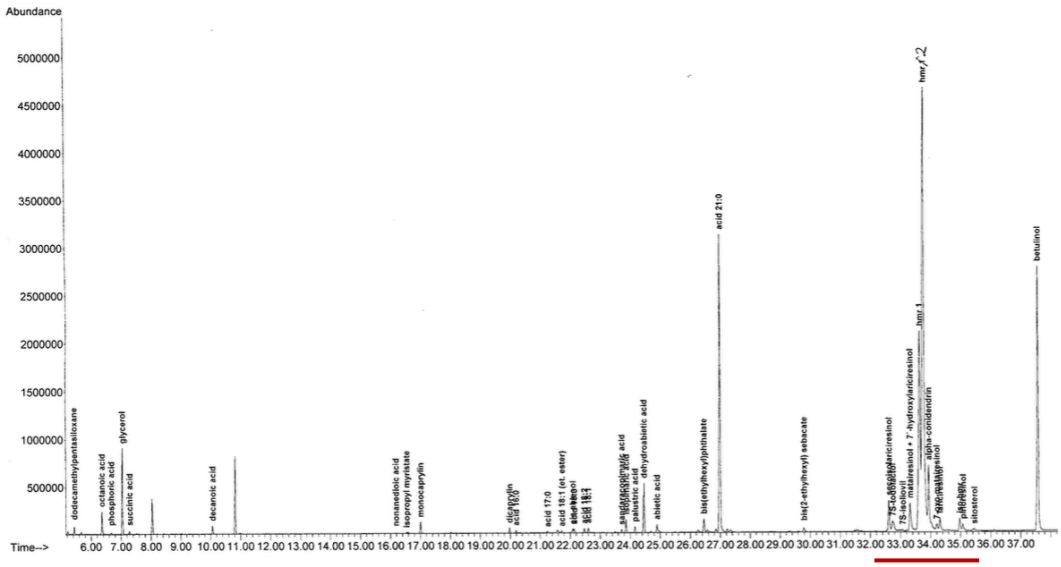

Figure 5 – GC-MS analysis identified HMR in its two isoforms (1/2) as the main lignans in *Picea abies* knotwood extract. Other lignans found were, for example, α-conidendrin, pinoresinol, and secoisolariciresinol. The chromatogram represents the compounds found in the dry PE and their relative abundance, from which the concentrations could be calculated. Each peak (compound) was identified with the help of an available library of known compounds in *P. abies* knotwood extract as well as the internal standards. Redline below the

chromatogram indicates the retention time when individual lignans from the extract were eluted.

The identified compounds in the PE from GC-MS analysis are listed in Table 1. The total lignan concentration of the dry extract sample was calculated to 31.3% (313 mg/g). Of the individual lignans, hydroxymatairesinol (HMR) was found to have the highest concentration in the dry extract (22.43% in total when combining the two isomers). Other lignans such as α-conidendrin (1.86%), matairesinol (1.68%), and secoisolariciresinol (1.40%) reached much lower concentrations compared to that of HMR. 71.66% of the total lignan amount consisted of the two HMR isomers, indicating that HMR is the dominant active lignan in the extract. Trace amounts of other non-active compounds in the extract found were e.g. a variety of fatty acids, diterpenes, as well as diglycerides. High molar mass material such as oligolignans and other large complexes could not be eluted and identified through the used chromatography method due to the low volatility and weight of the complexes.

Table 1 - Results from GC-MS analysis of dry *Picea abies* knotwood extract listing the compounds and their percental concentration in the dry extract. The concentration in percent can be changed to mg/g by multiplying with 10.

Compound	**% of dry extract**
Glycerol	**0.317**
Fatty acids:	
Octanoic acid (Caprylic acid)	0.135
Decanoic acid	0.0837
Monocaprylin	0.147
Dicaprylin	0.0997
Palmitic acid	0.0624
Heptadecanoic acid	0.0316
Ethyl oleate	0.0780
Linoleic acid	0.161
Oleic acid	0.142
Total of fatty acids:	**0.940**
Diterpenes (all rosin acids except for abienol):	
Cis-abienol	0.255
Sandracopimaric acid	0.108
Isopimaric acid	0.317
Palustric aicd	0.152
Dehydroabietic acid	0.932
Abietic acid	0.247

Total of diterpenes:	**2.01**
Lignans:	
Secoisolariciresinol	1.40
7S-Todolactol	0.245
Matairesinol	1.68
HMR1	7.83
HMR2	14.6
Alfa-Conidendrin	1.86
7-Oxomatairesinol	0.868
Lariciresinol	0.639
iso-HMR	0.937
Pinoresinol	0.302
9´-Hydroxylariciresinol?	0.338
Unknown lignan	0.606
Total of lignans:	**31.3**
Beta-Sitosterol	0.472
Bis(2-ethylhexyl)phtalate (plasticizer)	0.399
Bis(2-ethylhexyl)sebacate (plasticizer)	0.254
Unidentified compounds (long column)	0.618
Diglycerides	5.28
Sterylesters	1.89
Total of GC-eluted compounds:	**43.5**
High molar mass materia (non-GC eluted)	**56.5**

The GC-MS data provided the necessary information needed to calculate the actual extract concentration of the PE and its diluted stock solution (used in the *in vitro* experiments), as well as the total amount of lignans and HMR in these. The ready-made diluted stock solution used in this thesis study for *in vitro* experiments contained 8.75 wt% PE diluted in 1,3-propanediol according to the manufacturer Oy Granula Ab Ltd. Since 18% of the PE was found to be solvent after drying (water + ethanol leftover from knotwood extraction process), it could be subtracted from 8.75 wt% to obtain the actual concentration of the PE in the diluted stock (18% of 8.75 wt% = 7.175 wt%).

Since HMR was found to be the dominant lignan in the knotwood extract, its concentration in the diluted stock solution needed to be determined in order to grasp how much of the active compound was present in each cell sample. The results from GC-MS analysis revealed that the total HMR concentration in the dry extract was 22.43% when combining the isomers (Table 1). In the diluted stock solution, the concentration of HMR thus equals 0.2243 x 7.175 wt% = 1.609 wt%. When converting 1.609 wt% to mM by using the density of 1,3-propanediol + PE (δ = 1.07 g/ml), an HMR concentration of 45.985 mM was obtained (Fig. calculation). The lignan concentrations in the *in vitro* results in this thesis hereafter refer to the concentration

of HMR lignan in the samples, instead of the extract as a whole since HMR is the major active compound.

$$\frac{1.609\ g\ \times \frac{1\ mol}{374.389\ g}}{100\ g\ \times \frac{1\ L}{1070\ g}} = 0.04598 \frac{mol}{L} = 45.985\ mM$$

4.2. *P. abies* knotwood extract suppresses PC-3 cell proliferation in a concentration-dependent manner

48h live-cell imaging of PC-3 cells revealed significant differences in the proliferation between the extract-treated and control cells. The inhibitory effect on cell proliferation was observed to be concentration-dependent (Fig. 6A). Even though the proliferation of PC-3 cells was halted already with the lowest concentration (24 μM), only the highest concentration used in the study showed statistically significant differences when measuring the end-point cell confluency after 48h (Fig. 6B). Relatively notable variations were however observed in the confluency curves when comparing the same samples between the independent experiments, which resulted in larger sample variation in the statistics (Fig. 6B). Nonetheless, when treating the cells with 60 μM, the proliferation curves were always strikingly plane, and no clear cell proliferation could be observed during the 48h treatment time in these samples. Interestingly, these cells seemed to always have a slight drop in their confluency between 5 and 25h of treatment (Fig. 6A). This might have been caused by an initial shock to the extract, which may have initiated some cell death. 1,3-Propanediol, which was used for the dilution of the pure knotwood extract by the manufacturer, did not have a significant effect on the growth of PC-3 cells even in high concentrations as expected.

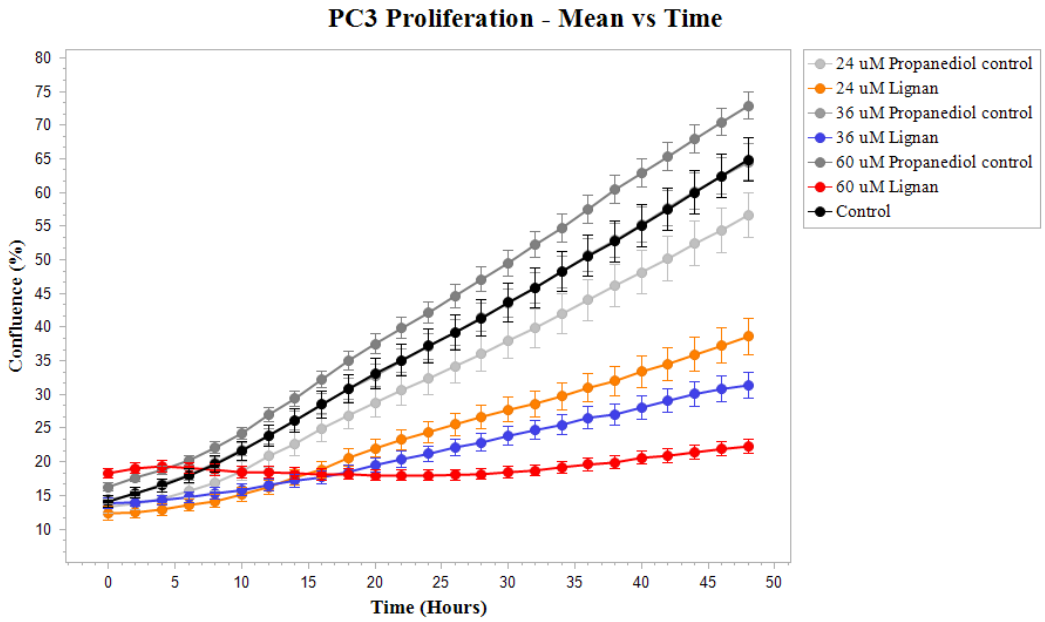

Figure 6A - *P. abies* knotwood extract represses PC-3 proliferation in a concentration-dependent manner. The graph represents an example result of confluency (percent of well surface covered in adherent cells) from live-cell imaging during 48h treatment, where each point is the mean of triplicate samples (±SD). Clear inhibition of proliferation was observed, wherein the cells treated with the highest extract concentration showed minimal growth during the treatment period. The corresponding solvent control (1,3-propanediol) treatments did not demonstrate any significant effect on PC-3 cells as expected. The control sample (black line) contained PC-3 cells grown in complete growth medium without any treatment.

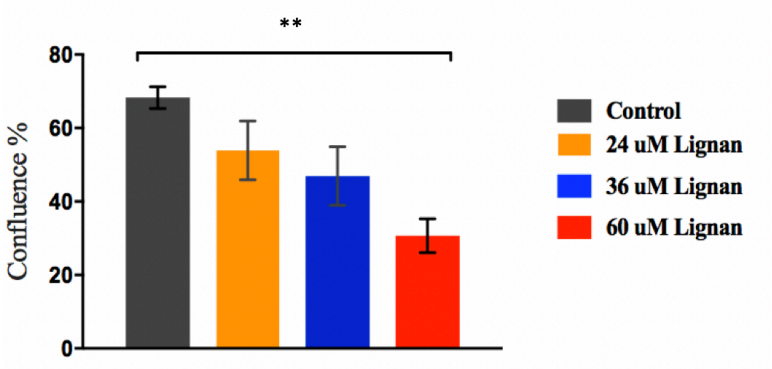

Figure 6B – Quantification of end-point data (at 48h) from live-cell imaging revealed a statistically significant concentration-dependent proliferation inhibition of *P. abies* knotwood extract on PC-3 cells. The quantification is a representation of the mean confluence of treated cells from three independent experiments of triplicate samples ±SEM. The control sample represents PC-3 cells grown in complete medium without any treatment. Statistical analysis was calculated with one-way ANOVA followed by Dunnett's multiple comparison test using the GraphPad Prism 6 software. ** Represents a significance value of $P < 0.01$.

Imaging material of up to 48h treated PC-3 cells indicated that the cells did not seem to undergo apoptosis or necrosis upon treatment, but rather experienced a halt in cell division (Fig. 7A-B). Slight morphological changes could nonetheless be observed (Fig. 7A). These observations lead to the postulation that HMR and/or other lignans in the extract inhibit key regulatory signaling pathways involved in cell proliferation and cell cycle progression but do not necessarily induce apoptosis in these conditions. Additionally, the treated cells were observed to be not as mobile compared to untreated once, which begs the question if cytoskeletal and adhesion proteins are affected upon treatment with lignans.

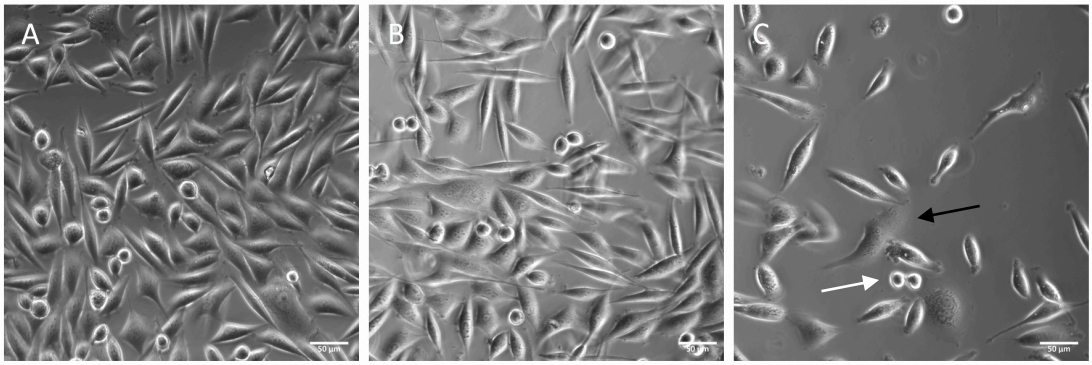

Figure 7A - PC-3 cells treated with the extract (24 μM) for 48h were not observed to undergo cell death through apoptosis or necrosis under normal culture conditions. The treated cells, however, did exhibit some morphological differences such as being slightly wider and more spread out (black arrow). Additionally, fewer actively dividing cells were seen in the treated cells (white arrow), correlating with previous data showing proliferation suppression. A) 1,3-Propanediol control B) Medium control C) 24 μM extract treatment. Phase-contrast images were taken with 20x magnification using Zeiss Axio Vert A1 microscope. The scalebar in the images represents a measure of 50 μm.

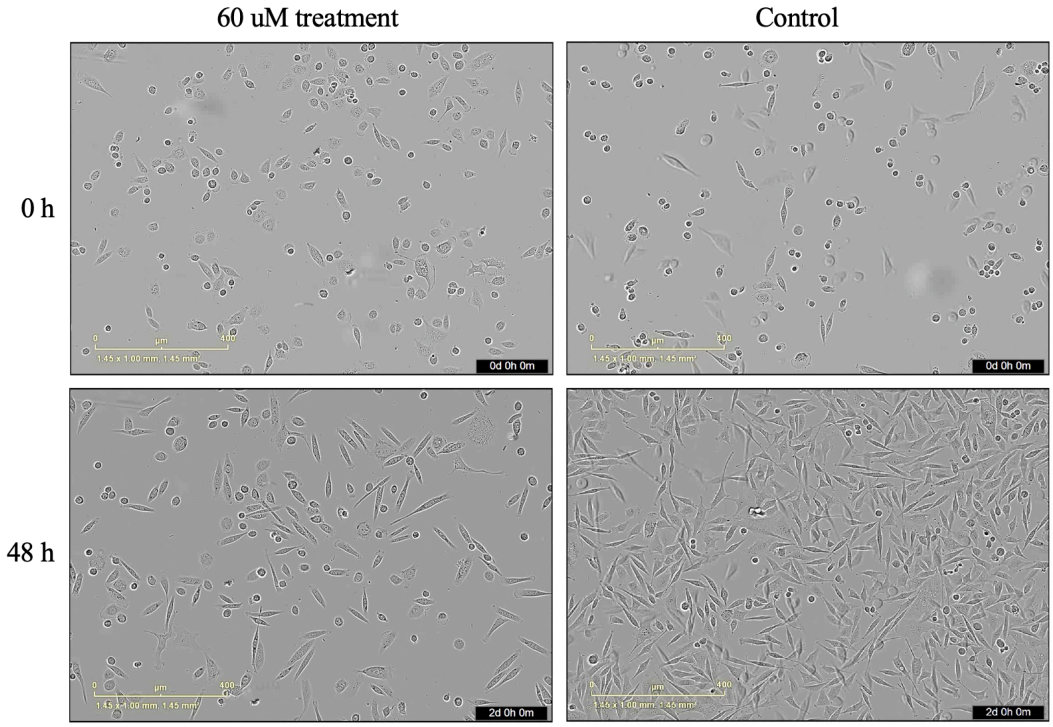

Figure 7B - Live-cell imaging during 48h revealed significant suppression of PC-3 cell proliferation upon extract treatment. As with phase-contrast images, no clear signs of apoptosis or other cell death was observed during treatment in normal culture conditions. Images were taken using IncuCyte S3 imaging system with a 10x objective. The scalebar in the images represents a measure of 400 μm.

4.3. *P. abies* knotwood extract initiates cell cycle arrest in G0/G1-phase in PC-3 cells

Since cell proliferation was observed to be halted in *P. abies* knotwood extract-treated PC-3 cells, cell cycle analysis using propidium iodide staining was conducted in order to distinguish in which phase the cells were arresting. After 24h treatment with both 24 μM and 60 μM, PC-3 cells were significantly accumulating in the G0/G1-phase of the cell cycle, which also correlated with a reduced number of cells in the S- and the G2/M-phases (Fig. 8). The non-treated control samples were relatively normally distributed between the different phases (Fig. 8). PI staining showed no clear difference in the number of sub-G0/G1 content (dead cells) between the treatment groups (data not shown). Intriguingly, there was no statistically significant difference

in the quantity of cells arresting in the G0/G1-phase between the two extract concentrations, indicating that already lower concentrations of the extract are able to achieve substantial cell cycle arrest. However, previous data of treated PC-3 cells showed only a slight statistically non-significant inhibition of proliferation with 24 μM compared to the effects of 60 μM treatment during 48h. It is probable that the cells are capable of overcoming the cell cycle arrest caused by low lignan concentrations after initial 24h treatment, but higher concentrations are needed to completely shut down proliferation during continued treatment periods. Cell cycle arrest in the G0/G1-phase nonetheless indicated that the lignans in the extract were able to suppress proliferation by affecting key factors regulating cell cycle progression from the G1- to the S-phase.

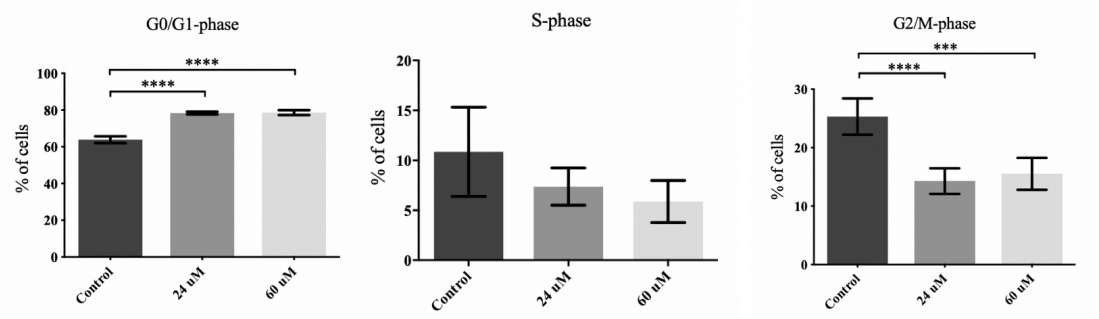

Figure 8 – *Picea abies* extract induces cell cycle arrest in the G0/G1-phase in PC-3 cells. After 24h incubation, a significant amount of the cells treated with the extract were accumulating in the first phases of the cell cycle, which was also seen a reduced amount of cells in the S- and the G2/M-phases. Interestingly, no difference between the two concentrations used was observed, indicating that maximum cell cycle inhibition is reached already with low extract concentrations during 24h treatment, but higher concentrations are needed for a more sustained inhibition during longer treatment times. The graphs represent the percentage of cells in each cell cycle phase from three independent experiments ±SEM. Statistical analysis was carried out by one-way ANOVA followed by Tukey's multiple comparison test using GraphPad Prism 6 software. *** and **** represent a significance value where $P < 0.001$ and $P < 0.0001$, respectively. No significance was found between treatment groups in the S-phase.

4.4. Insulin-induced Akt and ERK signaling are affected by *P. abies* knotwood extract in a concentration-dependent manner

On the grounds that proliferation of PC-3 cells was affected by the *P. abies* knotwood extract, key signaling nodes downstream of the insulin receptor governing cell growth and survival were analyzed through western blot. The insulin signaling pathway was chosen due to it being closely related to that of IGF, which in previous studies has been shown to be affected by some lignans.

Western blot analysis displayed that pAkt levels and its downstream element pGsk-3β were enhanced with the addition of insulin in PC-3 cells (Fig. 9A) Yet, insulin did not for unknown reasons stimulate ERK phosphorylation in PC-3 cells, unlike seen in previous studies with IGF-I. Treatment with the extract in concentrations of 60 μM, however, substantially inhibited Akt phosphorylation of both Ser473 and Thr308, as well as pGsk-3β (Ser9) already at 15 min (Fig. 9A). Interestingly, treatment with 24 μM, on the contrary, resulted in an increase in both Akt and ERK phosphorylation (Fig. 9A), which was also confirmed with quantification of pAkt data (Fig. 9B). This was unexpected since previous results showed a distinct inhibition of proliferation and cell cycle arrest with 24 μM (Fig. 6-8), suggesting that kinase signaling would similarly be restrained as seen with 60 μM treatment. There may be many reasons why such contradictory observations were seen downstream of the insulin receptor. Since the extract contains a variety of compounds, it is highly likely that some may be targeting other receptors than the IR, such as IGFR or hybrids of IR/IGFR, and this way stimulate phosphorylation of Akt and ERK in a concentration-dependent way, in a similar fashion as the effects of enterolignans on estrogen receptors. The enhanced kinase activity may alternatively be caused by intracellular feedback loops or other unidentified mechanisms that are influenced by lignans, nevertheless leading to cell cycle arrest in these conditions. Another possible scenario of this could be thorough binding to the GPER receptor, which is known to cause sustained ERK phosphorylation.

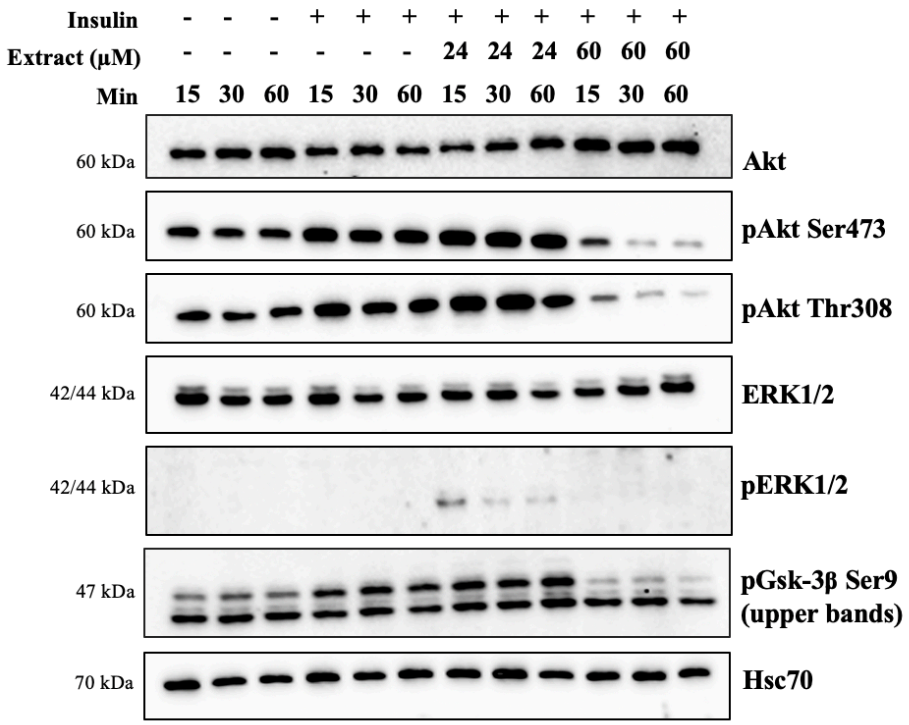

Figure 9A – *P. abies* extract initiates cell cycle arrest of PC-3 cells by altering kinase activity in the insulin signaling pathway. Inhibition of Akt phosphorylation affects the activity of its downstream target pGsk-3β, which in turn e.g. influences cell cycle progression by regulating cyclin activity. Interestingly, 24 µM treatment increased both pAkt and pERK1/2 phosphorylation, which was perhaps caused through a concentration-dependent agonistic activity of another signaling pathway. Hsc70 was used to control for sample loading.

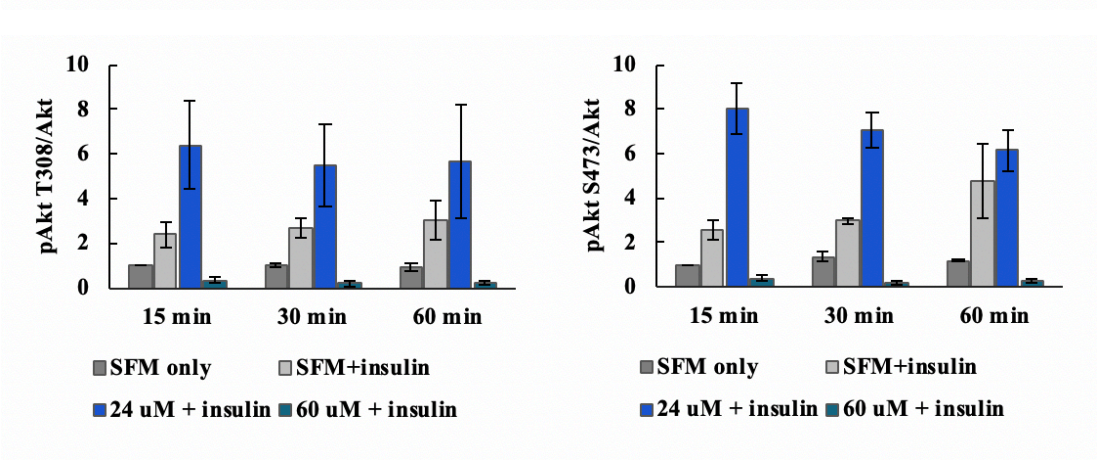

Figure 9B - Quantification of western blot analysis illustrates evident inhibition of downstream targets of insulin I) pAkt Thr308 and II) pAkt Ser474 after treatment with 60 µM extract. Additionally, an increase in Akt phosphorylation during 24 µM treatment could be observed, indicating a likely concentration-dependent effect that is possible to be caused through other signaling pathways. The histograms represent the relative pAkt/Akt ratio

normalized to "15 min SFM only" negative control samples from three independent experiments (mean ± SEM; n ≥ 3). Due to somewhat large variances in the data sets from the blots, no reliable statistical significance values could be calculated (using multiple t-test). The data nonetheless demonstrates a definite trend of Akt phosphorylation upon treatment with the extract.

To rule out that the observed enhanced pAkt and pERK activity was not caused by the solvent (1,3-propanediol) or lower concentrations of extract, identical cell signaling analysis as previously was performed using western blot. Treatment with the lowest concentration (12 μM) did not yield in a significant difference in Akt or ERK activity (Fig. 9C). This was somewhat predicted since a concentration in this range did not significantly suppress proliferation in live-cell imaging (data not shown). 1,3-Propanediol, which was used as the solvent for the dilution of pure knotwood extract, likewise did not affect phosphorylation levels as expected (Fig. 9C).

Since the activity of the major kinase Akt was observed to be inhibited by the knotwood extract, indicators of apoptosis were additionally studied through western blot analysis using the same protocol as previously. Phosphorylation of Bad (Ser136), as well as poly (ADP-ribose) polymerase (PARP) cleavage, did not show distinct changes upon short treatment time, however, no reliable repeated experimental data could be obtained due to low primary antibody binding (data not shown).

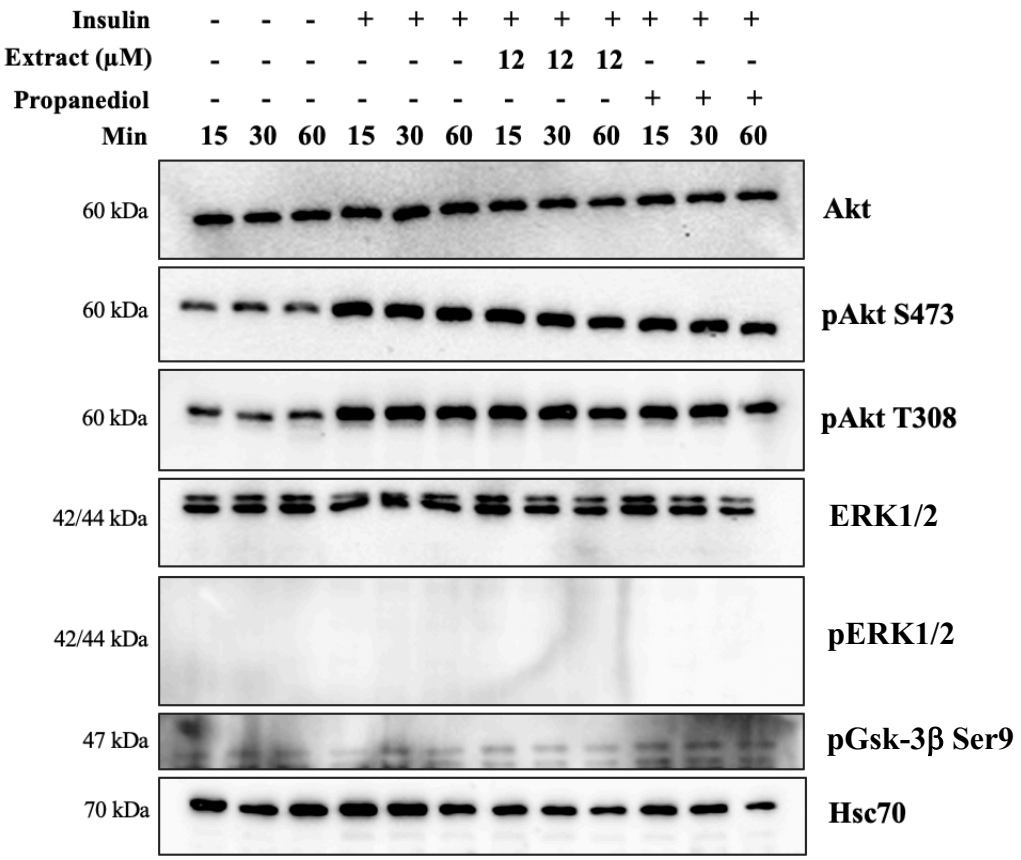

Figure 9C - Downstream signaling targets of insulin receptor were not affected by treatment with 12 μM extract or 1,3-propanediol in PC-3 cells. Neither an increase nor decrease in Akt or ERK levels or their phosphorylation could be observed with 12 μM treatment or 1,3-propanediol, indicating that the stimulatory effects seen with 24 μM are indeed concentration-dependent and occur only within a certain concentration range.

Due to *P. abies* knotwood extract inducing cell cycle arrest in the G0/G1-phase (Fig. 8), key checkpoint regulator p21 was analyzed through western blot as previously described except this time treating the serum-starved cells for 24h instead of 15-60 min, after which insulin was added for 30 min prior to preparing cell lysates. Results from this experiment displayed a slight increase in p21 expression (in addition to basal low levels) upon treatment with 24 μM extract as confirmed with quantification data (Fig. 10). This enhanced expression is likely a key consequence of affected Akt activity, which proceeds to halt the cell cycle in the G0/G1-phase. Due to the complexity of p21 activity in cancer, cell cycle arrest in the G1-phase can, however, be achieved through different signaling mechanisms other than p21, such as through p70S6K1, an altered cyclin D or CDK activity, retinoblastoma (Rb) protein, or alternatively via p27 or p16. Nonetheless, together these datasets suggest that lignans

in the *P. abies* knotwood extract inhibit and alter the insulin/IR system and its downstream signaling mediators.

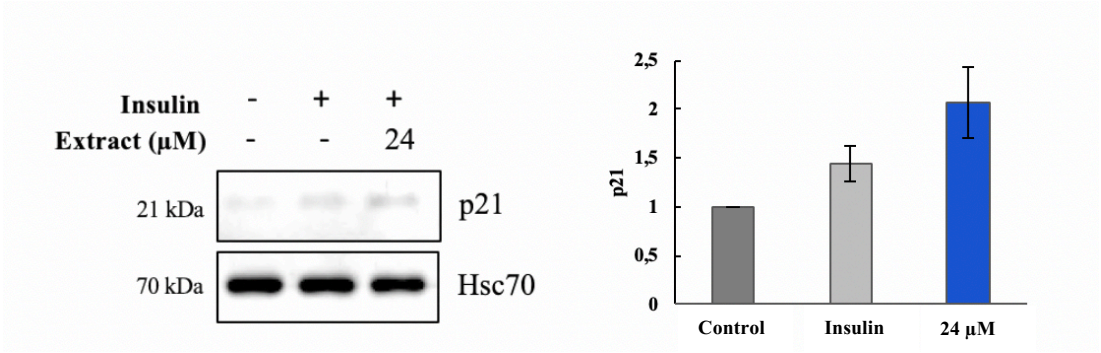

Figure 10 - *P. abies* extract induces G0/G1-phase cell cycle arrest in PC-3 cells possibly through enhanced p21 expression. p21 expression was slightly increased in PC-3 cells treated with *P. abies* extract in culture conditions where serum-starved PC-3 cells were treated for 24h with extract in serum-free medium, after which insulin was added for 30 min prior to preparing whole cell lysates. Quantification of p21 was normalized to Hsc70, which was used to control for sample loading. The histogram represents data from three independent experiments (mean ± SEM; n ≥ 3). No statistical significance was achieved, but a clear trend in p21 expression was observed in the data.

4.5. Growth factor depletion sensitizes PC-3 cells to *P. abies* knotwood extract resulting in rapid cell death

Remarkably, it was observed that the serum-starved PC-3 cells reacted in a vastly different fashion to the extract when treated for a longer period (hours instead of minutes) in growth factor-depleted conditions as compared to treatment in a growth factor-rich environment. With these treatment parameters, the cells were revealed through phase-contrast imaging to experience morphological changes as well as to rapidly undergo cell death within a few hours of addition of extract even in low concentrations (Fig. 11A). This observation was confirmed by a cytotoxicity assay, where the cells were treated in these same growth factor-depleted conditions for 24h prior to measuring cell viability (Fig. 11B). The IC_{50}-value of the extract was calculated from this data to be equivalent to 20.02 µM in these treatment circumstances. Together, this data demonstrates apparent cytotoxicity of the *P. abies* knotwood extract on PC-3 cells in growth factor-limited conditions. Furthermore, these results indicate that the antineoplastic activity of lignans on androgen-

independent prostate cancer is linked to alterations in the energy metabolism of the cell, which vastly differs from that of healthy cells, and thus possibly at least partly revealing the underlying selective mechanism of lignan activity on cancer cells in general.

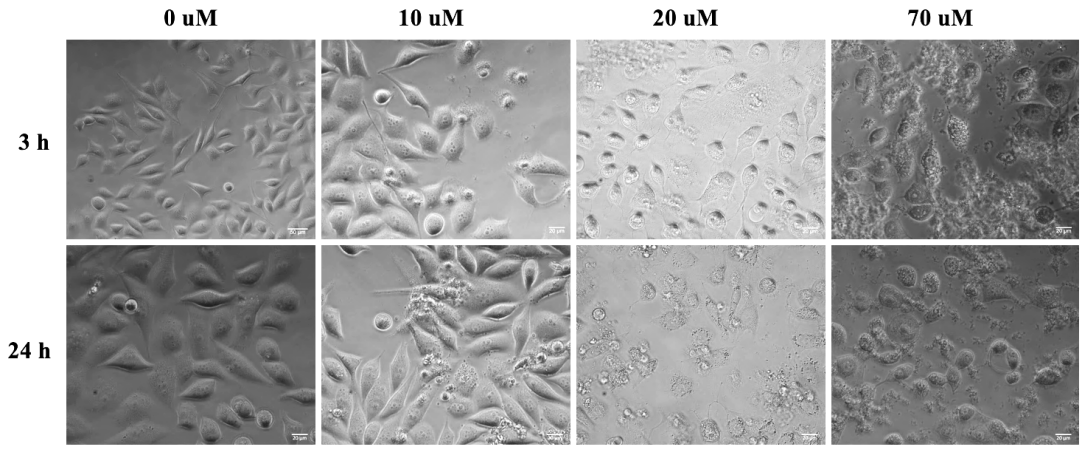

Figure 11A – Growth factor-depletion sensitized PC-3 cells to *P. abies* knotwood extract induced cell death. Phase-contrast images of serum-starved PC-3 cells treated for 3h and up to 24h with extract display an increase in dead cells in a concentration and time-dependent manner as compared to treatment in growth factor-rich conditions. Cells exhibit distinct morphological changes such as rounding upon treatment and appear to undergo cell death in an uncontrolled fashion rather than apoptosis considering the rupture of the outer membrane and the overall significant quantity of cell debris as seen in images with 70 μM treatment. Images were taken with 20x or 40x magnification using Zeiss Axio Vert A1 microscope. The scalebars in the images represent a measurement of 20 μm in all images except in 0 μM (3h), where the scalebar is set to 50 μm due to a lower magnification.

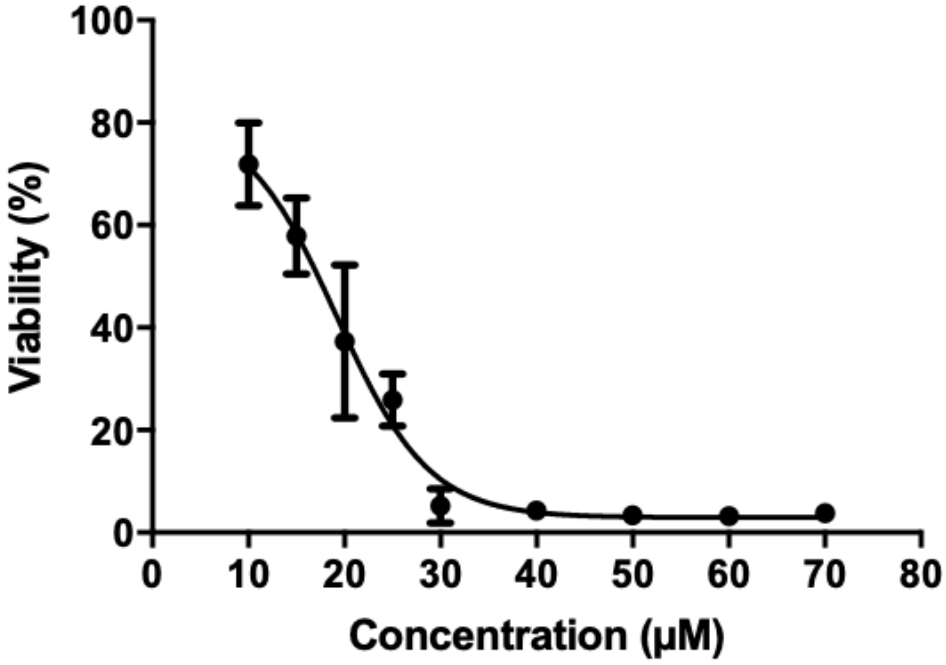

Figure 11B – *P. abies* knotwood extract induces significant cell death in PC-3 cells under growth factor-scarce conditions. A cytotoxicity assay measuring cell viability of serum-starved PC-3 cells treated for 24h with extract in serum-free medium revealed the IC_{50}-value to be 20.02 µM in these treatment conditions. Cell death could be observed already at low concentrations of <10 µM and the toxicity became more profound with increasing doses, indicating that the cells become sensitized to the extract when growth factors are limited. Growth factor-depletion *in vitro* mimics to an extent the conditions *in vivo*, such as in a solid tumor where the amount of available circulating growth factors is poor. Each treatment was plated in quintuple replicates on a 96-well plate and repeated three independent times. The graph represents mean values of viability (±SEM) from the independent experiment repeats normalized to the non-treated control sample where viability was set to 100%. The IC_{50}-value was calculated using GraphPad Prism 6 software.

5. Discussion and Conclusions

Prostate cancer has become one of the leading causes of death among men worldwide, being the second most common cancer in men overall. Due to unhealthy dietary and lifestyle habits which increase the likelihood of disease, the epidemic is only growing.

As the disease progresses, the cancer cells turn more aggressive by developing insensitivity to androgens, which normally regulate the growth and proliferation of prostate tissue. The androgen-independent cells proliferate without the need for androgens and usually turn metastatic, causing treatment options to be scarce and often ineffective, frequently leading to relapses and an untimely death of the patient. Hence, novel effective therapies for the disease are needed.

In this thesis, the antineoplastic effects of a *Picea abies* knotwood extract against the androgen-independent prostate carcinoma cell line PC-3 are demonstrated. The main active compounds in the extract were known to be lignans, which are potent antioxidants found throughout the plant kingdom that have previously shown diverse health-promoting benefits for prevention and treatment for various diseases. A GC-MS analysis of a knotwood extract sample showed that 31.3% (313 mg/g) of the pure knotwood extract consisted of various lignans, of which 22.43% (224.3 mg/g) was HMR in its two isoforms. Other common lignans found in the extract were CONI (1.86%), MAT (1.68%) and SECO (1.4%), yet in much lower concentrations compared to that of HMR. Trace amounts of other molecules found in the extract were, for instance, a variety of diterpenes, fatty acids, and diglycerides. Additionally, the extract consisted of some high molar mass material, which was not identified through GC-MS due to their low volatility.

Live-cell imaging revealed the extract to significantly suppressed PC-3 cell proliferation in a concentration-dependent manner during 48h incubation in normal culture conditions. To further address how the cell cycle was affected during treatment, flow cytometry analysis using propidium iodide staining was carried out. In agreement with previous studies done on enterolactone activity on PC-3 cells (Chen et al., 2009), the extract induced cell cycle arrest in the G0/G1-phase during 24h treatment using concentrations of 24 μM and 60 μM, yet, no apparent changes in the number of dead cells could be detected. It remains unclear, however, if PC-3 continue to reside in a quiescence-like state in growth factor-rich conditions during prolonged extract treatment (>48h), or if the cells are able to overcome cell cycle arrest and continue proliferating. Alternatively, prolonged treatment may eventually induce cell death.

PC-3 cells are known to express high levels of IGF-1R compared to normal prostate tissue cells or androgen-sensitive prostate cancer cells, and thereby stimulate proliferation and survival through IGF-1 or insulin binding which activates the major PI_3K/Akt and MAPK signaling pathways. Western blot analysis revealed that the extract targeted insulin receptor-mediated signaling and altered the phosphorylation of

its downstream kinases Akt and ERK in a concentration-dependent manner. Insulin-induced phosphorylation of Akt (Thr308 and Ser473) was substantially inhibited with 60 μM extract within the first 15 min of treatment. Furthermore, pGsk-3β, a downstream target of Akt, was also seen to be inhibited as a consequence of inactive Akt. No changes in ERK phosphorylation was detected using this concentration. Interestingly, however, a lower concentration of extract (24 μM) caused an increase in both pAkt and pERK levels. Even though no statistical significance could be calculated from the quantification of western blot data, a clear trend of effects on signaling caused by the extract were witnessed. Phosphorylation of ERK is usually not observed with insulin stimulation in this specific androgen-independent prostate cancer cell-line compared to those which are androgen-dependent. For this reason, a clear activation of pERK signaling with 24 μM treatment lead to the postulation that the compounds in the extract not only inhibit certain kinase activity but can also increase them in a dose-dependent manner. It is known that lignans such as enterolactone (the mammalian metabolite of e.g. HMR) can function either as an antagonist or an agonist for example on estrogen receptors α and β depending on the concentration, as well as target NF-κB signaling and membrane receptors such as IGF-1R or GPER. Furthermore, enterolactone has also been found to alter other signaling pathways in a variety of cancers other than the PI_3K/Akt transduction pathway, such as ERK/NF-κB/Snail, as well as gene expression related factors, thus demonstrating the wide mechanisms of action. It is therefore very probable that even though the extract comprises mostly of HMR as its active compound, the other lignans and oligolignans presumably also target not only the insulin receptor-mediated signaling pathway in PC-3 cells but also others, likely causing a synergistic effect on the cell behavior. Therefore, the observed *in vitro* antineoplastic effect of the *P. abies* knotwood extract on PC-3 cells cannot only be accounted to HMR, but rather considered as a collective result of the extract as a whole.

As the cell cycle arrested in the G0/G1-phase, the expression levels of p21 were analyzed through western blot by incubating the PC-3 cells in serum-free medium with the extract for 24h prior to the addition of insulin for receptor activation. Expression of p21 had previously been analyzed with shorter treatment times of 15-60 min (data not shown), but this resulted in no apparent p21 changes. However, since observable changes in gene expression levels in general tends to require longer times (hours instead of minutes), a 24h treatment period was chosen instead for this specific

experiment. Slight increases in p21 were thereby observed, which might explain the arrest in the first cell cycle phase due to p21 being a CDK inhibitor. PC-3 cells are deficient in p53 protein due to an allele deletion in combination with a mutation in the stop codon-producing frame, indicating that p21 expression is not significantly affected by p53 activity but rather through alternative signaling pathways such as through PI_3K/Akt. Since no apparent cell death had been detected in the previous experiments where cells had been treated in growth factor-rich conditions, it was surprising to note that treatment in growth factor-depleted circumstances caused the cancer cells to react to the extract by rapidly dying. Phase-contrast imaging and cell viability during 24h treatment was assessed, revealing PC-3 cells to be highly sensitized to the extract in a growth factor-diminished environment. Already low concentrations of the extract showed morphological changes and cell death, and the IC_{50}-value was determined to be 20.02 μM.

Since the cells had started to rapidly die during the 24h treatment prior to analyzing the p21 expression levels from whole-cell lysates, no viable cells could be obtained from 60 μM treated samples. Treatment with 24 μM had also killed off some of the cells, due to the concentration being close to the IC_{50}-value. For this reason, it is somewhat questionable whether the data collected for p21 expression is valid since cells were rapidly dying instead of only halting proliferation. Nonetheless, extract treatment showed a slight trend for an increased p21 expression. It would be interesting to analyze if p21 levels change upon lower extract concentrations during a shorter treatment time of a few hours with and without serum starvation. Other indicators of cell cycle arrest such as cyclin activity could additionally be analyzed in order to gain a more comprehensive understanding of the affected signaling events regulating proliferation.

Indicators of apoptosis initiation via pBad (Ser136), Bid (BH3 interacting-domain death agonist) or PARP cleavage partly modulated through Akt signaling could not be observed during short treatment in insulin-containing medium in pilot experiments (data not shown). This was somewhat expected since no clear increase in apoptotic cells was seen during 48h live-cell imaging. However, there were some faults in this experimental design and hence the data was presumably not accurate. A slightly longer treatment time (hours instead of minutes) might be needed for PARP cleavage or other apoptotic markers. Additionally, from a practical standpoint, the SDS-PAGE gels used for separating these proteins from the cell lysates should have had higher an SDS concentration since e.g. Bid has a very low molecular weight (22

kDa full length, 15 kDa cleaved). Furthermore, the antibodies used for studying these proteins were not of good quality, making it difficult to analyze the bands due to inadequate binding. Previous studies have nonetheless observed mitochondrial-mediated apoptosis in androgen-sensitive prostate cancer cells (LNCaP) upon the mammalian lignan metabolite enterolactone treatment *in vitro*, where cytochrome c release and PARP cleavage was prominent (Li-Hua Chen, Jing Fang, Huaixing Li, Wendy Demark-Wahnefried, & Xu Lin, 2007). For future reference, analyzing for instance pBad and Bid levels in PC-3 cells treated with extract in growth factor-depleted conditions could perhaps indicate if the cell death mechanism is indeed initiated through PI_3K/Akt mediated mitochondrial membrane depolarization. It is, however, possible that the observed cell death caused by the extract is generated through other mechanisms than pro-apoptotic signaling since it was unclear if the cells were dying through apoptosis or necroptosis. Necroptosis, which is a form of caspase-independent programmed necrotic cell death, is a fairly recently described process which remains largely undisclosed. Phase-contrast images indicated that the cells were dying in an uncontrolled fashion since the cells were swelling up, their plasma membranes seemed to rupture and substantial amounts of cell debris was floating in the medium, which are all hallmarks of necroptotic cell death in comparison to apoptosis. The mechanisms of action regarding apoptosis and necroptosis have been found to be intertwined and are highly influenced by the activity of autophagy and ATP availability as well as other related metabolic factors. Apoptosis is regarded as a fairly costly process in terms of energy usage due to the systematic compartmentalization, DNA fragmentation, and breakdown of organelles. Especially PCa cells, which rely heavily on glucose uptake for sustained ATP production through glycolysis, usually overexpress glucose transporters (GLUTs) in order to preserve the required ATP/ADP ratio. Generally, during nutrient withdrawal, autophagy is activated for the cell to compensate for the lack of available energy and to inhibit apoptosis. Even though autophagy could typically prevent cell death in PCa cells to an extent in low nutrient conditions, a halt in ATP production through reduced glucose uptake upon extract treatment may perhaps ultimately shift the fate from survival or apoptosis to necroptotic signaling. For this reason, it can be postulated that growth factor-withdrawal in combination with altered PI_3K/Akt and MAPK signaling generated by extract treatment might reduce GLUT activity or induce a sort of energy crisis, thereby sensitizing the PC-3 cells to undergo necroptosis instead of apoptosis or survival in general due to ATP production being significantly decreased by the

shutdown of hyperactive Akt signaling (Fig. 12). This hypothesis nonetheless remains to be further investigated.

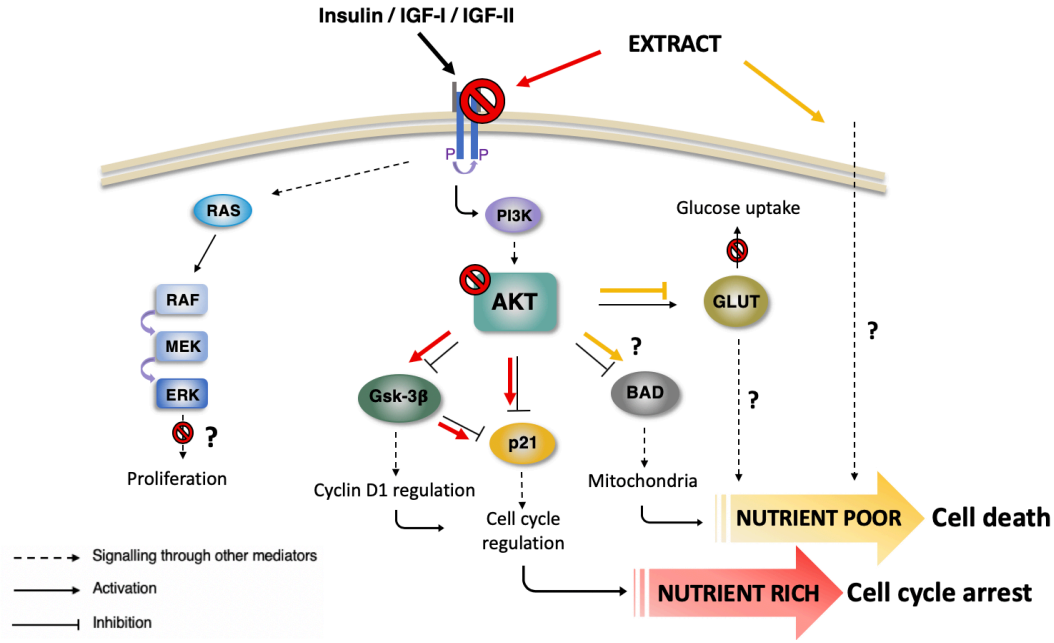

Figure 12 – Proposed mechanism of action for *P. abies* knotwood extract on PC-3 cells. Both inhibition of proliferation through cell cycle arrest and rapid cell death were observed upon extract treatment depending on the amount of available growth factors in the medium. Insulin-mediated Akt phosphorylation and its downstream target pGsk-3β were substantially inhibited, leading to elevated p21 expression and cell cycle arrest in the G0/G1-phase without inducing cell death (red arrows). ERK phosphorylation was also affected during lower extract treatment, however, its implications on proliferation or other mechanisms remain undetermined. Interestingly, growth factor-depletion sensitized PC-3 cells to rapid cell death in a necroptosis-like fashion already at lower extract concentrations (yellow lines). Inhibition of Akt phosphorylation may affect its downstream targets such as the pro-apoptotic protein Bad, which in turn can activate mitochondrial-mediated cell death. Furthermore, inhibited Akt signaling might also reduce glucose uptake through GLUT regulation. It is therefore postulated that growth factor-depletion in combination with inhibited PI_3K/Akt signaling may induce necroptotic signaling instead of survival or apoptosis by essentially attenuating energy production. The mechanism of action for the observed cell death remains, however, undisclosed, and it is possible that the lignans in the extract activate necroptosis or apoptosis through other mechanisms outside of PI_3K/Akt and MAPK signaling, as indicated with question marks in the figure.

In the human body, the amount of circulating available growth factors is relatively scarce compared to *in vitro* conditions where cells are continuously grown

in supplement-rich medium. For this reason, it is highly probable that the lignans in the extract are also able to induce cell death *in vivo* rather than just inhibit proliferation if a high enough concentration is reached at the tumor microenvironment. The main issue of using lignans as anticancer agents is the relatively high concentrations needed for cytotoxicity *in vivo* compared to used cytostatic drugs on the market. Combination with other known antineoplastic agents may, therefore, be a considerable treatment option for aggressive PCa. Due to lignans being selectively toxic only to cancer cells without causing harmful off-target effects, combinatory treatment should theoretically not have any disadvantages. Further, future studies may reveal if lignans are able to sensitize cancer cells to known drugs due to having a variety of cell surface targets. In fact, *in vitro* studies have already shown promising results in prostate cancer research, where e.g. lignans were shown to sensitize androgen-dependent LNCaP cells to TRAIL-induced apoptosis (Peuhu et al., 2010). Combinational therapy with lignans would thus benefit the patients greatly by reducing potential side-effects if available strong cytotoxic drugs no longer would need to be used in such high concentrations due to targeting cancers through multiple mechanisms. Furthermore, the cost-efficiency of PCa treatment using this method would consequently be significantly more favorable compared to the current system.

Since lignans are known to have selective toxicity to cancer cells while exerting a variety of other beneficial properties in the body such as lowering cholesterol levels, acting as anti-inflammatory agents, and functioning in free radical scavenging, dietary supplementation of the extract is likely to improve the overall health of the individual through many ways and to, importantly, function as a form of preventative care, especially for people who do not otherwise reach sufficient lignan levels through their diet. Dietary supplementation of ten drops (ca 0.12 ml/drop) per day of the diluted *P. abies* knotwood extract used in this study would increase both HMR and ENL plasma concentrations significantly. In a hypothetical sense, if a typical adult weighing 70 kg would ingest ten drops of the diluted extract, the concentration of HMR in the body would be equivalent of 11.26 nM, if calculated using a simple dilution equation assuming that the body is roughly 70% water and that one drop of the ready-made diluted extract (0.12 ml) contains 45.98 μM HMR (as calculated from GC-MS data). Naturally, the pharmacokinetics impact the outcome significantly since lignans are taken up in the bloodstream as well as being converted to ENL and END by the gut microbiome. Additionally, the extract contains also other lignans, although in much lower concentrations compared to HMR. The lignan

concentrations used in this thesis study are, nonetheless, not far from what can be reached through nutritional supplementation. For example, sesame is rich in HMR (17 mg/100g) and larger quantities are usually obtained from sesame paste (tahini). HMR is also one of the dominant lignans in foods such as barley, wheat, and oat. It is, therefore, possible to reach relatively high HMR concentrations in the body just by maintaining a healthy diet. For medicinal purposes, however, supplementation of HMR directly or through an extract like the one used in this study, plasma concentration over 100-fold the norm could potentially be reached. Interestingly, a study published in 2013 showed that ingestion of up to 72 mg/day for 8 weeks of a ready-made HMR supplement by postmenopausal women resulted in a 1238% increase in plasma HMR levels and reduced menopausal symptoms without causing negative side-effects (Udani et al., 2013).

Collectively, the results in this thesis evidently demonstrate the antineoplastic activity of *P. abies* knotwood extract on androgen-independent prostate cancer *in vitro*. To the writer's knowledge, outside of using individual lignans, this is the first time an extract of this type has been tested on PC-3 cells. Lignans, in general, have shown promising results in research during the past decades and their potential in cancer therapy is vast. Future studies using *in vivo* methods will hopefully broaden the understanding of lignans and their use in prostate cancer treatment and prevention.

6. Summary in Swedish – Kvistnötsextrakt från gran (*Picea abies*) som behandling mot androgenoberoende prostatacancer: *in vitro*-studie om insulinsignalering och tillväxt hos PC-3-celler

Prostatacancer (PCa) är en av de allmännaste cancertyperna i världen, och drabbar speciellt äldre män i över 60-års ålder. I Finland uppkommer ca 5 400 nya diagnostiserade fall årligen och mängden förutsägs öka stadigt under de kommande decennierna. Främst externa faktorer såsom livsstil, fettrik diet och för lite fysisk ansträngning ökar risken för att insjukna. Flera relativt effektiva läkemedel och terapier finns tillgängliga för PCa i dess förstadier, men en mer avancerad och metastaserad form sänker överlevnadsprognosen signifikant eftersom inga botemedel finns på marknaden för tillfället. På grund av detta finns det ett stort behov av nya

terapimetoder för behandling av avancerad PCa. Utöver detta, försöker man utveckla billigare, icke-invasiva och mer exakta metoder inom diagnostiken av PCa, speciellt eftersom förstorad prostatavävnad kan även tyda på andra sjukdomar än cancer och därmed leda till felaktig diagnos.

Friska prostataceller är beroende av de manliga könshormonerna (androgenerna) för att reglera tillväxt och överlevnad. Vid PCa kan cancercellerna generellt indelas i två fenotyper utgående från cellernas behov av androgener för tillväxt: de androgenberoende alternativt de androgenoberoende. Hormonterapi är exempelvis en vanlig förstahandsmetod inom sjukvården för androgenberoende PCa, eftersom en minskad mängd fria androgener i kroppen kan följaktligen dämpa tillväxten av cancercellerna. Denna form av terapi fungerar trots allt inte mot den aggressivare cancerformen där cellerna har muterats och erhållit egenskaper som möjliggör deras överlevnad och förökning utan androgener. I denna studie användes den androgenoberoende prostatacancercellinjen PC-3, som ursprungligen producerades från en patient med metastaserad PCa. Eftersom cellinjen inte längre kräver androgener för tillväxt, kompenserar cellerna detta genom att vara känsligare mot och beroende av andra tillgängliga tillväxtfaktorer såsom insulin och insulinliknande tillväxtfaktor (IGF). Utöver tillväxt reglerar insulinsignaleringen i allmänhet exempelvis autofagi, mitokondriemedierad överlevnad och apoptos, energiproduktion samt upptagning av glukos från omgivningen genom glukostransportproteiner. De två huvudsakliga signaleringsräckorna som påverkas av insulin är PI_3K/Akt- och MAPK-signalering.

Lignaner är polyfenoler producerade som sekundära metaboliter i växter, där deras antioxidant samt antimikrobiella egenskaper antas fungera som en del av växtens immunförsvar. Lignaner hittas främst i fiberrika växter, nötter, frön, och bönor. Av de över 200 identifierade lignanerna, är matairesinol (MAT), secoisolariciresinol (SECO), pinoresinol (PINO), och hydroxymatairesinol (HMR) några av de allmännaste typerna i kosten. Speciellt linfrön har utretts vara rika i lignaner. Intressant nog, har det visats att granens kvistnötter (*Picea abies*) har signifikant höga halter av HMR-lignanen, samt att mängden är betydligt större än i någon annan tillsvidare studerad växt. Flera av de lignaner som fås via kosten konverteras av tarmens bakterieflora till s.k. däggdjurslignaner enterolakton och enterodiol, som har påvisats ha liknande fysiologisk verkan som de andra prekursorlignanerna. I allmänhet har lignaner bevisats ha flera fördelaktiga egenskaper inom förebyggandet och behandlingen av olika sjukdomar genom att exempelvis fungera som starka

antioxidanter, ha antiinflammatorisk verkan, samt sänka kolesterolvärden i blodet. Utöver detta har lignaner studerats inom cancerforskning eftersom de kan motverka cancerceller selektivt utan att skada friska celler. Lignanerna hör till så kallade fytoöstrogener eftersom de kan reglera aktiviteten av östrogenreceptorn genom antagonistisk eller agnostisk verkan. Utgående från detta har det visats att lignaner är speciellt effektiva mot hormonrelaterade cancerformer såsom prostata- och bröstcancer, och kan eventuellt även fungera som förebyggande medel för att förhindra cancer överlag. Största delen av publicerad forskning har dock fokuserat på att studera anticancer effekten av individuella lignaner såsom SECO eller metaboliterna enterolakton och enterodiol, medan extrakt som helheter innehållande blandningar av lignaner har tidigare inte undersökts som medel mot prostatacancer till skribentens kännedom.

Utgående från detta, var målet med denna avhandling att studera inverkan av ett kvistnötsextrakt från *P. abies* på tillväxten och insulinsignaleringen hos PC-3 *in vitro*. Extraktet analyserades till en början med hjälp av gaskromatografi–masspektrometri för att koncentrationsbestämma dess lignaner. Analysen påvisade att extraktet innehöll totalt 31,3 % lignaner, varav HMR i dess två isomerer var den dominerande lignanen med en koncentration på 224,3 mg/g i torrextrakt. En utspädd stocklösning i 1,3-propandiol av extraktet innehöll därmed 45,985 mM HMR. För att studera effekten av extraktet på tillväxten av PC-3-celler, användes en mikroskopimetod där de behandlade levande cellerna visualiserades under 48 timmar. Behandlingen i näringsrik omgivning bevisade att cellernas tillväxt dämpades signifikant på ett koncentrationsberoende sätt utan att celldöd initierades. Lösningsmedlet 1,3-propandiol påverkade inte celltillväxten såsom förväntat. Flödessytometrisk analys demonstrerade vidare att cellcykeln hämmades i G0/G1-fasen vid extraktbehandling. Eftersom insulinsignaleringen är väsentlig för PC-3-cellernas förökning och överlevnad, studerades extraktets påverkan på denna signaleringsräcka genom att först svälta cellerna från andra tillväxtfaktorer, varefter de behandlades med extraktet samt insulin för att aktivera receptormedierad signalering. Western blot –analys av PC-3-celler som behandlades för 15–60 min avslöjade en tydlig hämning av insulinstimulerad Akt-fosforylering samt dess sekundära målmolekyl glykogensyntas-kinas-3β då cellerna behandlades med koncentrationer på 60 μM. Dessutom ökade expressionen av cellcykelregleraren p21 en aning under en behandling på 24h, vilket vidare avspeglade eventuellt varför cellcykeln stannade specifikt i G0/G1-fasen. Kontroversiellt nog, verkade extraktet

stimulera både Akt- och MAPK-signalering vid lägre koncentrationer på 24 μM, även om både cellcykeln och tillväxten hämmades med denna mängd extrakt. Då PC-3-cellerna behandlades i förhållanden utan insulin eller andra tillväxtfaktorer, reagerade cellerna med att initiera snabb celldöd redan vid låga extraktkoncentrationer. Detta var oväntat, eftersom tidigare resultat i tillväxtfaktorrika förhållanden inte hade hänvisat till celldöd av något slag. Utgående från faskontrastbilder verkade det ytterligare som om cellerna dog genom nekroptos istället för apoptos. Nekroptos är en kontrollerad form av nekrotisk celldöd som har bevisats vara särskilt kopplad till aktiviteten av autofagi och energiproduktionen i cellen. Utgående från resultaten i denna studie, samt tidigare forskning av lignaner och nekroptos, kan man spekulera ifall en tillväxtfaktorfattig omgivning tillsammans med förändrad PI_3K/Akt- och MAPK-signalering till följd av extraktbehandling orsakar en sorts energikris och därmed sensibiliserar PC-3-cellerna till nekroptos istället för apoptos eller överlevnad överhuvudtaget. Denna hypotes bör dock vidare studeras för att få en grundlig förståelse om hur lignanerna kan fungera som anticancermedel, men speciellt för att utreda ifall påverkan på energimetabolismen i allmänhet är orsaken till den selektiva toxiciteten hos lignanerna.

Resultaten i denna avhandling påvisar att kvistnötsextraktet från *P. abies* har en tydlig tillväxthämmande effekt på PC-3-celler som följd av förändrad insulinmedierad intracellulär signalering. Cancercellernas varierande respons på extraktets lignaner verkar vara beroende på mängden tillgängliga tillväxtfaktorer i omgivningen och dess inverkan på energimetabolismen, vilket är speciellt i central roll med tanken på exempelvis tumörer där mängden cirkulerande tillväxtfaktorer och näring är begränsad. Utgående från detta är det möjligt att lignanerna är mer toxiska mot cancerceller i levande organismer än i cellkulturer där omgivningens näringsmänger är konstant reglerade. Potentialen hos lignaner i sin helhet inom förebyggandet och behandlingen av sjukdomar är trots allt enorm. Speciellt eventuella kombinationsterapier av lignaner med nuvarande mediciner kommer förhoppningsvis att studeras vidare för att minska bieffekter samt för att förbättra kostnadseffektiviteten inom prostatacancervården.

7. Acknowledgements

At the beginning of my summer internship in the Eriksson lab back in 2018, I was still undecided about my plans for a subject for my master's thesis. After working on the lignan project during that summer and having gathered a fair amount of interesting data, it was a natural step forward to continue the project with a thesis in mind. After having overcome some initial troubleshooting of experiment setups, I was able to gather the needed data for my thesis thanks to many people helping me along the way.

Initially, I would like to thank John Eriksson for encouraging me to continue my summer internship to do a full master's thesis, as well as guide me along the way during the whole project. A big thank you also to Annika Smeds from the Laboratory of Wood and Paper Chemistry at Åbo Akademi University for assisting me with GC-MS and the data analysis we acquired from it. Tech support for my many live-cell imaging experiments using IncuCyte was kindly provided by Jouko Sandholm. Many days and evenings were spent partly in frustration and success when doing western blots, but thankfully my colleagues in the Eriksson lab gave me valuable advice during many trial and error experiments in the wet lab as well as with data analyses. Flow cytometry was a method which I did not have much previous experience in, and hence I would like to thank especially Alia Joko for guiding me along the learning process.

Even though this thesis project took longer to complete than I had initially planned, I am grateful for the experience and having had the chance to work in such a great team who kept the spirits high even on days when lab equipment was malfunctioning or repeat experiments failed to give the required data. Last but in no means least, many thanks also to my friends and family, as well as my partner in crime at home who have all kept me entertained with conversations during coffee brakes and encouraged me to complete the project to the best of my abilities.

8. References

Abbas, T., & Dutta, A. (2009). P21 in cancer: Intricate networks and multiple activities. *Nature Reviews Cancer, 9*(7), 460-461. doi:10.1038/nrc2657

Adlercreutz, H. (2007). Lignans and human health. *Critical Reviews in Clinical Laboratory Sciences, 44*(5-6), 483-525. doi:10.1080/10408360701612942

Aksamitiene, E., Kiyatkin, A., & Kholodenko, B. N. (2012). Cross-talk between mitogenic ras/MAPK and survival PI3K/akt pathways: A fine balance. *Biochemical Society Transactions, 40*(1), 139-146. doi:10.1042/BST20110609

Andjelkovich, M., Cohen, P., Alessi, D. R., Hemmings, B. A., & Cross, D. A. E. (1995). Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. *Nature, 378*(6559), 785-789. doi:10.1038/378785a0

Antonino Belfiore. (2007). The role of insulin receptor isoforms and hybrid insulin/IGF-I receptors in human cancer. *Current Pharmaceutical Design, 13*(7), 671-686. doi:10.2174/138161207780249173

Bai, J., Li, Y., & Zhang, G. (2017). Cell cycle regulation and anticancer drug discovery. *Cancer Biology & Medicine, 14*(4), 348-362. doi:10.20892/j.issn.2095-3941.2017.0033

Bakin, R. E., Gioeli, D., Sikes, R. A., Bissonette, E. A., & Weber, M. J. (2003). Constitutive activation of the ras/mitogen-activated protein kinase signaling pathway promotes androgen hypersensitivity in LNCaP prostate cancer cells. *Cancer Research, 63*(8), 1981.

Basu, P., & Maier, C. (2018). Phytoestrogens and breast cancer: In vitro anticancer activities of isoflavones, lignans, coumestans, stilbenes and their analogs and derivatives. *Biomedicine & Pharmacotherapy, 107*, 1648-1666. doi:10.1016/j.biopha.2018.08.100

Beurel, E., Grieco, S. F., & Jope, R. S. (2015). Glycogen synthase kinase-3 (GSK3): Regulation, actions, and diseases. *Pharmacology and Therapeutics, 148*, 114-131. doi:10.1016/j.pharmthera.2014.11.016

Biasiotto, G., Zanella, I., Predolini, F., Archetti, I., Cadei, M., Monti, E., . . . Di Lorenzo, D. (2018). 7-hydroxymatairesinol improves body weight, fat and sugar metabolism in C57BJ/6 mice on a high-fat diet. *The British Journal of Nutrition, 120*(7), 751-762. doi:10.1017/S0007114518001824

Borriello, S. P., Stechell, K. D. R., Axelson, M., & Lawson, A. M. (1985). Production and metabolism of lignans by the human faecal flora. *Journal of Applied Bacteriology, 58*(1), 37-43. doi:10.1111/j.1365-2672.1985.tb01427.x

Bott, S. R. J., Arya, M., Kirby, R. S., & Williamson, M. (2005). p21WAF1/CIP1 gene is inactivated in metastatic prostatic cancer cell lines by promoter methylation. *Prostate Cancer and Prostatic Diseases, 8*(4), 321-326. doi:10.1038/sj.pcan.4500822

Boucher, J., Kleinridders, A., & Kahn, C. R. (2014). Insulin receptor signaling in normal and insulin-resistant states. *Cold Spring Harbor Perspectives in Biology, 6*(1), a009191. doi:10.1101/cshperspect.a009191

Bray, F., Ferlay, J., Soerjomataram, I., Siegel, R. L., Torre, L. A., & Jemal, A. (2018). Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA: A Cancer Journal for Clinicians, 68*(6), 394-424. doi:10.3322/caac.21492

Buck, K., Zaineddin, A. K., Vrieling, A., Linseisen, J., & Chang-Claude, J. (2010). Meta-analyses of lignans and enterolignans in relation to breast cancer risk. *The American Journal of Clinical Nutrition, 92*(1), 141-153. doi:10.3945/ajcn.2009.28573

Cary, K. C., Singla, N., Cowan, J. E., Carroll, P. R., & Cooperberg, M. R. (2014). Impact of androgen deprivation therapy on mental and emotional well-being in men with prostate cancer: Analysis from the CaPSURE™ registry. *Journal of Urology, The, 191*(4), 964-970. doi:10.1016/j.juro.2013.10.098

Chang, L., Chiang, S., & Saltiel, A. R. (2004). Insulin signaling and the regulation of glucose transport. *Molecular Medicine (Cambridge, Mass.), 10*(7-12), 65-71. doi:10.2119/2005-00029.Saltiel

Chen, L., Fang, J., Sun, Z., Li, H., Wu, Y., Demark-Wahnefried, W., & Lin, X. (2009). Enterolactone inhibits insulin-like growth factor-1 receptor signaling in human prostatic carcinoma PC-3 cells. *The Journal of Nutrition, 139*(4), 653-659. doi:10.3945/jn.108.101832

Chen, W., Chen, C., Ren, G., Huang, Y., Qin, L., & Chen, L. (2016). The treatment effects of flaxseed-derived secoisolariciresinol diglycoside and its metabolite enterolactone on benign prostatic hyperplasia involve the G protein-coupled estrogen receptor 1. *Applied Physiology, Nutrition, and Metabolism, 41*(12), 1303-1310. doi:10.1139/apnm-2016-0332

Chen, X., Thakkar, H., Tyan, F., Gim, S., Robinson, H., Lee, C., . . . Srivastava, R. K. (2001). Constitutively active akt is an important regulator of TRAIL sensitivity in prostate cancer. *Oncogene, 20*(42), 6073-6083. doi:10.1038/sj.onc.1204736

Chikara, S., Lindsey, K., Borowicz, P., Christofidou-Solomidou, M., & Reindl, K. M. (2017). Enterolactone alters FAK-src signaling and suppresses migration and invasion of lung cancer cell lines. *BMC Complementary and Alternative Medicine, 17*(1), 30. doi:10.1186/s12906-016-1512-3

Cho, N., Choi, M., Kim, B., Cho, Y., Moon, K. C., & Kang, G. H. (2006). BRAF and KRAS mutations in prostatic adenocarcinoma. *International Journal of Cancer, 119*(8), 1858-1862. doi:10.1002/ijc.22071

Das, M., & Devi, K. P. (2019). A mini review on the protective effect of lignans for the treatment of neurodegenerative disorders. *Journal of Nutrition, Food and Lipid Science, 2019*(1) doi:10.33513/NFLS/1901-06

De Angelis, R., MSc, Sant, M., MD, Coleman, M. P., Prof, Francisci, S., PhD, Baili, P., MSc, Pierannunzio, D., PhD, . . . Capocaccia, R., MSc. (2014). Cancer survival in europe 1999–2007 by country and age: Results of EUROCARE-5—a

population-based study. *Lancet Oncology, The, 15*(1), 23-34. doi:10.1016/S1470-2045(13)70546-1

Diehl, J. A., Cheng, M., Roussel, M. F., & Sherr, C. J. (1998). *Glycogen synthase kinase-3β regulates cyclin D1 proteolysis and subcellular localization* Cold Spring Harbor Laboratory Press.

Eklund, P. C., Långvik, O. K., Wärnå, J. P., Salmi, T. O., Willför, S. M., & Sjöholm, R. E. (2005). Chemical studies on antioxidant mechanisms and free radical scavenging properties of lignans. *Organic & Biomolecular Chemistry, 3*(18), 3336-3347. doi:10.1039/b506739a

Embi, N., Rylatt, D. B., & Cohen, P. (1980). Glycogen synthase kinase-3 from rabbit skeletal muscle. separation from cyclic-AMP-dependent protein kinase and phosphorylase kinase. *European Journal of Biochemistry, 107*(2), 519-527. doi:10.1111/j.1432-1033.1980.tb06059.x

Falschlehner, C., Emmerich, C. H., Gerlach, B., & Walczak, H. (2007). TRAIL signalling: Decisions between life and death. *International Journal of Biochemistry and Cell Biology, 39*(7-8), 1462-1475. doi:10.1016/j.biocel.2007.02.007

Ferreira, A. M., Morais, E. S., Leite, A. C., Mohamadou, A., Holmbom, B., Holmbom, T., . . . Silvestre, A. J. D. (2017). Enhanced extraction and biological activity of 7-hydroxymatairesinol obtained from norway spruce knots using aqueous solutions of ionic liquids. *Green Chemistry, 19*(11), 2626-2635. doi:10.1039/C7GC01091E

Finnish Cancer Registry. (2019). Syöpä 2017. Retrieved from https://syoparekisteri.fi/assets/files/2019/10/sy%C3%B6p%C3%A42017_raportti.pdf

Freedland, S. J., Eastham, J., & Shore, N. (2009). Androgen deprivation therapy and estrogen deficiency induced adverse effects in the treatment of prostate cancer. *Prostate Cancer and Prostatic Diseases, 12*(4), 333-338. doi:10.1038/pcan.2009.35

Galletti, G., Leach, B. I., Lam, L., & Tagawa, S. T. (2017). Mechanisms of resistance to systemic therapy in metastatic castration-resistant prostate cancer. *Cancer Treatment Reviews, 57*, 16-27. doi:10.1016/j.ctrv.2017.04.008

Galluzzi, L., Kroemer, G., Vandenabeele, P., & Vanden Berghe, T. (2010). Molecular mechanisms of necroptosis: An ordered cellular explosion. *Nature Reviews Molecular Cell Biology, 11*(10), 700-714. doi:10.1038/nrm2970

Gao, N., Zhang, Z., Jiang, B., & Shi, X. (2003). Role of PI3K/AKT/mTOR signaling in the cell cycle progression of human prostate cancer. *Biochemical and Biophysical Research Communications, 310*(4), 1124-1132. doi:10.1016/j.bbrc.2003.09.132

Giuliano, C., Siani, F., Mus, L., Ghezzi, C., Cerri, S., Pacchetti, B., . . . Blandini, F. (2020). Neuroprotective effects of lignan 7-hydroxymatairesinol (HMR/lignan) in a rodent model of parkinson's disease. *Nutrition, 69*, 110494. doi:10.1016/j.nut.2019.04.006

Goodall, M., Fitzwalter, B., Zahedi, S., Wu, M., Rodriguez, D., Mulcahy-Levy, J., . . . Thorburn, A. (2016). The autophagy machinery controls cell death switching between apoptosis and necroptosis. *Developmental Cell, 37*(4), 337-349. doi:10.1016/j.devcel.2016.04.018

Grosso, G., Godos, J., Lamuela-Raventos, R., Ray, S., Micek, A., Pajak, A., . . . Galvano, F. (2017). A comprehensive meta-analysis on dietary flavonoid and lignan intake and cancer risk: Level of evidence and limitations. *Molecular Nutrition & Food Research, 61*(4), 1600930-n/a. doi:10.1002/mnfr.201600930

Gwak, H., Kim, Y., An, H., Dhanasekaran, D. N., & Song, Y. S. (2017). Metformin induces degradation of cyclin D1 via AMPK/GSK3β axis in ovarian cancer. *Molecular Carcinogenesis, 56*(2), 349-358. doi:10.1002/mc.22498

Hanahan, D., & Weinberg, R. (2011). Hallmarks of cancer: The next generation. *Cell, 144*(5), 646-674. doi:10.1016/j.cell.2011.02.013

Harmatha, J., & Dinan, L. (2003). Biological activities of lignans and stilbenoids associated with plant-insect chemical interactions. *Phytochemistry Reviews, 2*(3), 321-330. doi:10.1023/B:PHYT.0000045494.98645.a3

Heidegger, I., Massoner, P., Sampson, N., & Klocker, H. (2015). The insulin-like growth factor (IGF) axis as an anticancer target in prostate cancer. *Cancer Letters, 367*(2), 113-121. doi:10.1016/j.canlet.2015.07.026

Holmbom, B., Eckerman, C., Eklund, P., Hemming, J., Nisula, L., Reunanen, M., . . . Willför, S. (2003). Knots in trees – A new rich source of lignans. *Phytochemistry Reviews, 2*(3), 331-340. doi:10.1023/B:PHYT.0000045493.95074.a8

Hu, C., Yuan, Y. V., & Kitts, D. D. (2007). Antioxidant activities of the flaxseed lignan secoisolariciresinol diglucoside, its aglycone secoisolariciresinol and the mammalian lignans enterodiol and enterolactone in vitro. *Food and Chemical Toxicology, 45*(11), 2219-2227. doi:10.1016/j.fct.2007.05.017

Hullar, M. A. J., Lancaster, S. M., Li, F., Tseng, E., Beer, K., Atkinson, C., . . . Lampe, J. W. (2015). Enterolignan-producing phenotypes are associated with increased gut microbial diversity and altered composition in premenopausal women in the united states. *Cancer Epidemiology, Biomarkers & Prevention : A Publication of the American Association for Cancer Research, Cosponsored by the American Society of Preventive Oncology, 24*(3), 546-554. doi:10.1158/1055-9965.EPI-14-0262

Jeong, S., Dasgupta, A., Jung, K., Um, J., Burke, A., Park, H. U., & Brady, J. N. (2007). PI3K/AKT inhibition induces caspase-dependent apoptosis in HTLV-1-transformed cells. *Virology, 370*(2), 264-272. doi:10.1016/j.virol.2007.09.003

Kaighn, M. E., Narayan, K. S., Ohnuki, Y., Lechner, J. F., & Jones, L. W. (1979). Establishment and characterization of a human prostatic carcinoma cell line (PC-3). *Investigative Urology, 17*(1), 16.

Kriel, J., & Loos, B. (2019). The good, the bad and the autophagosome: Exploring unanswered questions of autophagy-dependent cell death. *Cell Death and Differentiation, 26*(4), 640-652. doi:10.1038/s41418-018-0267-4

Krueckl, S. L., Sikes, R. A., Edlund, N. M., Bell, R. H., Hurtado-Coll, A., Fazli, L., . . . Cox, M. E. (2004). Increased insulin-like growth factor I receptor expression and signaling are components of androgen-independent progression in a lineage-derived prostate cancer progression model. *Cancer Research, 64*(23), 8620-8629. doi:10.1158/0008-5472.CAN-04-2446

Lavoie, J. N., L'Allemain, G., Brunet, A., Müller, R., & Pouysségur, J. (1996). Cyclin D1 expression is regulated positively by the p42/p44MAPKand negatively by the p38/HOGMAPKPathway. *Journal of Biological Chemistry, 271*(34), 20608-20616. doi:10.1074/jbc.271.34.20608

Li, N., Zhao, M., Liu, T., Dong, L., Cheng, Q., Wu, J., . . . Zhang, S. (2017). A novel soybean dirigent gene GmDIR22 contributes to promotion of lignan biosynthesis and enhances resistance to phytophthora sojae. *Frontiers in Plant Science, 8*, 1185. doi:10.3389/fpls.2017.01185

Liberti, M. V., & Locasale, J. W. (2016). The warburg effect: How does it benefit cancer cells? *Trends in Biochemical Sciences, 41*(3), 211-218. doi:10.1016/j.tibs.2015.12.001

Li-Hua Chen, Jing Fang, Huaixing Li, Wendy Demark-Wahnefried, & Xu Lin. (2007). Enterolactone induces apoptosis in human prostate carcinoma LNCaP cells via a mitochondrial-mediated, caspase-dependent pathway. *Molecular Cancer Therapeutics, 6*(9), 2581-2590. doi:10.1158/1535-7163.MCT-07-0220

Lilja, H., Vickers, A. J., & Ulmert, D. (2008). Prostate-specific antigen and prostate cancer: Prediction, detection and monitoring. *Nature Reviews Cancer, 8*(4), 268-278. doi:10.1038/nrc2351

Liou, G., & Storz, P. (2010). Reactive oxygen species in cancer. *Free Radical Research, 44*(5), 479-496. doi:10.3109/10715761003667554

Li-Quan, W., Meselhy, R. M., Yan, L. I., Guo-Wei, Q., & Masao, H. (2000). Human intestinal bacteria capable of transforming secoisolariciresinol diglucoside to mammalian lignans, enterodiol and enterolactone. *Chemical & Pharmaceutical Bulletin, 48*(11), 1606.

Liu, Q., Luo, L., & Zheng, L. (2018). Lignins: Biosynthesis and biological functions in plants. *International Journal of Molecular Sciences, 19*(2), 335. doi:10.3390/ijms19020335

Lothar Rössig, Cornel Badorff, Yvonne Holzmann, Andreas M. Zeiher, & Stefanie Dimmeler. (2002). Glycogen synthase kinase-3 couples AKT-dependent signaling to the regulation of p21Cip1 degradation. *Journal of Biological Chemistry, 277*(12), 9684-9689. doi:10.1074/jbc.M106157200

Loubière, C., Goiran, T., Laurent, K., Djabari, Z., Tanti, J., & Bost, F. (2015). Metformin-induced energy deficiency leads to the inhibition of lipogenesis in prostate cancer cells. *Oncotarget, 6*(17) doi:10.18632/oncotarget.3404

Lunt, S. Y., & Vander Heiden, M. G. (2011). Aerobic glycolysis: Meeting the metabolic requirements of cell proliferation. *Annual Review of Cell and Developmental Biology, 27*(1), 441-464. doi:10.1146/annurev-cellbio-092910-154237

Maehama, T., & Dixon, J. E. (1999). PTEN: A tumour suppressor that functions as a phospholipid phosphatase. *Trends in Cell Biology, 9*(4), 125-128. doi:10.1016/S0962-8924(99)01519-6

Mali, A. V., Joshi, A. A., Hegde, M. V., & Kadam, S. S. (2018). Enterolactone modulates the ERK/NF-κB/snail signaling pathway in triple-negative breast cancer cell line MDA-MB-231 to revert the TGF-β-induced epithelial–mesenchymal transition. *Cancer Biology & Medicine, 15*(2), 137-156. doi:10.20892/j.issn.2095-3941.2018.0012

Markulin, L., Corbin, C., Renouard, S., Drouet, S., Gutierrez, L., Mateljak, I., . . . Lainé, E. (2019). Pinoresinol-lariciresinol reductases, key to the lignan synthesis in plants. *Planta, 249*(6), 1695-1714. doi:10.1007/s00425-019-03137-y

Martinez-Outschoorn, U. E., Peiris-Pagés, M., Pestell, R. G., Sotgia, F., & Lisanti, M. P. (2017). Cancer metabolism: A therapeutic perspective. *Nature Reviews. Clinical Oncology, 14*(1), 11-31. doi:10.1038/nrclinonc.2016.60

McMenamin, M. E., Soung, P., Perera, S., Kaplan, I., Loda, M., & Sellers, W. R. (1999). Loss of PTEN expression in paraffin-embedded primary prostate cancer correlates with high gleason score and advanced stage. *Cancer Research, 59*(17), 4291.

Möller, A., Olsson, H., Grönberg, H., Eklund, M., Aly, M., & Nordström, T. (2019). The Stockholm3 blood-test predicts clinically-significant cancer on biopsy: Independent validation in a multi-center community cohort. *Prostate Cancer and Prostatic Diseases, 22*(1), 137-142. doi:10.1038/s41391-018-0082-5

Montagnani Marelli, M., Moretti, R. M., Procacci, P., Motta, M., & Limonta, P. (2006). Insulin-like growth factor-I promotes migration in human androgen-independent prostate cancer cells via the alphavbeta3 integrin and PI3-K/akt signaling. *International Journal of Oncology, 28*(3), 723.

Nicholson, K. M., & Anderson, N. G. (2002). The protein kinase B/akt signalling pathway in human malignancy. England: *Elsevier Inc*. doi:10.1016/S0898-6568(01)00271-6

Osses, D. F., Roobol, M. J., & Schoots, I. G. (2019). Prediction medicine: Biomarkers, risk calculators and magnetic resonance imaging as risk stratification tools in prostate cancer diagnosis. *International Journal of Molecular Sciences, 20*(7), 1637. doi:10.3390/ijms20071637

Peñalvo, J. L., Haajanen, K. M., Botting, N., & Adlercreutz, H. (2005). Quantification of lignans in food using isotope dilution gas chromatography/mass spectrometry. *Journal of Agricultural and Food Chemistry, 53*(24), 9342-9347. doi:10.1021/jf051488w

Peterson, J., Dwyer, J., Adlercreutz, H., Scalbert, A., Jacques, P., & McCullough, M. L. (2010). Dietary lignans: Physiology and potential for cardiovascular disease risk reduction. *Nutrition Reviews, 68*(10), 571-603. doi:10.1111/j.1753-4887.2010.00319.x

Peuhu, E., Rivero-Mueller, A., Stykki, H., Torvaldson, E., Holmbom, T., Eklund, P., . . . Eriksson, J. E. (2010). Inhibition of akt signaling by the lignan matairesinol

sensitizes prostate cancer cells to TRAIL-induced apoptosis. *Oncogene, 29*(6), 898-908. doi:10.1038/onc.2009.386

Piispanen, R., Willför, S., Saranpää, P., & Holmbom, B. (2008). Variation of lignans in norway spruce (picea abies [L.] karst.) knotwood: Within-stem variation and the effect of fertilisation at two experimental sites in finland. *Trees, 22*(3), 317-328. doi:10.1007/s00468-007-0186-3

Qie, S., & Diehl, J. (2016). Cyclin D1, cancer progression, and opportunities in cancer treatment. *Journal of Molecular Medicine, 94*(12), 1313-1326. doi:10.1007/s00109-016-1475-3

Radogna, F., Dicato, M., & Diederich, M. (2015). Cancer-type-specific crosstalk between autophagy, necroptosis and apoptosis as a pharmacological target. *Biochemical Pharmacology, 94*(1), 1-11. doi:10.1016/j.bcp.2014.12.018

Reddy, V. P., Aryal, P., Robinson, S., Rafiu, R., Obrenovich, M., & Perry, G. (2020). Polyphenols in alzheimer's disease and in the gut-brain axis. *Microorganisms, 8*(2)

Sachdev, D., & Yee, D. (2007). Disrupting insulin-like growth factor signaling as a potential cancer therapy. *Molecular Cancer Therapeutics, 6*(1), 1-12. doi:10.1158/1535-7163.MCT-06-0080

Saggar, J. K., Chen, J., Corey, P., & Thompson, L. U. (2010). The effect of secoisolariciresinol diglucoside and flaxseed oil, alone and in combination, on MCF-7 tumor growth and signaling pathways. *Nutrition and Cancer, 62*(4), 533-542. doi:10.1080/01635580903532440

Saigal, C. S., Gore, J. L., Krupski, T. L., Hanley, J., Schonlau, M., & Litwin, M. S. (2007). Androgen deprivation therapy increases cardiovascular morbidity in men with prostate cancer. *Cancer, 110*(7), 1493-1500. doi:10.1002/cncr.22933

Sánchez-Elordi, E., Sterling, R. M., Santiago, R., de Armas, R., Vicente, C., & Legaz, M. E. (2020). Increase in cytotoxic lignans production after smut infection in sugar cane plants. *Journal of Plant Physiology, 244*, 153087. doi:10.1016/j.jplph.2019.153087

Saxton, R. A., & Sabatini, D. M. (2017). mTOR signaling in growth, metabolism, and disease. *Cell, 168*(6), 960-976. doi:10.1016/j.cell.2017.02.004

Setchell, K. D. R., Brown, N. M., Zimmer-Nechemias, L., Wolfe, B., Jha, P., & Heubi, J. E. (2014). Metabolism of secoisolariciresinol-diglycoside the dietary precursor to the intestinally derived lignan enterolactone in humans. *Food Funct, 5*(3), 491-501. doi:10.1039/C3FO60402K

Shazli N. Malik, Michael Brattain, Paramita M. Ghosh, Dean A. Troyer, Thomas Prihoda, Roble Bedolla, & Jeffrey I. Kreisberg. (2002). Immunohistochemical demonstration of phospho-akt in high gleason grade prostate cancer. *Clinical Cancer Research, 8*(4), 1168.

Siddle, K. (2012). Molecular basis of signaling specificity of insulin and IGF receptors: Neglected corners and recent advances. *Frontiers in Endocrinology, 3*, 34. doi:10.3389/fendo.2012.00034

Singh, R., Letai, A., & Sarosiek, K. (2019). Regulation of apoptosis in health and disease: The balancing act of BCL-2 family proteins. *Nature Reviews. Molecular Cell Biology, 20*(3), 175-193. doi:10.1038/s41580-018-0089-8

Smeds, A. I., Eklund, P. C., Sjöholm, R. E., Willför, S. M., Nishibe, S., Deyama, T., & Holmbom, B. R. (2007). Quantification of a broad spectrum of lignans in cereals, oilseeds, and nuts. *Journal of Agricultural and Food Chemistry, 55*(4), 1337-1346. doi:10.1021/jf0629134

Smeds, A. I., Eklund, P. C., & Willför, S. M. (2016). Chemical characterization of high-molar-mass fractions in a norway spruce knotwood ethanol extract. *Phytochemistry, 130*, 207-217. doi:10.1016/j.phytochem.2016.05.006

Snyder, A. G., Hubbard, N. W., Messmer, M. N., Kofman, S. B., Hagan, C. E., Orozco, S. L., . . . Oberst, A. (2019). Intratumoral activation of the necroptotic pathway components RIPK1 and RIPK3 potentiates antitumor immunity. *Science Immunology, 4*(36), eaaw2004. doi:10.1126/sciimmunol.aaw2004

Spilioti, E., Holmbom, B., Papavassiliou, A. G., & Moutsatsou, P. (2014). Lignans 7-hydroxymatairesinol and 7-hydroxymatairesinol 2 exhibit anti-inflammatory

activity in human aortic endothelial cells. *Molecular Nutrition & Food Research, 58*(4), 749-759. doi:10.1002/mnfr.201300318

Suzuki, S., & Umezawa, T. (2007). Biosynthesis of lignans and norlignans. *Journal of Wood Science, 53*(4), 273-284. doi:10.1007/s10086-007-0892-x

Taichi Kimura, Takeshi Kuwata, Satoshi Ashimine, Manabu Yamazaki, Chisako Yamauchi, Kanji Nagai, . . . Atsushi Ochiai. (2010). Targeting of bone-derived insulin-like growth factor-II by a human neutralizing antibody suppresses the growth of prostate cancer cells in a human bone environment. *Clinical Cancer Research, 16*(1), 121-129. doi:10.1158/1078-0432.CCR-09-0982

Tummers, B., & Green, D. R. (2017). Caspase-8: Regulating life and death. *Immunological Reviews, 277*(1), 76-89. doi:10.1111/imr.12541

Udani, J. K., Brown, D. J., Tan, M. O. C., & Hardy, M. (2013). Pharmacokinetics and bioavailability of plant lignan 7-hydroxymatairesinol and effects on serum enterolactone and clinical symptoms in postmenopausal women: A single-blinded, parallel, dose-comparison study. *Journal of the American College of Nutrition, 32*(6), 428-435. doi:10.1080/07315724.2013.849578

Umezawa, T. (2003). Diversity in lignan biosynthesis. *Phytochemistry Reviews, 2*(3), 371-390. doi:10.1023/B:PHYT.0000045487.02836.32

Vanden Berghe, T., Linkermann, A., Jouan-Lanhouet, S., Walczak, H., & Vandenabeele, P. (2014). Regulated necrosis: The expanding network of non-apoptotic cell death pathways. *Nature Reviews. Molecular Cell Biology, 15*(2), 135-147. doi:10.1038/nrm3737

von Karstedt, S., Montinaro, A., & Walczak, H. (2017). Exploring the TRAILs less travelled: TRAIL in cancer biology and therapy. *Nature Reviews. Cancer, 17*(6), 352-366. doi:10.1038/nrc.2017.28

Wang, G., Zhao, D., Spring, D. J., & DePinho, R. A. (2018). Genetics and biology of prostate cancer. *Genes & Development, 32*(17-18), 1105-1140. doi:10.1101/gad.315739.118

Watson, P. A., Arora, V. K., & Sawyers, C. L. (2015). Emerging mechanisms of resistance to androgen receptor inhibitors in prostate cancer. *Nature Reviews. Cancer, 15*(12), 701-711. doi:10.1038/nrc4016

Willför, S., Hemming, J., Reunanen, M., Eckerman, C., & Holmbom, B. (2003). Lignans and lipophilic extractives in norway spruce knots and stemwood. *Holzforschung, 57*(1), 27-36. doi:10.1515/HF.2003.005

Wu, J. D., Haugk, K., Woodke, L., Nelson, P., Coleman, I., & Plymate, S. R. (2006). Interaction of IGF signaling and the androgen receptor in prostate cancer progression. *Journal of Cellular Biochemistry, 99*(2), 392-401. doi:10.1002/jcb.20929

Yang, D., Xiao, C., Su, Z., Huang, M., Qin, M., Wu, W., . . . Liu, X. (2017). (-)-7(S)-hydroxymatairesinol protects against tumor necrosis factor-α-mediated inflammation response in endothelial cells by blocking the MAPK/NF-κB and activating Nrf2/HO-1. *Phytomedicine, 32*, 15-23. doi:10.1016/j.phymed.2017.04.005

Yoneda, T. (1998). Cellular and molecular mechanisms of breast and prostate cancer metastasis to bone. *European Journal of Cancer, 34*(2), 240-245. doi:10.1016/S0959-8049(97)10132-0

Yoo, J., Lee, H. J., Kang, K., Jho, E. H., Kim, C. Y., Baturen, D., . . . Nho, C. W. (2010). Lignans inhibit cell growth via regulation of wnt/β-catenin signaling. *Food and Chemical Toxicology, 48*(8), 2247-2252. doi:10.1016/j.fct.2010.05.056

Yoon, M. (2017). The role of mammalian target of rapamycin (mTOR) in insulin signaling. *Nutrients, 9*(11), 1176. doi:10.3390/nu9111176

Zaidi, S., Gandhi, J., Joshi, G., Smith, N. L., & Khan, S. A. (2019). The anticancer potential of metformin on prostate cancer. *Prostate Cancer and Prostatic Diseases, 22*(3), 351-361. doi:10.1038/s41391-018-0085-2

9. Supplementary information

Antibody	Source	Manufacturer	Dilution	MW (kDa)
pBad (Ser136)	Rabbit	CST	1:500	23
Akt	Rabbit	CST	1:1000	60
pAkt (Ser473)	Rabbit	CST	1:1000	60
pAkt (Thr308)	Rabbit	CST	1:1000	60
ERK1/2 (p-44/42 MAPK)	Rabbit	CST	1:1000	44/42
pERK1/2 (phospho-p-44/42 MAPK)	Rabbit	CST	1:1000	44/42
Hsc70	Rat	Enzo	1:1000	70
pGsk-3β (Ser9)	Rabbit	CST	1:1000	46
p21	Rabbit	CST	1:1000	21
PARP	Mouse	CST	1:1000	116 (89)

Antibody	Manufacturer	Dilution
Rat IgG	GE Healthcare	1:10000
Rabbit IgG	CST	1:10000
Mouse IgG2A	SouthernBiotech	1:10000