LETHAL WEAPONS
– Novel approaches for receptor-targeted cancer cell elimination

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Åbo Akademi
University
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To the memory of my father

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II Peuhu E, Remes M, Holmbom T, Eklund P, Sjöholm R and Eriksson JE (2010): Screening of lignans for sensitization to TRAIL-induced apoptosis – Novel mechanisms for nortrachelogenin anticancer activity. *Manuscript.*

III Rosenholm JM, Meinander A*, Peuhu E*, Niemi R, Eriksson JE, Sahlgren C and Lindén M (2009): Targeting of porous hybrid silica nanoparticles to cancer cells. *ACS Nano* 3:197–206.

IV Rosenholm JM*, Peuhu E*, Eriksson JE, Sahlgren C and Lindén M (2009): Targeted Intracellular delivery of hydrophobic agents using mesoporous hybrid silica nanoparticles as carrier systems. *Nano Letters* 9:3308–3311.

V Rosenholm JM, Peuhu E, Bate-Eya LT, Eriksson JE, Sahlgren C, and Lindén M (2010): Cancer-cell specific induction of apoptosis using mesoporous silica nanoparticles as drug-delivery vectors. *Small* 6:1234-41.

* equal contribution

ABBREVIATIONS

4E-BP1	4E-binding protein 1
5-FU	5-fluorouracil
ADT	Androgen-deprivation therapy
AICD	Activation-induced cell death
AIDS	Acquired immunodeficiency syndrome
AIF	Apoptosis-inducing factor
ALPS	Autoimmune lymphoproliferative syndrome
Apaf-1	Apoptotic protease activity factor 1
APC	Adenomatous polyposis coli
AR	Androgen receptor
ATM	Ataxia telangiectasia mutated
ATP	Adenosine triphosphate
Bad	Bcl-2-associated death protein
Bak	Bcl-2-homologous antagonistic/killer
Bax	Bcl-2-associated X protein
Bcl-2	B-cell lymphoma gene 2
BH	Bcl-2 homology
Bid	BH3-interacting domain death agonist
Bik	Bcl-2-interacting killer
Bim	BH3-interacting mediator of cell death
BIR	Baculovirus IAP-repeat
Bmf	Bcl-2-modifying factor
ca-Akt	Constitutively active Akt
CAR	Carinol
CARD	Caspase-associated recruitment domains
CARS	Carissanol
CD95L	CD95 ligand
cIAP	Cellular IAP
CKI	Casein kinase I
CKII	Casein kinase II
CME	Clathrin-mediated endocytosis
CRD	Cystein-rich domain
CRPC	Castration-resistant prostate cancer
CvME	Caveolin-mediated endocytosis
CytD	Cytochalasin D
DAPI	40,6-diamidino-2-phenylindole
DcR	Decoy receptor
DED	Death effector domain
DIABLO	Direct IAP-binding protein with low pI
DiI	1,1′-dioctadecyl-3,3,3′,3′-tetramethindocarbocyanine perchlorate
DiO	3,3-dioctadecyl-oxacarbocyanine perchlorate
DISC	Death-inducing signaling complex
DNA	Deoxyribonucleic acid
DR	Death receptor
EDAR	Ectodysplasin receptor
EGF	Epidermal growth factor
EGFP	Enhanced green fluorescence protein
EGFR	EGF receptor
EMT	Epithelial-mesenchymal transition
END	Enterodiol
EndoG	Endonuclease G
ENL	Enterolactone
EPR	Enhanced permeability and retention
ERK	Extracellular signal-regulated protein kinase
FAb	Fragment antigen binding
FADD	Fas-associated death domain protein
FGFR	Fibroblast growth factor receptor
FITC	Fluorescein isothiocyanate
FLICE	Fas-associated death domain-like interleukin-1β-converting enzyme
FLIP	FLICE-inhibitory protein
FoxO	Forkhead Box O
Gab-1	Grb-2 associated binder 1
GDP	Guanosine diphosphate
GF	Growth factor
GFR	GF receptor
GFR	Growth factor
GLUT4	Glucose transporter type 4
GPCR	G-protein coupled receptor
GSK-3β	Glycogen synthase kinase β
GTP	Guanosine triphosphate
HEK	Human embryonic kidney
HMR	7-hydroxymatairesinol
Hrk	Harakiri
HTRA2	High temperature requirement-protein A2
IκB	Inhibitor kappaB
IAP	Inhibitor of apoptosis
IGF-I	Insulin-like growth factor I

IGF-IR	IGF-I receptor
IKK	IκB kinase
IRS-1	Insulin receptor substrate 1
izTRAIL	Isoleucine zipper TRAIL
JNK	c-Jun N-terminal kinase
LPS	Lipopolysaccharide
LSB	Laemmli sample buffer
MAPK	Mitogen-activated protein kinase
MAT	Matairesinol
mCD95L	Membrane CD95L
Mcl-1	Myeloid cell leukemia 1
MDM2	Murine double-minute 2
mDR5	Mouse DR5
MFI	Mean fluorescence intensity
MOMP	Mitochondrial outer membrane permeabilization
MRI	Magnetic resonance imaging
MSN	Mesoporous silica nanoparticle
mTOR	Mammalian target of rapamycin
N-APP	The extracellular fragment of β-amyloid precursor protein
NDGA	Nordihydroguaiaretic acid
NF-κB	Nuclear factor kappaB
NGFR	Neural growth factor receptor
NK	Natural killer
NLS	Nuclear localization signal
NTG	Nortrachelogenin
OMM	Outer mitochondrial membrane
PAMAM	Poly(amidoamine)
PARP	Poly (ADP-ribose) polymerase
PBS	Phosphate-buffered saline
PDGF	Platelet-derived growth factor
PDGFR	PDGF receptor
PDK1	Phosphoinositide-dependent protein kinase 1
PE	Phycoerythrin
PEG	poly(ethylene glycol)
PEI	Poly(ethylene imine)
PH	Pleckstrin homology
PHLPP	Pleckstrin homology domain leucine-rich repeat protein phosphatase
PI3K	Phosphatidylinositol 3-OH-kinase
PIDD	p53-induced protein with death domain
PIP_2	Phophatidylinositol-4,5-phosphate
PIP_3	Phophatidylinositol-3,4,5-phosphate
PKB	Protein kinase B
PKC	Protein kinase C
PLA	Poly(lactic acid)
PLAD	Pre-ligand assembly domain
PLGA	Poly(lactic-co-glycolic acid)
PP2A	Protein phosphatase 2A
PSA	Prostate-specific antigen
PTB	Phosphotyrosine-binding
PTEN	Phosphate and tensin homolog located on human chromosome number 10
PUMA	p53-upregulated modulator of apoptosis
RAIDD	RIP-associated ICH-1/CED-3 homologue with death domain
RBD	Ras-binding domain
RES	Reticuloendothelial system
RIP-1	Receptor-interacting protein 1
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RTK	Receptor tyrosine kinase
S6K	S6-kinase
SAR	Structure-activity relation
sCD95L	Soluble CD95L
SDG	Secoisolariciresinol diglucoside
SDS	Sodium dodecyl sulphate
SECO	Secoisolariciresinol
SEM	Standard error of mean
SH2	Src-homology 2
Sos	Son of sevenless
STAT	Signal transducer and activator of transcription
sTNFα	soluble TNFα
TAK1	Transforming growth factor β-activated kinase 1
TKI	Tyrosine kinase inhibitor
TL1A	TNF-like ligand 1A
TMRM	Tetramethylrhodamine methyl ester
TNFα	Tumor necrosis factor α
TNFR	Tumor necrosis factor receptor
TRADD	TNFR-associated death domain
TRAF2	TNFR-associated factor 2
TRAIL	TNF-related apoptosis-inducing ligand
UPR	Unfolded-protein response
VEGF	Vascular-endothelial growt factor
VEGFR	VEGF receptor
XIAP	X-linked IAP

ABSTRACT

The currently used forms of cancer therapy are associated with drug resistance and toxicity to healthy tissues. Thus, more efficient methods are needed for cancer-specific induction of growth arrest and programmed cell death, also known as apoptosis. Therapeutic forms of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) are investigated in clinical trials due to the capability of TRAIL to trigger apoptosis specifically in cancer cells by activation of cell surface death receptors. Many tumors, however, have acquired resistance to TRAIL-induced apoptosis and sensitizing drugs for combinatorial treatments are, therefore, in high demand. This study demonstrates that lignans, natural polyphenols enriched in seeds and cereal, have a remarkable sensitizing effect on TRAIL-induced cell death at non-toxic lignan concentrations. In TRAIL-resistant and androgen-dependent prostate cancer cells we observe that lignans repress receptor tyrosine kinase (RTK) activity and downregulate cell survival signaling via the Akt pathway, which leads to increased TRAIL sensitivity. A structure-activity relationship analysis reveals that the γ-butyrolactone ring of the dibenzylbutyrolactone lignans is essential for the rapidly reversible TRAIL-sensitizing activity of these compounds. Furthermore, the lignan nortrachelogenin (NTG) is identified as the most efficient of the 27 tested lignans and norlignans in sensitization of androgen-deprived prostate cancer cells to TRAIL-induced apoptosis. While this combinatorial anticancer approach may leave normal cells unharmed, several efficient cancer drugs are too toxic, insoluble or unstable to be used in systemic therapy. To enable use of such drugs and to protect normal cells from cytotoxic effects, cancer-targeted drug delivery vehicles of nanometer scale have recently been generated. The newly developed nanoparticle system that we tested *in vitro* for cancer cell targeting combines the efficient drug-loading capacity of mesoporous silica to the versatile particle surface functionalization of hyperbranched poly(ethylene imine), PEI. The mesoporous hybrid silica nanoparticles (MSNs) were functionalized with folic acid to promote targeted internalization by folate receptor overexpressing cancer cells. The presented results demonstrate that the developed carrier system can be employed *in vitro* for cancer selective delivery of adsorbed or covalently conjugated molecules and furthermore, for selective induction of apoptotic cell death in folate receptor expressing cancer cells. The tested carrier system displays potential for simultaneous delivery of several anticancer agents specifically to cancer cells also *in vivo*.

INTRODUCTION

Approximately one per mille of the 50–70 trillion cells of an adult body is replaced on a daily basis to serve the biological goals of our life, namely survival and reproduction. The balance between cell division and cell death enables us to maintain the relatively constant body size, and to succeed in this, each cell must obediently serve the benefit of the organism, even if it means sacrificing the life of the cell. A process called apoptosis is a mode of programmed cell death that enables cells to die in a well-coordinated manner and the remains to be cleared away by the neighboring cells. The signals that trigger this cellular suicide program can originate from within the cell in response to cellular injury, such as accumulating DNA damage, or from the exterior of the cell in the form of death ligands. In an adult, death ligands are utilized primarily by the cells of the immune system to activate apoptosis in cells the body no longer needs, or in cells that are considered dangerous to the organism. The activation proceeds by binding of the death ligands to cell surface proteins, termed death receptors, capable of transmitting the signal to the interior of the cell. Intracellular signaling leads to amplification of the received message and ultimately to enzymatic decomposition of the cellular structures.

In cancer, individual cells continue to grow and divide despite the internal and external signals that demand the cell to commit suicide. This behavior stems from critical mutations in the cancer cell genome that promote cell survival and growth independent of external stimuli and disrupt mechanisms that normally limit cell division or induce apoptotic cell death. These internal differences in cell signaling can also be manifested at the cancer cell surface as altered expression of molecules, like receptors. Such patterns in cell surface composition can be identified and used for distinction of cancer cells from healthy cells. However, current cancer therapies rely heavily on the induction of apoptosis in dividing cells with the consequence of also damaging non-cancerous cells in tissues that are being rapidly replaced. The second major problem in dealing with cancer is that the cancer cells adapt to new circumstances and, therefore, evade applied treatments by becoming resistant. These critical issues are in the core of the challenges that cancer is currently posing to the aging populations and new more powerful and cost-efficient treatments as well as improved modes of cancer drug administration are desperately sought after.

REVIEW OF LITERATURE

1. CANCER AS A THERAPEUTIC CHALLENGE

1.1 Hallmarks of cancer

In contrast to common perception, cancer is not one disease but a large group of variable diseases that should be treated in different ways in order to be cured. Cancers originate from distinct cell types and gradually harbor specific genome alterations during the course of cancer development and progression. Inherited mutations can predispose to cancer, but most of the diagnosed cancers occur through transformation of cells during our life span. These genetic and epigenetic changes that have capacity to transform cells include gain-of-function mutations in proto-oncogenes, loss-of-function mutations in tumor suppressor genes and mutations that lead to inactivation of genes that control genomic stability (reviewed by Hahn & Weinberg 2002). Cellular proto-oncogenes function in regulation of normal cell growth and proliferation, but can be turned into growth promoting oncogenes, through processes such as gene amplification, chromosomal translocation, enhanced transcription, or impairment of negative regulation. Inactivation of genes that restrain cell proliferation and survival, designated as tumor suppressor genes, can occur through mutations that alter the gene expression, post-translational modifications or functionality of the gene product. Reflecting the variety of genomic changes leading to cancer, all the disease entities take their own clinical course and respond to specific therapy forms, while being resistant towards others.

In order to grow and invade, transforming cells must acquire several functional capabilities (Figure 1). These critical changes in cell physiology include impaired induction of programmed cell death (also called apoptosis), self-sufficiency for growth signals, insensitivity to antigrowth signals, sustained angiogenesis, limitless replicative potential, deregulating cellular energetics, avoiding immune destruction, tissue invasion and metastasis, as well as enabling characteristics, genomic instability and tumor-promoting inflammation (reviewed by Hanahan & Weinberg 2000, Hanahan 2010, *oral communication*). The one feature that makes cancer a lethal disease is tumor metastasis. In order to grow, tumors must acquire blood or lymphatic vessels to the tumor site for sufficient supply of oxygen and nutrients and removal of metabolites. Concomitantly, this process called angiogenesis enables tumor metastasis, but first the primary tumor cells need to obtain migratory and invasive properties. A developmental process called epithelial-mesenchymal transition (EMT) is thought to play a central role also in initiation of metastasis, as it transforms well-organized epithelial cells into cells of mesenchymal phenotype with loose cell-cell adhesion and lost cell polarity (reviewed by Thiery *et al.* 2009). After local invasion and entry into the vasculature, imperative steps for metastasis include survival in the circulation, infiltration to distant organs, and finally colonization (reviewed by Chambers *et al.* 2002 and Nguyyen *et al.* 2009). All these processes are potential targets for cancer therapeutics. In addition, recent advances have highlighted the important role of tumor-associated tissues in promotion of cancer survival and metastasis, suggesting that also non-cancer tissues must be taken into consideration when developing new cancer therapeutics (reviewed by Joyce & Pollard 2009).

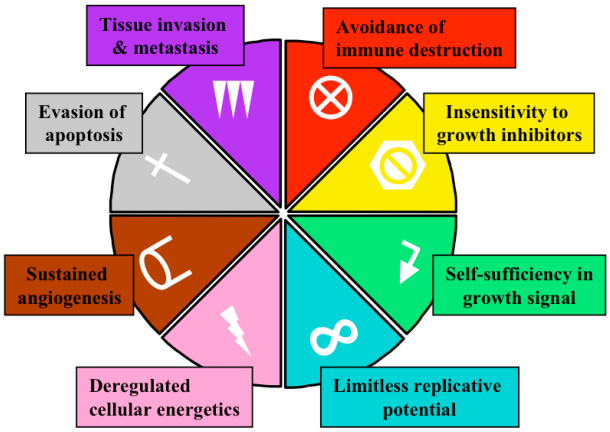


Figure 1. The hallmarks of cancer. Modified from (Hanahan & Weinberg 2000).

1.1.1 Conventional chemotherapy

Treatment of solid tumors has largely employed surgery, radiation, photodynamic treatment, hyperthermia and chemotherapy. Many of the traditional chemotherapeutics are aimed at the particular weakness of cancer cells, which is their proliferation. Most cells in an adult body have entered post-mitotic state and do not proliferate any longer. Therefore antimitotic drugs, like tubulin-targeting taxanes and vinca alkaloids, can be employed to eliminate dividing cancer cells. Many treatments also take advantage of the vulnerabilities that the cells have once they have discarded critical checkpoint controls. In such cells, induction of DNA and chromosome damage does not cause cell cycle arrest but entry to mitosis occurs, which is likely to lead to cell death by mitotic catastrophe (reviewed by Castedo *et al.* 2004). Cancer treatments with alkylating agents or platinum drugs are based on these effects. Antimetabolites, such as methotrexate and 5-fluorouracil (5-FU), interfere with DNA and RNA synthesis and thereby prevent cancer cell growth and survival. Antitumor antibiotics, like doxorubicin, actinomycin-D and mitoxantrone, inhibit enzymes that are involved in DNA replication, and topoisomerase inhibitors, such as topotecan and etoposide, inhibit enzymes that separate DNA strands. Treatment with these drug types results in inhibited cell proliferation and induction of cell death. In some cases also corticosteroids, prednisolone and dexamethasone, are used as anticancer treatments due to their anti-inflammatory activity. All the mentioned anticancer agents have been successful in clinic alone or in combination to other compounds, but many patients either do not respond or alternatively develop resistance towards the therapy.

1.1.2 Challenges in development of cancer therapeutics

The side effects caused by chemotherapy are multiple and vary from anemia and nausea to problems in the function of vital organs. Drugs that target cell proliferation particularly harm the tissues that are under constant renewal, such as skin, hair, intestine and bone marrow. Similarly, treatments that damage the genome in order to induce tumor cell death can instead result in unrepaired DNA damage and cell loss or even cell transformation in other tissues (reviewed by Allan & Travis 2005). The risk of bystander toxicity is naturally increased when multiple cancer treatments are used in combination to achieve good drug response.

Tumors that initially respond well to a particular therapy often develop drug resistance. Deregulation of apoptosis and other cell death programs contributes to malignant growth both during the initial tumor development and acquisition of resistance to chemotherapy (reviewed by de Bruin & Medema 2008). During cancer initiation, progression and treatment tumor cells acquire genomic instability that creates new genetic and epigenetic changes that further provide selection advantage in altered environment. This plasticity of cancer cell populations drives the relapse of tumors. The molecular mechanisms behind cancer drug resistance include inhibited import of the drug molecules, acquired ability to pump the drugs out or ability to metabolize and detoxify the administered drugs (reviewed by Gottesman *et al.* 2002). For example, expression of the ABC transporter P-glycoprotein enables cancer cells to pump out a variety of different drug molecules, thereby lowering their intracellular concentrations to sub-toxic levels (Chen *et al.* 1986). Resistance can also arise from improved mechanisms for DNA repair or incapacity of the cell to induce apoptosis due to loss of critical effectors in the signaling cascade (Gottesman *et al.* 2002). To overcome these mechanisms of resistance, application of two or three unrelated cancer drugs simultaneously is often required.

Increasing evidence supports the existence of a particular population of tumor cells called cancer stem cells that maintain their ability for self-renewal, generate differentiated cells as their progeny by asymmetric cell division and most importantly, have the capacity of giving rise to new tumors in animal hosts (Clarke *et al.* 2006). It has also been reported that reprogramming of somewhat differentiated tumor cells back to cancer stem cells may occur, and that the signals within the tumor microenvironment have a critical role in this process (Gupta *et al.* 2009). As cancer stem cells are more resistant to conventional therapeutics than the rest of the tumor, optimal cancer therapy is likely to require treatments that successfully eliminate both cancer stem cells and differentiated cancer cells. In order to better identify the subgroups of patients that are most likely to profit from particular therapy forms, researchers are trying to find new molecular markers. For example, in the case of colon cancer individualized data from tumor gene expression arrays can be used for identifying gene signatures that help to predict the patient's response to certain drugs as well as prognosis (reviewed by Gangadhar & Schilsky 2010). When combined to this type of molecular diagnostics, multiagent therapy might turn out to be the most effective weapon against cancer.

1.1.3 Targeted cancer therapies

Cancer treatment is slowly heading towards the assignment of tailor-made drug therapies to each individual patient and the targeted elimination of cancer without inducing undesirable side effects. Targeted cancer therapy is based on using drugs or other substances to identify and eliminate cancer cells while doing little damage to normal cells. In order to distinguish cancer cells from normal cells, cancer-specific features need to be identified, but finding such molecular targets is a big challenge to cancer research. Cancer cells often rely on hyperactive forms of growth and survival-promoting proteins for their proliferation and escape from apoptosis (Hahn & Weinberg 2002). Inhibition of these oncoproteins by small-molecule drugs, monoclonal antibodies and other means has been pursued vigorously in order to develop targeted cancer therapeutics (reviewed by Gschwind *et al.* 2004 and Engelman 2009). Several new therapeutic forms, such as drugs that target the vascular endothelial growth factor receptors (VEGFRs) in tumor angiogenesis or inhibit epidermal growth factor receptors (EGFRs) in breast cancer, have

already been discovered, while many future advances against cancer are likely to arise from this field.

Cancer patients can also be treated with immunotherapies that stimulate the natural immune system to more efficiently identify and attack cancer cells. While the purpose of active immunotherapies, such as therapeutic vaccines, is to stimulate the host's defenses against the disease, passive immunotherapies are based on administering components, such as antibodies, that are created outside the body (reviewed by Melief & Burg 2008, Higano *et al.* 2009). In addition, advances in targeted gene therapy might succeed in curing the disease by replacing the mutated sequences in cancer cell genome. For example, reintroduction of the wild-type tumor suppressor p53 gene, which is mutated in no less than half of all human tumors, has been shown to benefit cancer therapy of head and neck cancers (reviewed by Huang *et al.* 2009).

1.1.4 Treatment of prostate cancer

Prostate cancer is the most common malignancy and the second leading cause of cancer mortality in men of the western world (Jemal *et al.* 2008). The majority of prostate cancer patients are aged and diagnosed with clinically localized, low-risk prostate cancer that can be effectively treated with surgery and radiation. However, prognosis is poor for locally advanced or metastatic disease that has progressed to castration-resistant prostate cancer (CRPC) (reviewed by Shepard & Raghavan 2010). Improved diagnostics with serum prostate specific antigen (PSA) as a relatively good marker for prostate cancer risk have resulted in earlier diagnosis of prostatic carcinomas. For advanced prostate cancer, the typical sites of metastasis are lymph nodes and bone, and the disease-related symptoms are often connected to bone metastasis.

Despite extensive research efforts, androgen-deprivation therapy (ADT) by surgical castration or nowadays more commonly by chemical castration with antiandrogens remains the most effective therapy for advanced metastatic prostate cancer. Recently, novel higher affinity androgen receptor inhibitors have been developed and proven effective in preclinical prostate tumor models (Tran *et al.* 2009). Successful clinical trials with an inhibitor of the enzyme that catalyzes androgen biosynthesis, cytochrome P17, have unexpectedly demonstrated that CRPC often remains hormone-dependent (reviewed by Attard *et al.* 2009). Upregulated expression of androgen receptor in seemingly hormone-independent tumor xenografts might compensate for the low level of androgens upon ADT (Chen *et al.* 2004). Despite the good initial response, ADT is of limited benefit because of the difficult adverse effects of androgen deprivation, especially on the cardiovascular system, and the CRPC that occurs in most patients within some years (Shepard & Raghavan 2010). However, chemotherapy with docetaxel provides significant survival benefit in patients with CRPC (Tannock *et al.* 2004, Petrylak *et al.* 2004), and treatment regimens together with several other chemotherapeutics, such as cabazitaxel, prednisone and sartraplatin, are being investigated in clinical studies as second-line therapy for advanced prostate cancer (Shepard & Raghavan 2010). New therapeutic agents that are not directed against rapidly proliferating cells are urgently needed in treatment of prostate cancer, because the proliferative fraction of prostate cancer cells is usually less than 10% (Berges et al. 1995) and only a fraction of the patients with metastatic prostate cancer responds to current chemotherapeutics. Targeted therapy forms currently in clinical trials for advanced prostate cancer include antiangiogenic and bone-targeted agents, and immunotherapies, like the sipuleucel-T vaccine, as well as specific inhibitors for critical

survival signaling proteins, such as the Src kinases, mammalian target of rapamycin (mTOR) and insulin-like growth factor receptor I (reviewed by Fizazi *et al.* 2010).

1.2 Growth factor-induced survival signaling in prostate cancer

Proliferation of normal cells depends on the presence of mitogenic growth factors (GFs) in the surroundings of the cell. Stimulation of the cell surface growth factor receptors (GFRs) by these ligands leads to activation of intracellular signaling pathways and regulation of cell differentiation, growth, proliferation, survival, migration and metabolism. Cancer cells, in contrast, frequently harbor mechanisms that make them self-sufficient in terms of these signals through acquisition of mutations in key proteins of the pathways. Such oncoproteins include GFRs and their downstream signaling molecules, like the small GTPase Ras, which is found mutated in perhaps one quarter of all human tumors (Hahn & Weinberg 2002).

GFs can function in autocrine, paracrine or endocrine manner with some GFs displaying more local regulatory roles [epidermal growth factor (EGF), platelet-derived growth factor (PDGF)] and others regulating growth and metabolism at the whole organism level [insulin, insulin-like growth factor I (IGF-I)] (reviewed by Pollak 2009). It has been estimated that upregulation of the GFR signaling and the downstream phosphatidylinositol-3-OH kinase (PI3K) pathway occurs approximately in 30–50% of prostate cancers (reviewed by Morgan *et al.* 2009). Thus, the PI3K signaling pathway constitutes a key target for prostate cancer therapy (reviewed by Engelman 2009, Morgan *et al.* 2009, Wong *et al.* 2010). In addition, extensive crosstalk exists between the androgen receptor signaling and the PI3K signaling pathways. Activation of PI3K signaling through GFRs may play a critical role in allowing prostate tumors to maintain continued proliferation in low-androgen environments, thereby promoting the development of CRPC (reviewed by Mulholland *et al.* 2006).

1.2.1 Receptor tyrosine kinase activation by growth factors

Growth factor receptors are receptor tyrosine kinases (RTKs) that convey signals through activation of a conserved tyrosine kinase domain. All the 58 currently known human RTKs divided into 20 subfamilies have a similar molecular architecture, with ligand-binding domains in the extracellular region, a single transmembrane helix, and a cytoplasmic region that contains the protein tyrosine kinase domain as well as additional regulatory regions (reviewed by Lemmon & Schlessinger 2010). Binding of the ligand stabilizes the interaction between the individual receptor molecules in a RTK dimer, which leads to sequential *trans*-autophosphorylation of the tyrosine kinase domains and subsequent release of the autoinhibitory interactions in each receptor (Lemmon & Schlessinger 2010). Autophosphorylation of the tyrosine kinase domain creates phosphotyrosine-based binding sites for a number of cytoplasmic signaling molecules containing Src homology-2 (SH2) or phosphotyrosine-binding (PTB) domains (Songyang *et al.* 1993, Kavanaugh & Williams 1994). They may bind directly to RTKs or via docking proteins, such as Grb-2, IRS-1 (Insulin receptor substrate-1) and Gab-1 (Grb-2 associated binder-1), which are recruited to and phosphorylated by RTKs (reviewed by Schlessinger 2000). The assembled complexes initiate signaling cascades leading to enhanced protein synthesis and transcriptional regulation of target genes involved in cell survival, growth, and differentiation.

The two major signaling cascades triggered upon RTK stimulation comprise the PI3K/Akt pathway and the mitogen-activated protein kinase (MAPK) pathway (Figure 2), although activation of other signaling proteins, such as phospholipase Cγ and signal transducer and activator of transcription (STAT) also occurs (Schlessinger 2000). The MAPK pathway involves binding of the adaptor Grb-2 directly or via Shc to the tyrosine phosphorylated RTK, and subsequent recruitment of the guanine nucleotide exchange factor son of sevenless (Sos) close to the plasma membrane (reviewed by Margolis & Skolnik 1994). Sos activates membrane-tethered small GTPase Ras proteins by converting them from inactive GDP-bound state to active GTP-bound state. Activated Ras proteins further stimulate c-Raf-1 that phosphorylates mitogen-activated protein kinase kinases 1 and 2 (MEK1/2). MEK, in turn, phosphorylates extracellular signal-regulated protein kinase 1 and 2 (ERK1/2) that again phosphorylates a variety of substrates in the cytoplasm and in the nucleus.

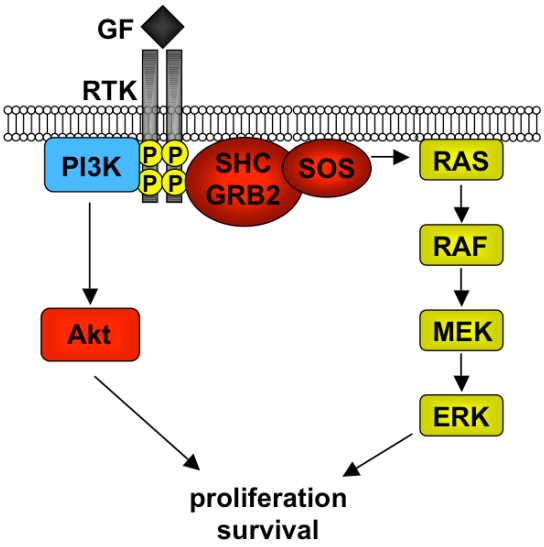


Figure 2. Growth factor (GF) – stimulated receptor tyrosine kinases (RTK) activate the PI3K and MAPK pathways. Auto-phosphorylated RTK recruits adaptor proteins as well as enzymes like PI3K and Sos. Activation of the small GTPase Ras by Sos induces the MAPK pathway via activation of Raf. Thereafter, sequential phosphorylations lead to activation of MEK and ERK. PI3K, in turn, activates the Akt kinase. Both pathways promote cell survival and proliferation.

Aberrant RTK activation in human cancers is principally mediated by autocrine activation, chromosomal translocations, RTK overexpression or gain-of-function mutations (Lemmon & Schlessinger 2010). Growth of normal and malignant prostate cells is also regulated by RTKs, such as IGF-I receptor (IGF-IR), EGFR, fibroblast growth factor receptor (FGFR), PDGF receptor (PDGFR) and VEGFR (reviewed by Hellawell & Brewster 2002). In the prostate, stromal cells produce most of the GFs, which act on the GFRs on epithelial cells. In prostate cancer, the production of most GFs is upregulated and autocrine pathways emerge in epithelial cells for example for IGF-I and FGF (Hellawell & Brewster 2002). Most prostate cancers arise from the epithelial compartment, where EGF and IGF-I have important functions in promotion of cell growth, proliferation, differentiation and survival.

1.2.2 Phosphatidylinositol-3-OH kinase generates lipid second messengers

Among RTK-induced signaling pathways, the PI3K pathway has been identified as a key mechanism in carcinogenesis and survival of many different types of cancer (reviewed by Engelman 2009). When PI3Ks are activated by G-protein coupled receptors (GPCRs), RTKs or other cell surface receptors that induce tyrosine kinase activity, they phosphorylate the 3′-hydroxyl group of plasma membrane inositol lipids. According to their substrate preference and sequence homology, PI3Ks can be divided into three classes (I-III), of which Class I PI3Ks primarily generate phosphatidylinositol-3,4,5-trisphosphate (PIP_3, Figure 3) by phosphorylation of phosphatidylinositol-4,5-bisphosphate (PI-4,5-P_2). These PI3Ks are further grouped into two subfamilies with distinct receptors to which they are recruited. Class IA PI3Ks are activated by growth factor RTKs, whereas the class IB PI3Ks are activated upon recruitment to GPCRs. The class IA PI3Ks seem to be most involved in cancer (Engelman 2009).

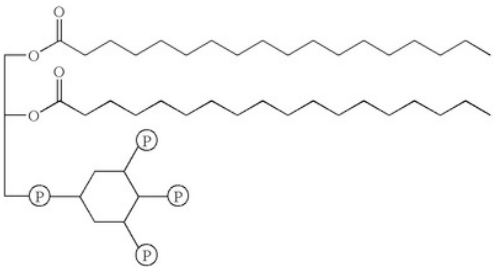


Figure 3. Phosphatidylinositol-3,4,5-trisphosphate (PIP_3).

Phosphate and tensin homolog located on human chromosome number 10 (PTEN) phosphatase counteracts the class I PI3K activity by removing phosphates from PIP_3 promoting formation of PIP_2 (Maehama & Dixon 1998). The function of this tumor suppressor is commonly disturbed in prostate cancer, resulting in constitutive activation of the PI3K pathway (Li *et al.* 1997, Vlietstra *et al.* 1998). Overwhelmingly, loss of heterozygosity or mutation at the PTEN locus occurs in up to 35% and 12% of prostate cancer samples, respectively (Engelman *et al.* 2006). The activity of the PI3K pathway can also be enhanced by increased RTK activation or genetic mutation and amplification of key components of the pathway (Engelman 2009).

The class IA PI3Ks are heterodimers consisting of a p85 regulatory subunit and a p110 catalytic subunit, which both come in numerous isoforms. Three longer p85 regulatory subunits, p85α, p85β and p55γ, as well as two shorter isoforms, p55α and p50α, interact via their p110-binding domain with the three catalytic subunits, p110α, p110β and p110δ. In addition to the C-terminal catalytic domain and other regulatory structures, the p110 subunits contain a Ras-binding domain (RBD) that mediates their activation by the small GTPase Ras. The class IA PI3Ks are recruited to the plasma membrane by the SH2 domains of the p85 regulatory subunit that bind tyrosine-phosphorylated consensus sequences at either active RTKs or adaptor proteins that bind to them (Carpenter *et al.* 1993). Inhibition of the catalytic subunit p110 by the regulatory p85 subunit is released upon binding, which leads to enzymatic activation of PI3K (Yu *et al.* 1998). The produced PIP_3 functions as a lipid second messenger that recruits to plasma membrane a wide array of a pleckstrin homology (PH) domain -containing signaling molecules (reviewed by DiNitto *et al.* 2003). The protein serine/threonine kinase Akt (also known as PKB) is a

primary target of PIP_3 (Franke *et al.* 1997, Klippel *et al.* 1997). The PI3K pathway activation has been associated with advanced pathological tumor stage, increased incidence of lymph node metastases and development of androgen-independent growth, which is why PI3K and its downstream signaling molecules offer critical drug targets for therapeutic intervention in prostate cancer (reviewed by Morgan *et al.* 2009).

1.2.3 Akt activity promotes cell growth and survival

c-Akt is the cellular homolog of the transforming oncogene of the AKT8 retrovirus (Staal 1987). The mammalian Akt family of serine/threonine kinases comprises three isoforms (Akt1–3) that are highly conserved throughout evolution, but present different expression profiles and may have overlapping and distinct roles in cancer (reviewed by Altomare & Testa 2005). By phosphorylation of target proteins, Akt regulates a wide range of cellular functions, such as protein synthesis, cell metabolism, cell cycle progression and cell survival (reviewed by Datta *et al.* 1999) (Figure 4). PI3K activation leads to accumulation of $PIP3_3$, which recruits the normally cytoplasmic Akt as well as phosphoinositide-dependent protein kinase 1 (PDK1) to the plasma membrane through PH domain interactions (Franke *et al.* 1997). This colocalization leads to Akt phosphorylation by PDK1 at the Tyr308 (Alessi *et al.* 1997) and by mTOR complex 2 at Ser473 (Sarbassov *et al.* 2005). These two phosphorylation events are required for full activation of the Akt kinase and subsequent phosphorylation of downstream targets.

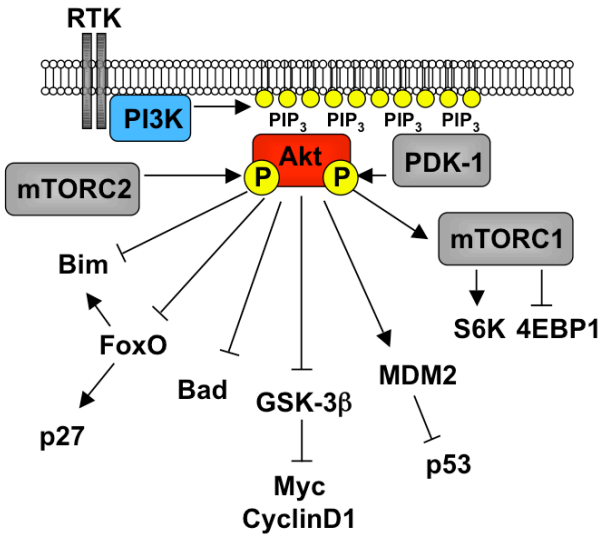


Figure 4. Examples of Akt-regulated signaling pathways for cell survival, growth and proliferation. See text for details. Adapted from (Engelman *et al.* 2009).

Akt activates mTOR complex 1 that stimulates cellular translation machinery by phosphorylation of S6-kinase (S6K) and 4E binding protein 1 (4E-BP1) (reviewed by Raught *et al.* 2001), and inactivates glycogen synthesis by phosphorylation of glycogen synthase kinase 3β (GSK-3β) (Cross *et al.* 1995). Phosphorylation of GSK-3β also results

in stabilization the cell cycle proteins c-Myc and cyclin D1 that in the absence of RTK activation become degraded (reviewed by Liang & Slingerland 2003). In muscle and fat, Akt upregulates expression of GLUT4 glucose transporter, thereby promoting glucose uptake (Kohn *et al.* 1996). Activation of Akt favors G1/S cell cycle transition by blocking the transcription of cell cycle inhibitors, such as $p27^{KIP1}$, through phosphorylation of Forkhead Box O (FoxO) transcription factors (Liang & Slingerland 2003). Other targets of FoxO that become suppressed upon Akt activation include the proapoptotic molecules BH3-interacting mediator of cell death (Bim) and CD95 ligand. Akt can promote cell survival also directly by phosphorylation of the proapoptotic proteins Bad (Bcl-2-antagonist of cell death) and procaspase-9 leading to their inactivation (Datta *et al.* 1997, Cardone *et al.* 1998). Furthermore, Akt-mediated phosphorylation of mouse double minute 2 (MDM2) leads to proteasomal degradation of p53, which supports cell cycle progression and inhibits p53-induced apoptosis upon cellular stress (Mayo & Donner 2001). Akt is also able to activate the IkB kinase (IKK), a positive regulator of the nuclear factor kappaB (NF-κB) transcription factors, which promote the transcription of antiapoptotic genes (Kane *et al.* 1999).

In addition to ubiquitin-dependent Akt degradation and caspase-dependent Akt cleavage, Akt signaling can be attenuated by the protein phosphatases PP2A and pleckstrin homology domain leucine-rich repeat protein phosphatase (PHLPP) that dephosphorylate Akt (reviewed by Liao & Hung 2010). More than 50% of prostate carcinomas exhibit Akt hyperactivity and the therapeutic use of Akt inhibitors is currently being investigated (Altomare & Testa 2005). For example, simultaneous targeting of several Akt isoforms or inhibition of both mTOR complexes by pharmaceutical compounds may be beneficial in prostate cancer treatment (reviewed by Sarker *et al.* 2009).

2. REGULATION OF APOPTOTIC CELL DEATH

2.1 Activators and inhibitors of apoptosis

Apoptosis is the predominant mechanism of programmed cell death that animals employ for coordinated elimination of excess or damaged cells during development and maintenance of tissue homeostasis (Kerr *et al.* 1972). However, as errors occur in biological systems, also regulation of apoptosis may fail. Excessive cell loss due to apoptotic cell death is known to occur in various human pathologies, including cerebral ischemia (reviewed by Broughton *et al.* 2009), AIDS (reviewed by Muthumani *et al.* 2003), and neurodegenerative disorders, such as Alzheimer's disease (reviewed by Rohn 2010). Excessive cell accumulation because of impaired apoptosis is key component of cancer development (reviewed by Hanahan & Weinberg 2000), and contributes to the etiology of several autoimmune diseases (reviewed by Maniati *et al.* 2008).

At the cellular level, apoptosis can be triggered by a variety of stimuli, including DNA damage, chromosomal abnormalities, growth factor withdrawal, viral infection, oncogene activation, substrate detachment, hypoxia and death receptor activation. Apoptosis is an energy-dependent process that occurs in sequential steps of morphological events (Wyllie *et al.* 1980). The dying cell undergoes nuclear and cytoplasmic condensation, blebbing of the plasma membrane, and fragmentation into membrane-enclosed particles. Professional

phagocytes, including neutrophils, monocytes, macrophages and dendritic cells, or the neighboring cells rapidly identify and engulf these apoptotic bodies, thereby preventing induction of inflammatory response. The opposite happens when a cell dies a necrotic cell death. Swelling of the cell and rupture of the plasma membrane result in release of the cell contents into surrounding tissues, which is why necrosis can cause inflammation. Necrosis has traditionally been defined as an accidental and uncontrolled process, but accumulating evidence now suggests that also necrosis can occur in a more regulated manner through specific signaling events (reviewed by Golstein & Kroemer 2007). Whether a cell dies by apoptosis, necrosis or by other characterized modalities of programmed cell death between these two classical types, depends on the nature and severity of the stimulus, as well as the cell type. Crosstalk between these cell death pathways even enables switching from one mode to another in case the functional requirements for a particular cell death modality are not met. Of the various forms of programmed cell death, apoptosis is the major regulator of embryogenesis and organismal homeostasis.

2.1.1 Caspases – The executers of apoptosis

The biochemical markers of apoptosis include fragmentation of nuclear DNA, cell surface exposure of phosphatidylserine phospholipids, and activation of caspases, the specialized cysteine-dependent aspartate proteases that execute the apoptotic cell death (reviewed by Hengartner 2000). In contrast, the caspase-independent forms of programmed cell death, including autophagy and necroptosis, are executed typically by other cellular proteases, such as cathepsins and calpains (reviewed by Bröker *et al.* 2005). Caspases are expressed as inactive zymogens, procaspases, which can be activated by specific proteolysis. Upon apoptotic stimuli, the hierarchical activation of caspases is set off by the activation of the initiator caspases, including caspase-2, -8, -9, and -10, by induced proximity at multiprotein platforms (Muzio *et al.* in 1998, Chen *et al.* 2002, Boatright *et al.* 2003, Bouchier-Hayes *et al.* 2009). Upon dimerization at the activating complexes, initiator caspases undergo autocleavage that stabilizes the active enzyme (Pop *et al.* 2007). The stimulated initiator caspases proteolytically activate the downstream effector caspases, including caspase-3, -6 and -7. As the effector procaspases already form constitutive dimers, the only requirement for their activation is the cleavage that allows the caspase to adopt the active conformation (reviewed by Riedl & Shi 2004). The objective of these sequential activation events is the amplification of the original death signal. At the terminal phase of apoptosis, hundreds of structural and regulatory proteins are specifically cleaved by caspases, which leads to death of the cell (reviewed by Lüthi & Martin 2007). Caspase-1, the prototype of inflammatory caspases, which also include caspase-4, -5, -11, -12 and -13, contributes to the maturation of inflammatory cytokines instead of apoptosis-induction, and can be activated in the inflammasome complex (Martinon *et al.* 2002).

Caspases are subject to multiple regulatory mechanisms. Indeed, the regulation of initiator caspase activation needs to be careful, as the activation of these apical proteases will trigger the cascade of downstream caspase activation and apoptosis. The prodomains of initiator caspases contain conserved interaction modules that serve in formation of homotypic protein interactions and in generation of the first proteolytic signal in the apoptosis pathway. The two death effector domains (DEDs) on caspase-8 and caspase-10 mediate the recruitment and dimerization of these caspases at active cell surface death receptors (DRs) via the adaptor Fas-associated death domain protein (FADD) in activation of the extrinsic apoptosis pathway (Muzio *et al.* 1998). Caspase-9 and caspase-2 initiate apoptosis through the intrinsic pathway via their caspase-associated recruitment domains

(CARDs), which mediate homotypic interactions in formation of the activating platforms for these caspases (Hofmann *et al.* 1997). The extrinsic and the intrinsic apoptosis pathways serve to start the apoptotic process in response to signals of different origin. Extracellular cytokines called death receptor ligands engage the extrinsic pathway through the cell surface DRs, while mitochondria play a critical role in induction of apoptosis by cell intrinsic cues.

2.1.2 Caspase-inhibitory proteins suppress apoptotic signaling

The family of Fas-associated death domain-like interleukin-1β-converting enzyme (FLICE) -inhibitory proteins (FLIPs), comprising three mammalian isoforms, c-FLIP long ($FLIP_L$), c-FLIP short (c-$FLIP_S$) and c-$FLIP_R$, and a viral FLIP, has a critical function in regulation of DR-mediated initiator caspase activation (Thome *et al.* 1997, Irmler *et al.* 1997, Hu *et al.* 1997). As the c-FLIP proteins are homologous to caspase-8 and caspase-10, they contain also the two DEDs and can be recruited to activated DRs and dimerize with caspases (Irmler *et al.* 1997). c-FLIP, however, lacks both the catalytic active site and the residues that form the substrate-binding pocket of an active caspase. This feature makes c-FLIP enzymatically inactive, unable to promote caspase activation and thereby powerful dominant negative inhibitor of caspase-8 and caspase-10 activation. Not surprisingly, mouse embryonic fibroblasts from c-FLIP knockout animal are more sensitive to extrinsic apoptosis induction (Yeh *et al.* 2000). c-$FLIP_L$ has an inactive caspase-like catalytic domain at the C-terminus, while the shorter isoforms, c-$FLIP_S$ and c-$FLIP_R$, completely lack the catalytic domain and instead have unique splicing tails. Differential regulation and function of c-FLIP isoforms are based on these structural differences. The short c-FLIP isoforms simply block recruitment and activation of caspase-8, but c-$FLIP_L$:caspase-8 dimerization leads to partial cleavage of both proteins and localized proteolytic activity of caspase-8 (Krueger *et al.* 2001). c-$FLIP_L$ may exert an antiapoptotic role in caspase activation when expressed in large amounts, but when present in lower amounts, c-$FLIP_L$ is likely to promote caspase-8 and caspase-10 activity (Chang *et al.* 2002). Interestingly, c-FLIP is not only an inhibitor of caspase activation, but can also promote activation of NF-κB and ERK survival signaling pathways (Kataoka *et al.* 2000). As accumulating evidence indicates that overexpression of c-FLIP in cancer correlates with inhibition of DR-mediated apoptosis and resistance to chemotherapy, c-FLIP has become an attractive target for cancer therapy (reviewed by Safa *et al.* 2008).

Another critical group of proteins controlling the activity of both initiator and effector caspases is the inhibitor of apoptosis (IAP) protein family (reviewed by Gyrd-Hansen & Meier 2010). IAPs are defined by the presence of 1–3 baculovirus IAP repeat (BIR) protein domains that can bind to caspases. Some of IAPs contain a RING domain with the capability to function as an E3 ubiquitin ligase and to target proteins to proteasomal degradation. The X-linked IAP (XIAP) has been reported to directly inhibit the activity of the executioner caspases-3 and -7 and the initiator caspase-9 (Eckelman & Salvesen 2006). However, caspase-independent actions of IAPs by employment of the ubiquitin ligase activity promote the activation of NF-κB survival-signaling, which might be the most important contribution of XIAP and the cellular IAPs, cIAP1 and cIAP2, in regulation of cell fate as well as tumorigenesis (Gyrd-Hansen & Meier 2010). IAPs are recruited to NF-κB activating signaling complexes, where ubiquitination not only regulates degradation of target proteins, but also creates docking sites for interacting proteins (Gyrd-Hansen & Meier 2010).

2.1.3 Mitochondrial permeabilization releases proapoptotic factors

The mitochondria undergo two major alterations upon apoptosis-induction. These are the outer mitochondrial membrane permeabilization (MOMP), and the loss of the electrochemical gradient (mitochondrial polarity) that normally is present across the inner mitochondrial membrane. It is commonly accepted that MOMP precedes the mitochondrial membrane depolarization and dictates the point-of-no-return in cellular apoptosis. The mitochondrial apoptosis pathway, also known as intrinsic pathway, can be triggered by intracellular events through induction of MOMP. Permeabilization of the outer mitochondrial membrane (OMM) is currently thought to occur by formation of proteolipid pores, when the proapoptotic B-cell lymphoma gene 2 (Bcl-2) family proteins Bcl-2-associated X protein (Bax) and Bcl-2 homologous antagonistic/killer (Bak) homo-oligomerize within the OMM (Wei *et al.* 2001).

When MOMP is induced, apoptogenic proteins are released from the mitochondrial intermembrane space to the cytosol. These proteins include cytochrome *c*, SMAC (second mitochondria-derived activator of caspases)/DIABLO (direct inhibitor of apoptosis (IAP)-binding protein with low pI), AIF (apoptosis-inducing factor), EndoG (endonuclease G) and Omi/HTRA2 (high-temperature-requirement protein A2) (Susin *et al.* 1999, Du *et al.* 2000, Verhagen et al 2000, Li *et al.* 2001, Suzuki *et al.* 2001). In intact mitochondria cytochrome *c* resides in the mitochondrial intermembrane /intercristae spaces and functions as an electron transporter in the respiratory chain. Once released from the mitochondria, cytochrome *c* binds to the apoptotic protease activity factor-1 (Apaf-1) in the cytosol, which triggers the oligomerization of Apaf-1 into a ring-like structure in the presence of ATP (reviewed by Riedl & Salvesen 2007). Following formation of this structure called the apoptosome, the CARD domains on Apaf-1 molecules become exposed, cytosolic procaspase-9 monomers are recruited to apoptosome via CARD domain interactions, and caspase-9 is activated (Zou *et al.* 1999). The activation does not require caspase-9 cleavage but occurs by dimerization of the procaspase-9 monomers (Renatus *et al.* 2001). As an initiator caspase, caspase-9 will propagate the apoptotic signal by cleaving and activating effector caspases, like caspase-3, eventually causing cell death. The mitochondrial protein, Smac/DIABLO, which also is released form the mitochondria after MOMP, inhibits IAPs by direct inhibition and induction of their proteasomal degradation, thereby ensuring that the mitochondrial caspase cascade will eventually result in apoptosis of the cell (Chai *et al.* 2000).

The therapeutic effect of many conventional cancer treatments is based on their ability to initiate the mitochondrial apoptosis pathway by inducing DNA damage or the production of reactive oxygen species (ROS) (reviewed by Ozben 2007). Following oxidative, genotoxic or oncogenic stress, the tumor suppressor protein p53 is stabilized and transcriptionally regulates many genes involved in apoptosis, cell-cycle arrest, and other cellular functions (Zhao *et al.* 2000). In the cytosol, p53 promotes apoptosis independent of its transcriptional activity by localizing to the mitochondria (Marchenko *et al.* 2000) and interacting with the Bcl-2 family proteins that regulate mitochondrial integrity (reviewed by Yee & Vousden 2005). The stabilized p53 is also involved in activation of caspase-2 in response to genotoxic stress in a complex called the PIDDosome (Tinel & Tschopp 2004). Caspase-2 forms this complex together with RIP-associated ICH-1/CED-3 homologue with death domain (RAIDD) and p53-induced protein with death domain (PIDD). Unlike other initiator caspases, caspase-2 does not directly activate executioner caspases, but instead acts via MOMP to induce apoptosis (Guo *et al.* 2002).

2.1.4 Regulation of the mitochondrial integrity by the Bcl-2 protein family

Permeabilization of the OMM is controlled through interactions of the proapoptotic and antiapoptotic proteins of the Bcl-2 family, which is characterized by Bcl-2 homology (BH) domains in the protein structure (Figure 5) (Reviewed by Chipuk et al 2010). The functional activity of the Bcl-2 family proteins is based on homo- and heterodimerization by interaction of the BH3 domain α helix with a groove formed by the BH1 and BH2 domains (Sattler *et al.* 1997). The antiapoptotic Bcl-2 protein subfamily members, A1, Bcl-2, Bcl-w, Bcl-xL and Myeloid cell leukemia 1 (Mcl-1), contain four BH domains and act by sequestering the proapoptotic Bcl-2 proteins, thereby preventing their participation in apoptotic complexes (Cheng *et al.* 2001). The proapoptotic Bcl-2 proteins include the multidomain effector proteins, Bax and Bak, as well as the BH3 domain–only subfamily. The release of the effectors Bax and Bak leads to their homo-oligomerization at the OMM, and formation of pore complexes to promote MOMP. The BH3-only proteins are upstream sensors of cellular damage that can be divided to the "direct activators" and "sensitizers/de-repressors" (reviewed by Chipuk *et al.* 2010). The direct activators, Bim, BH3-interacting domain death agonist (Bid) and potentially also p53-upregulated modulator of apoptosis (PUMA), interact with both the antiapoptotic Bcl-2 proteins and the effectors Bax/Bak. These BH3-only proteins can directly induce Bax and Bak oligomerization and MOMP (Kim *et al.* 2006). The "sensitizers/derepressors", Bcl-2-associated death protein (Bad), Bcl-2-interacting killer (Bik), Bcl-2-modifying factor (Bmf), Harakiri (Hrk) and Noxa, predominantly bind to the antiapoptotic Bcl-2 proteins with the capacity to competitively liberate Bax and Bak from these repressors. In addition to the direct activator BH3-only proteins, p53 can bind and activate oligomerization of Bax and Bak (Chipuk *et al.* 2004, Leu *et al.* 2004).

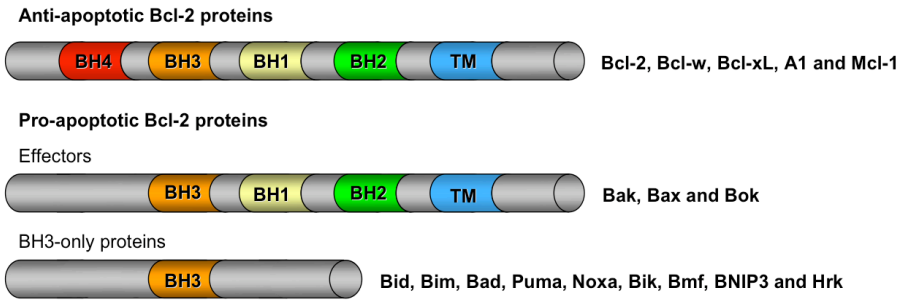


Figure 5. The Bcl-2 protein family. Schematic overview of the Bcl-2 homology (BH) domains and membrane-inserting domains (TM) in members of each Bcl-2 protein category. Adapted from (Tait & Green 2010).

Bcl-2 is a known oncoprotein that is able to co-operatively drive malignant progression in mouse models *in vivo* by promoting cancer cell survival (Vaux *et al.* 1988, Strasser *et al.* 1990, Schmitt *et al.* 2000). It is clear that also Bax and Bak are critical mediators of apoptosis, as their combined deletion causes severe developmental defects in mice due to abolishment of most apoptotic processes (Lindsten *et al.* 2000). However, the model that regards Bid and Bim as direct activators of Bax and Bak, has been challenged by another model according to which all the BH3-only proteins simply function as neutralizers of the prosurvival Bcl-2 family members and thereby promote the release of Bax and Bak (Willis *et al.* 2007). The induction of Bax and Bak oligomerization can reportedly occur also by non-protein factors, such as heat shock and detergents (Hsu & Youle 1997, Pagliari *et al.*

2005), and Bax and Bak –dependent apoptosis was observed to take place in the absence of Bid and Bim (Willis *et al.* 2007). These studies suggest that mechanisms alternative to Bid and Bim –mediated induction of Bax and Bak activation exist. The *in vivo* results of Merino *et al.* (2009) highlight that the direct interaction with Bax and the ability to engage the antiapoptotic Bcl-2 proteins are both important features in Bim BH3-only protein function, showing that these mechanisms of are not exclusive during MOMP induction.

The Bcl-2 family members are regulated at the level of transcription, as well as by post-translational mechanisms (reviewed by Chipuk *et al.* 2010). Different BH3-only proteins share little homology, and also seem to function as sensors for distinct cellular stresses. Phosphorylation of BH3-only proteins can lead to their sequestration by other proteins, and prevention of relocalization to the mitochondrial membrane. For example, growth factor stimulation promotes Bad phosphorylation at multiple residues, primarily by Akt, which leads to cytosolic sequestration and inactivation of Bad by the 14-3-3 proteins until Bad becomes dephosphorylated upon apoptotic stimuli (Zha *et al.* 1996, Wang *et al.* 1999). Also Bim is negatively regulated by Akt-mediated phosphorylation in response to survival signals (Qi *et al.* 2006), and by ERK1/2-mediated phosphorylation that targets Bim to proteasomal degradation (Ley *et al.* 2003). In contrast, phosphorylation of Bim by c-Jun N-terminal kinase (JNK) upon cellular stress leads to the release of Bim from the dynein motor proteins and relocalization to the mitochondria where Bim promotes cytochrome *c* release (Lei & Davis 2003).

2.1.5 Bid as a mediator between extrinsic and intrinsic apoptosis pathways

The BH3-only protein Bid is regulated by proteolytic cleavage and subsequent exposure of the BH3 domain (Figure 6). Activated caspase-8 can cleave Bid to its truncated form, the C-terminal tBid, which then accumulates into the mitochondria and promotes MOMP (Li *et al.* 1998, Luo *et al.* 1998). In order to activate the proapoptotic function of Bid, the cleavage and proteasome-dependent degradation of the autoinhibitory N-terminal portion of cleaved Bid need to occur (Chou *et al.* 1999, Tan *et al.* 1999, Tait *et al.* 2007). The chain of events leading to tBid-mediated activation of Bax starts with rapid tBid association with the mitochondrial membrane, followed by Bax or Bak recruitment, insertion, and oligomerization (Wei *et al.* 2001, Kuwana *et al.* 2002, Lovell *et al.* 2008). The translocation of the C-terminal tBid to the mitochondria has been reported to depend on the N-terminal myristoylation of tBid, which promotes its insertion to the mitochondrial membrane (Zha *et al.* 2000). Targeting of tBid for proteasomal degradation functions as a mechanism for limiting the mitochondrial apoptosis cascade (Breitschopf *et al.* 2000).

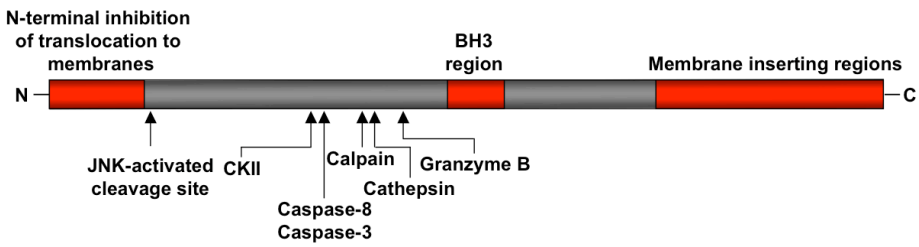


Figure 6. Schematic presentation of the Bid protein structure. Casein kinase II (CKII) phosphorylation site and cleavage sites for caspase-3, caspase-8, calpain, cathepsin, and granzyme B as well as the JNK-activated cleavage site are indicated. Modified from (Billen *et al.* 2009).

In addition to cleavage by caspase-8, Bid can be cleaved by caspase-3 downstream of cytochrome *c* release (Li *et al.* 1998, Slee *et al.* 2000). Bid may also be a physiologically relevant substrate for caspase-2 in heat shock (Li *et al.* 1998, Bonzon *et al.* 2006), granzyme B in T cell-mediated cytotoxicity (Sutton *et al.* 2000, Heibein *et al.* 2000), lysosomal proteases upon lysosomal permeabilization (Stoka *et al.* 2001, Reiners *et al.* 2002) and calpains in ischemia and cisplatin-induced apoptosis (Chen *et al.* 2001a, Mandic *et al.* 2002). Phosphorylation of Bid at multiple sites close to the cleavage region by enzymes of the casein kinase family, and potentially also by other kinases, inhibits the caspase-mediated cleavage of Bid and its proapoptotic activation (Desagher *et al.* 2001, Degli-Esposti *et al.* 2003). JNK is involved in generation of a large C-terminal fragment of Bid, jBid, which accumulates to mitochondria but induces selective release of apoptotic factors from the mitochondria due to the longer N-terminal part (Deng *et al.* 2003). Furthermore, ATM kinase-phosphorylated Bid seems to play a role in DNA damage-induced apoptosis and cell cycle arrest (Zinkel *et al.* 2005, Kamer *et al.* 2005). Careful control of the proapoptotic activity of Bid reflects its importance at the cross roads between the caspase-8-activating extrinsic apoptosis pathway and apoptosis signaling through the mitochondria. This link is of major significance, as many cell types require Bid-mediated activation of MOMP and cytochrome *c* release in order to commit to death receptor-mediated apoptosis.

2.2 Death receptor-mediated apoptosis

The signals from the extracellular ligands to the intracellular caspase machinery are transmitted via a group of cell surface proteins called the DRs. This route of signaling is known as the extrinsic cell death pathway. DRs belong to the tumor necrosis factor receptor (TNFR) superfamily that comprises 29 proteins characterized by one to six extracellular cystein-rich domains (CRDs) (reviewed by Aggarwal 2003). Although most TNFRs are type I transmembrane proteins, some of them are anchored to plasma membrane by glycophospholipid moieties or secreted as soluble molecules. Once activated, the members of the TNFR family mediate a multitude of cellular functions from differentiation to regulation of immunological responses (Aggarwal 2003). A specific feature of the TNFR members that are classified as DRs, is the relatively conserved cytoplasmic death domain (DD) of approximately 80 amino acid residues that is required for signal transduction (Itoh & Nagata 1993, Tartaglia *et al.* 1993). To date, six DRs have been identified: TNFR1 (p55), CD95 (Fas/APO-1), DR3 (TRAMP), DR4 (TRAIL-R1/TNFRSF10A), DR5 (TRAIL-R2/TNFRSF10B) and DR6 (Figure 7). Although ectodysplasin A receptor (EDAR) and nerve growth factor receptor (NGFR/p75NTR) also contain intracellular DD, these receptors are not typically designated as DRs. Several decoy receptors that lack functional signaling properties can compete with the DRs in ligand binding and inhibit DR signalling (reviewed by Askenazi & Dixit 1999).

2.2.1 Death receptor ligands are important regulators of the immune system

DRs are activated by their cognate ligands that belong to the TNF family of cytokines (Aggarwall 2003) (Figure 7). Of the 19 identified TNF superfamily ligands, five are known to activate DRs. Tumor necrosis factor α (TNFα) and lymphotoxin α (LTα) bind to TNFR1 (Dembic *et al.* 1990), CD95 ligand (CD95L, Fas-ligand) binds to CD95 (Suda *et al.* 1993), and TL1A binds to DR3 (Migone *et al.* 2002). Both CD95L and TL1A can also

interact with the soluble decoy receptor 3 (DcR3) (Pitti *et al.* 1998, Migone *et al.* 2002). Tumor necrosis factor-related apoptosis inducing-ligand (TRAIL/Apo2L) has been shown to bind five receptors, including two DRs, DR4 (Pan *et al.* 1997a) and DR5 (Pan *et al.* 1997b, Sheridan *et al.* 1997), and two decoy receptors, decoy receptor 1 (DcR1/TRIDD/TRAIL-R3/TNFRSF10C; Pan *et al.* 1997b, Sheridan *et al.* 1997) and decoy receptor 2 (DcR2/TRUNDD/TRAIL-R4/TNFRSF10D; Marsters *et al.* 1997). In addition to these four membrane-bound receptors, TRAIL binds with lower affinity to a soluble receptor, osteoprotegerin (OPG), with the consequence of blocking the antiosteoclastogenic activity of OPG and the apoptotic activity of TRAIL (Emery *et al.* 1998). The extracellular fragment of β-amyloid precursor protein (N-APP), characteristic to Alzheimer's disease pathology, was only recently discovered to bind to DR6 and trigger neuronal degeneration, thereby functioning as a long lost ligand for DR6 (Nikolaev *et al.* 2009).

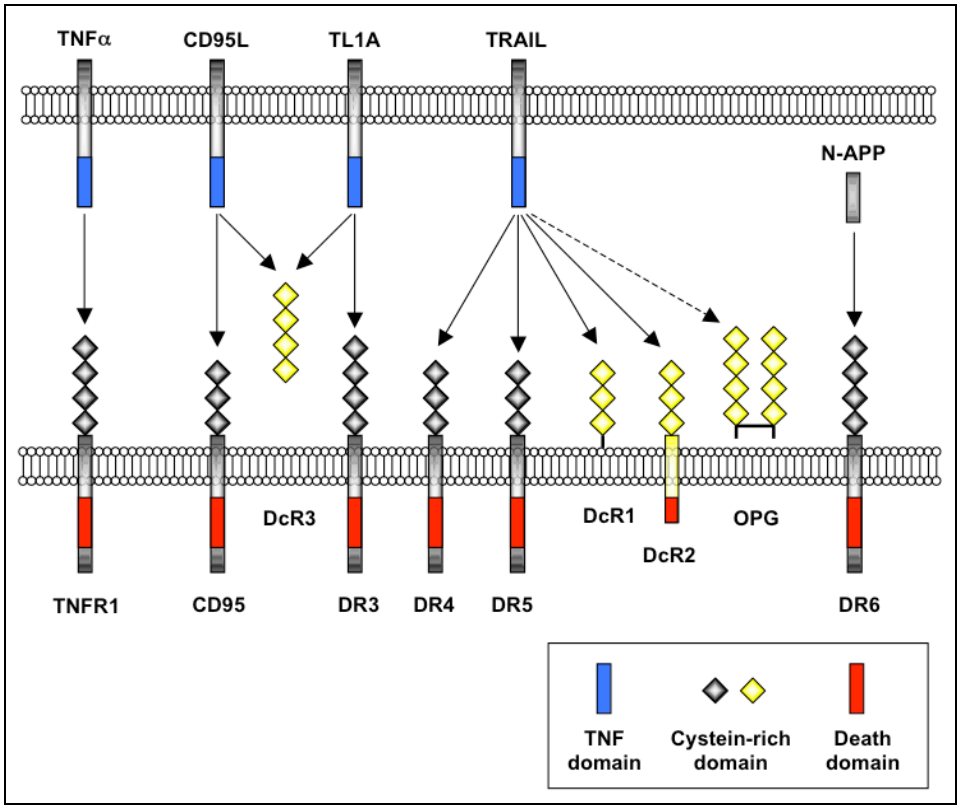


Figure 7. The interactions of TNF ligands with death receptors (DRs) and decoy receptors (DcRs). Single DR ligands can interact with the cystein-rich extracellular domains of several death domain (DD) containing DRs (grey) or DcRs (yellow) that do not have a functional DD. See text for details. Adapted from (Gonzalvez & Ashkenazi 2010).

All death receptor ligands, except for lymphotoxin α and N-APP, are expressed as type II transmembrane proteins predominantly on the cell surface of immune cells, such as T cells, B cells, monocytes, dendritic cells and natural killer (NK) cells (Aggarwal 2003). In addition to transmitting apoptosis-inducing signals, DR ligands can activate signaling pathways that promote growth and survival, but some ligands are more potent than others

with this respect. All TNF ligands can activate to varying degree the NF-κB transcription factor that regulates expression of a large number of target genes involved in cell proliferation and survival as well as apoptosis (reviewed by Hayden & Ghosh 2008). TNFα, for example, is better known as a proinflammatory cytokine that induces proliferation, differentiation and cellular activation, but also elicits activation of caspases and apoptosis in certain cell types. TNFα is involved in various physiological and pathological processes, with the main focus on immune responses and autoimmunity (Aggarwal 2003).

CD95L and TRAIL, in turn, represent the prototypic death receptor ligands that potently trigger cellular apoptosis. Like TNFα, CD95L also plays a critical role in regulation homeostasis in the immune system. The loss-of-function mutations *gld* or *lpr*, in mouse CD95L or the CD95 receptor, respectively, induce a severe lymphoproliferative phenotype, similar to the human autoimmune lymphoproliferative syndrome (ALPS) associated with mutations in the CD95 signaling pathway (Watanabe-Fukunaga *et al.* 1992, Takahashi *et al.* 1994, Rieux-Laucat *et al.* 1995, Fisher *et al.* 1995). Expression of CD95L and its receptor on activated T cells is involved in a process called activation-induced cell death (AICD) (Brunner *et al.* 1996) that mediates peripheral deletion of autoreactive T cells (reviewed by Bouillet & O'Reilly 2009). Expression of CD95L on sites of immune privilege, such as brain, uterus, eye and testis, attenuates induction of immune responses against foreign antigens at these locations (Griffith *et al.* 1995). Inducible cell surface translocation of CD95L from intracellular storage is employed in cytotoxic T cell and NK cell -mediated killing of infected or transformed target cells (Bossi & Griffiths 1999). The ability of CD95L to induce non-apoptotic signals has been evidenced for example by studies demonstrating, how CD95-signaling is able to enhance liver regeneration (Desbarts & Newell 2000), provide co-stimulation for activated T cells (Maksimow *et al.* 2006) and most recently, how CD95-mediated prosurvival signals promote tumor growth (Chen *et al.* 2010).

2.2.2 Regulation of death receptor ligand activity

The activity of death receptor ligands can be modulated through their recruitment to dynamic plasma membrane domains, called membrane rafts (reviewed by Simons & Gerl 2010). This membrane compartment, rich in sphingolipids and cholesterol, has been shown to accommodate multiple signaling receptors and their intracellular effectors. CD95L recruitment to membrane rafts enhances its killing activity possibly due to increased aggregation of both the ligand and the bound CD95 receptor at the target cell (Eramo *et al.* 1994, Muppidi & Siegel 1994, Nachbur *et al.* 2006). Regulation of TNFα and TRAIL activity by distribution to plasma membrane rafts has not yet been characterized, but translocation of their receptors to rafts influences the outcome of DR signaling. While raft-localized TNFR1 is more likely to induce survival signaling than apoptosis (Treede *et al.* 2009), DR4 and DR5 have enhanced ability to trigger apoptosis, once recruited to membrane rafts (Rossin *et al.* 2009).

Proteolytic shedding of the membrane-associated ligands by metalloproteases produces soluble forms of TNFα (Black *et al.* 1997) and CD95L (Tanaka *et al.* 1995) further expanding the possibilities for stringent regulation of death receptor ligand activity. In a recent *in vivo* study, O'Reilly and coworkers (2009) elucidated whether the soluble death ligands possess attenuated killing activity. They generated transgenic mice that specifically

lacked either the secreted from (sCD95L) or the transmembrane form of CD95L (mCD95L), and thereby demonstrated that only mCD95L was essential for cytotoxic activity, as only the mice lacking mCD95L developed severe lymphadenopathy and autoimmunity similar to CD95L-deficient mice. Soluble TNFα (sTNFα), on the other hand, has a different action profile than its transmembrane counterpart. Only sTNFα is able to sensitize T-cells for AICD (Muller *et al.* 2009), while non-cleavable TNFα can still kill tumor cells or virally infected cells (Perez *et al.* 1990, Borsotti *et al.* 2007). In contrast to metalloprotease-mediated cleavage of TNFα and CD95L, *in vitro* studies have demonstrated that TRAIL shedding might involve cellular cysteine proteases (Mariani & Krammer 1998). Further studies are required to elucidate if production of soluble TRAIL by shedding also occurs *in vivo*.

2.2.3 The initial phases of death receptor activation

Unlike their ligands, the DRs seem to be present on the surface of most normal and transformed cells. Increasing evidence suggests that several of the DRs and other TNFR family members arrange into preformed receptor complexes by homotypic interactions between the extracellular protein domains. These interactions have been shown to require the proximal N terminus of the receptor called the pre-ligand assembly domain (PLAD). Identification of PLAD interactions for most DRs, including TNFR1 (Chan *et al.* 2000), DR4, DR5 and DcR2 (Clancy *et al.* 2005) and CD95 (Papoff *et al.* 1999, Siegel *et al.* 2000), has changed the common view of ligand-induced trimerization. Inhibition of TRAIL-induced apoptosis by decoy receptor DcR2 was found to function through PLAD-mediated formation of mixed complexes with DR5 in CD8+ T cells (Clancy *et al.* 2005). As for CD95, ligand-independent association of the receptors in lipid rafts dictates sensitivity to CD95L-induced apoptosis in CD4+ T cells (Muppidi & Siegel 2004). These results demonstrate that pre-ligand assembly is an important step in the regulation of DR-mediated cellular responses, and may thereby provide an interesting target for pharmacological intervention (reviewed by Chan 2007).

It has become evident that certain cell signaling pathways require receptor internalization for full activation or generation of a different biological response. Receptor internalization-dependent and -independent signaling pathways also play a role in DR signaling. The seemingly contradicting biological activities of DRs, namely promotion of both survival and apoptosis, might be related to the dynamics of receptor localization at the plasma membrane and endocytosis into the intracellular compartments (reviewed by Schütze *et al.* 2008). For example, transmission of proapoptotic signals in response to TNFα has been shown to depend on internalization of the activated TNFR1 complex, while activation of NF-κB occurs when the receptor is located at the plasma membrane (Schneider-Brachert *et al.* 2004). Similarly, effective caspase-8 activation in response to CD95 ligation seems to require the post-translational modification of CD95 by palmitoylation, localization at plasma membrane lipid rafts, and internalization into the endosomes (Eramo *et al.* 2004, Feig *et al.* 2007). Inhibition of CD95 internalization, in turn, results in the activation of the MAPK and NF-κB signaling pathways (Lee *et al.* 2006).

DRs can be divided into two types according to the primary adaptor protein that they recruit upon ligand binding and formation of multiprotein signaling platforms. The adaptor molecules contain domains that bind to DRs and create docking sites for downstream signal transduction. CD95, DR4 and DR5 recruit FADD protein and primarily induce

proapoptotic signaling. Representing the other DR-type, TNFR1 and DR3 bind TNF receptor-associated death domain (TRADD) protein and show mainly proinflammatory and immune-stimulatory activity (Hsu *et al.* 1995). Cross talk between these two signaling modules enables, for example, TNFR1 to signal via FADD to induce apoptosis, and for CD95, DR4 and DR5 to stimulate prosurvival pathways through activation of NF-κB and MAP kinases in some circumstances (reviewed by Wilson *et al.* 2009).

2.2.4 TNFR1- and CD95-induced signaling cascades

The presence of TNFR1 on almost all nucleated cell types suggests a very versatile role for TNFR1 in regulation of biological functions (reviewed by Vandenabeele *et al.* 1995). The diversity of TNFR1 signaling is reflected by the capacity of TNFR1 to form two temporary and spatially distinct cytoplasmic signaling complexes that can signal either NF-κB activation form the cell surface or apoptosis from the internalized receptors (Micheau & Tschopp 2003). The TNFR1 signaling complex I is rapidly formed at the plasma membrane and composed of the adaptor proteins TRADD and TNF receptor-associated factor 2 (TRAF2), as well as DD-containing receptor-interacting protein 1 (RIP1) and other proteins. Several of the TNFR1-associated proteins, such as cIAP1/2 and TRAF2, have the ability to regulate the signaling output of the complex through their E3 ubiquitin ligase activity (reviewed by Wertz & Dixit 2010). Once ubiquitinated, RIP1 interacts with transforming growth factor-β-activated kinase 1 (TAK1), which subsequently activates the IKK complex (reviewed by Hayden & Ghosh 2008). This leads to inhibitor kappaB (IκB) phosphorylation, polyubiquitination and proteasomal degradation, liberating NF-κB to move into the nucleus where it regulates target gene transcription. Through TRAF2 interactions, TNFR1 can also activate MAP kinases, such as JNK or p38 (Lee *et al*. 1997, Yuasa *et al.* 1998). Thus, TNFR1 complex 1 mediates the proliferative and proinflammatory effects of TNFα. Internalization of the activated TNFR1 receptor complex is a prerequisite for the formation of the secondary intracellular complexes. TNFR1 complex IIA (RIP1/FADD/procaspase-8/10) and IIB (TRADD/FADD/procaspase-8/10) are formed with the participation of the adapter protein FADD and procaspases-8 and -10 in the cytosol (reviewed by Wilson *et al.* 2009). Activation of the initiator caspases in TNFR complex II leads to activation of caspase-3 and eventually apoptosis. The complex II –mediated TNFR1 pathway resembles the proapoptotic signaling cascades induced by CD95L and TRAIL.

Unlike its ligand, the CD95 receptor is expressed in a wide range of tissues, including the thymus, heart, liver and kidney (reviewed by Nagata 1997). It was initially identified as an antigen for a monoclonal antibody that induced apoptosis in tumor cells (Trauth *et al.* 1989, Itoh *et al.* 1991, Oehm *et al.* 1992), after which studies of CD95-induced apoptosis have greatly elucidated the mechanisms and regulation of DR signaling. CD95-induced apoptosis can be counteracted by expression of the soluble DcR3, which has been reported to occur in tumors through genomic DcR3 amplification (Pitti *et al.* 1998). Ligand binding to preformed CD95 complexes initiates rapid clustering of the receptors into micro-molecular aggregates and further into larger surface clusters that can even be visualized by fluorescence microscopy (Algeciras-Schimnich *et al.* 2002, Siegel *et al.* 2004, Feig *et al.* 2007) (Figure 8). In these structures, CD95 directly recruits FADD and caspase-8 via homotypic interactions to form the death-inducing signaling complex (DISC) at the cytoplasmic death domain (Boldin *et al.* 1995, Chinnaiyan *et al.* 1995, Kischkel *et al.* 1995, Muzio *et al.* 1996). DISC serves as a platform for activation of caspase-8 and caspase-10 by induced proximity (Medema *et al.* 1997, Vincenz & Dixit 1997, Muzio *et al.*

1998). Recruitment of c-FLIP to CD95–DISC regulates activation of caspase-8 and caspase-10 and participates in transmission of antiapoptotic CD95-signaling (reviewed by Thome & Tschopp 2001).

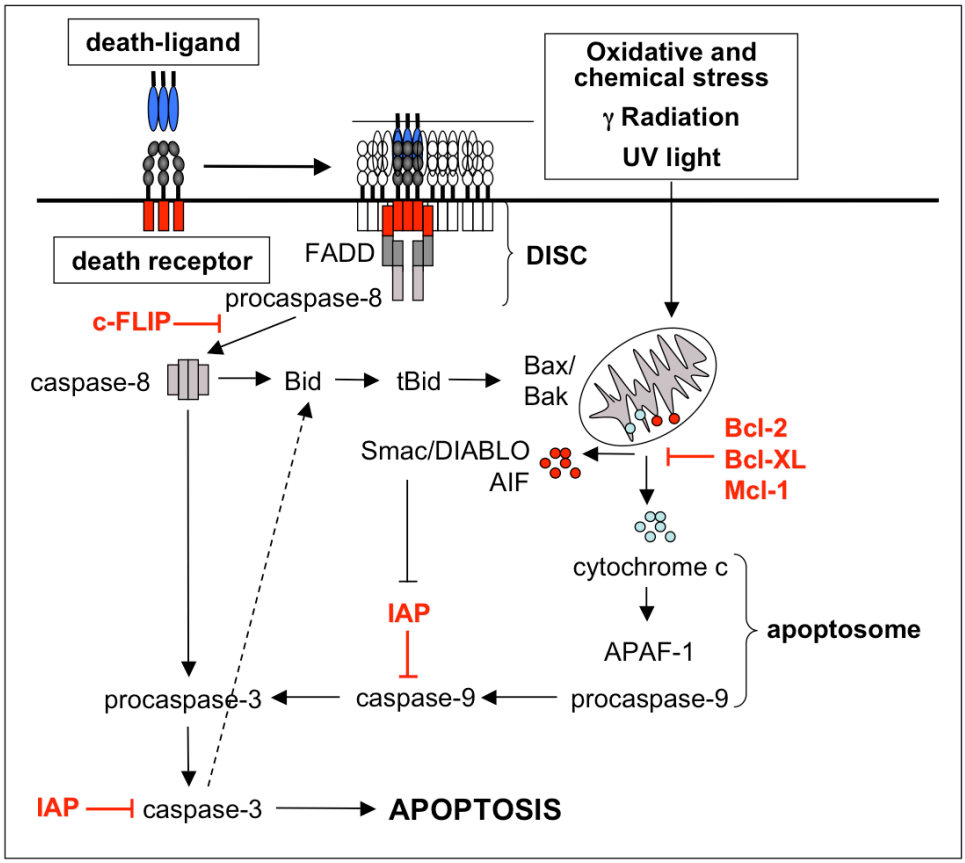


Figure 8. The extrinsic and intrinsic apoptotic signaling pathways. Programmed cell death can be triggered by the extrinsic pathway that is mediated by death receptors (DRs) or the intrinsic pathway that is induced upon cell damaging stresses. Binding of a death ligand leads to formation of a death-inducing signaling complex (DISC) at the intracellular death domain of the DR and activation of initiator caspases like caspase-8. In the DISC, c-FLIP is capable of inhibiting caspase activation. The proapoptotic (Bax, Bak) and antiapoptotic (Bcl-2, Bcl-xL, Mcl-1) Bcl-2 proteins regulate the integrity of the mitochondrial outer membrane Upon initiation of the intrinsic apoptosis pathway, apoptogenic factors, like cytochrome *c*, Smac/DIABLO and AIF, are released from mitochondria. Formation of apoptosome complex in the presence of cytosolic cytochrome *c* leads to activation of the initiator caspase-9. The initiator caspases cleave and activate effector caspases, like caspase-3, that execute the cellular apoptosis. Inhibitor of apoptosis proteins (IAPs) that inhibit caspases can, in turn, be inactivated by cytosolic Smac/DIABLO. The DR pathway can be coupled to the intrinsic apoptosis pathway by caspase-mediated cleavage of Bid protein to truncated Bid (tBid) that promotes mitochondrial permeabilization.

Cells that are sensitive to CD95L-induced apoptosis can be categorized into type I and II cells based on their requirement of MOMP for apoptosis-induction (Scaffidi *et al.* 1998, reviewed by Barnhart *et al.* 2003). Type I cells release large amounts of activated caspase-

8 upon CD95 stimulation through efficient DISC formation followed by direct cleavage and activation of caspase-3. Type II cells, by contrast, produce only little active caspase-8 at the DISC, and therefore require further amplification of the apoptotic signal through the activation of MOMP. Bid cleavage by active caspase-8 is likely to serve as the link between DISC formation and MOMP (Li *et al.* 1998, Luo *et al.* 1998). Consequently, overexpression of antiapoptotic Bcl-2 proteins rescues only type II cells from CD95-incuced apoptosis (Scaffidi *et al.* 1998). Hepatocytes from mice expressing the human Bcl-2 transgene were protected from CD95-mediated apoptosis, suggesting that they are type II cells, while thymocytes from the same mice remained susceptible to CD95L, and later became categorized as type I cells (Strasser *et al.* 1995, Lacronique *et al.* 1996). Further evidence for the type II nature of hepatocytes was provided in a study with Bid-deficient mice, which were found resistant to CD95-mediated hepatocellular apoptosis (Yin *et al.* 1999).

Most of the studied CD95L-sensitive type I tumor cells have mesenchymal-like features, whereas type II tumor cells display a more epithelial phenotype, suggesting that type I and II signaling might correlate with different stages of carcinogenesis during the EMT (Algeciras-Schimnich *et al.* 2003). However, many tumors are resistant to CD95-mediated apoptosis, and instead of promoting cell death, CD95-signaling may promote tumor growth *in vivo* (Chen *et al.* 2010). CD95L can induce cell motility and invasion in apoptosis-resistant tumor cell lines and clones that are selected under chemotherapy, suggesting that CD95 may even contribute to metastasis (Barnhart *et al.* 2004, Ametller *et al.* 2010). More research efforts are needed to understand the tumor-promoting role of CD95 and how it may influence the outcome of cancer therapy.

2.2.5 Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)

TRAIL was originally identified through sequence homology with TNFα and CD95L, as a novel proapoptotic member of the TNF superfamily (Wiley *et al.* 1995, Pitti *et al.* 1996). Like other members of the TNF superfamily, TRAIL is a homotrimeric molecule, and the principles of TRAIL trimer binding to three DR5 receptors, revealed by crystallography of the complex, resemble the receptor-ligand interactions observed for other TNFR superfamily members (Hymowitz *et al.* 1999). A unique feature of TRAIL is a zinc ion buried at the TRAIL trimer interface (Hymowitz *et al.* 2000). Binding of this zinc ion to a single cysteine residue on each monomer maintains the native structure and stability and, hence, the biological activity of TRAIL (Hymowitz *et al.* 2000).

2.2.5.1 The roles of TRAIL in the immune system

The physiological roles of TRAIL are not yet fully understood, but there are clear indications that TRAIL participates in regulating immune responses and in killing of potentially harmful cells. In addition to being widely expressed in hematopoietic cells, TRAIL has also been detected in other tissues, like prostate, lung, colon, placenta, and small intestine (Wiley *et al.* 1995). Expression of TRAIL can be induced in dendritic cells, macrophages, T cells and NK cells in an activation-dependent manner by cytokines, such as interferons (Kayagaki *et al.* 1999 Halaas *et al.* 2000, Liu *et al.* 2001, Sato *et al.* 2001). For example, viral infections upregulate TRAIL expression in T cells and NK cells, thereby enhancing the clearance of infected cells (Sato *et al.* 2001, Ishikawa *et al.* 2005).

Experimental work with transgenic mouse models has given new insight into the biological functions of TRAIL, including an important role in maintenance of self-tolerance. In mice, mDR5 (mTRAIL-R2, mKILLER) acts as a single ortholog of both human DR4 and DR5 (Wu *et al.* 1999), and two decoy receptors (mDcTRAILR1 and mDcTRAILR2), which lack the cytoplasmic domain, have been identified (Schneider *et al.* 2003). Mice lacking expression of either TRAIL or mDR5 develop normally, suggesting that TRAIL-induced signaling is not essential in embryonic development (Cretney *et al.* 2002, Sedger *et al.* 2002, Diehl *et al.* 2004, Finnberg et al. 2005). Unlike the mice with CD95 mutations, TRAIL or mDR5 knockout mice also seem to have normal resting immune cell populations (Cretney *et al.* 2003, Diehl *et al.* 2004, Cretney *et al.* 2008). Instead, mDR5-deficiency increases innate immune responses against certain types of pathogens, suggesting that TRAIL receptor activation may also negatively regulate innate immunity (Diehl *et al.* 2004).

Mice deficient in TRAIL demonstrate increased susceptibility to experimental autoimmune disease (Lamhamedi-Cherradi *et al.* 2003, Cretney *et al.* 2005), and treatment with antibodies that block endogenous TRAIL have been shown to exacerbate the disease condition in mouse models for arthritis and multiple sclerosis (Song *et al.* 2000, Hilliard *et al.* 2001, Cretney *et al.* 2005). These results strongly suggest that one of the functions of TRAIL *in vivo* is to inhibit autoimmune inflammation. Indeed, elevated serum levels of soluble TRAIL have been detected in patients with autoimmune diseases, such as lupus erythematosus and multiple sclerosis (Wandinger *et al.* 2003, Lub-de Hooge *et al.* 2005). The cellular mechanisms employed by TRAIL to prevent autoimmunity might be different from those used in TRAIL-mediated target cell killing. Instead of increasing T cell apoptosis, TRAIL was shown to inhibit T cell proliferative response and to induce cell cycle arrest (Song *et al.* 2000, Hilliard *et al.* 2001, Lunemann *et al.* 2002), demonstrating that TRAIL can also function through inhibition of immune cell activation and proliferation. Taken together, these findings indicate that TRAIL has an important regulatory role in both innate and adaptive immunity.

2.2.5.2 Molecular mechanisms of TRAIL-induced signaling

Binding of trimeric TRAIL to DR4 or DR5 promotes receptor clustering into larger aggregates and induces the formation of the DISC (Wagner *et al.* 2007) (Figure 9). Similar to the CD95 activation discussed above, recruitment of FADD at the cytoplasmic death domain of DR4 and DR5 and subsequent binding of caspase-8 and caspase-10 to FADD are essential steps for TRAIL-induced caspase activation and apoptosis (Bodmer *et al.* 2000, Kischkel *et al.* 2000, Kuang *et al.* 2000, Sprick *et al.* 2000). The ligand-induced receptor clustering and caspase-8 activation can be modulated by enzymatic *O*-glycosylation of conserved residues at the extracellular domain of DR4 and DR5 (Wagner *et al.* 2007). Recruitment and activation of caspase-10 at the DISC may be able to substitute for lack of caspase-8 in some model systems in induction of TRAIL-induced apoptosis (Kischkel *et al.* 2001, Wang *et al.* 2001), although the opposite has also been reported (Sprick *et al.* 2002). Furthermore, binding of c-FLIP to TRAIL-induced DISC modulates the outcome of DR4 and DR5 signaling. For example, selective knockdown of c-$FLIP_S$ and especially c-$FLIP_L$, in several cancer cell lines enhances the recruitment and activation of caspase-8 at the TRAIL–DISC as well as sensitizes the cells to TRAIL-induced apoptosis (Sharp *et al.* 2005). Ubiquitylation of caspase-8 at TRAIL–DISC has been shown to promote its translocation to intracellular ubiquitin-rich foci, where activation of the caspase-8 is enhanced (Jin *et al.* 2009). Bid cleavage, Bax/Bak-dependent

release of cytochrome c and caspase-9 activation have been shown to occur downstream of caspase-8 activation, when cells are treated with TRAIL (LeBlanc et al. 2002, Ravi & Bedi 2002, Kandasamy et al. 2003) (Figure 8).

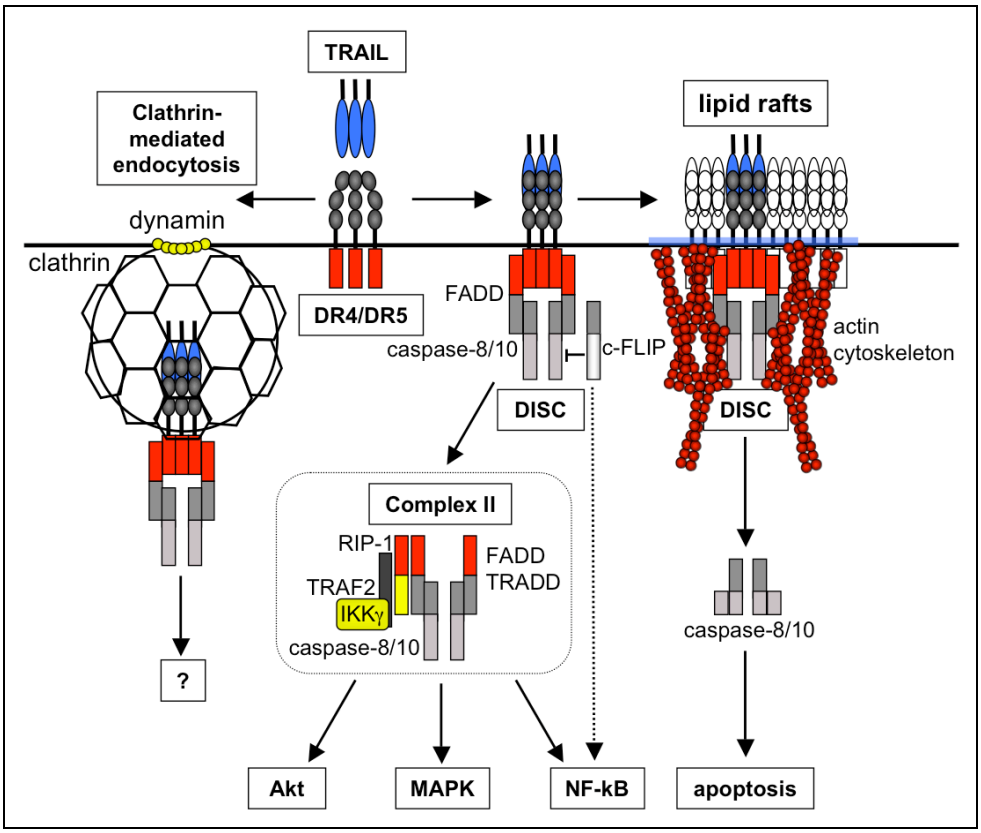


Figure 9. Current model for TRAIL-induced signaling. Activation of pre-ligand assembled DR4 and DR5 receptors by TRAIL induces formation of DISC with FADD and caspase-8/10, and translocation to lipid raft membrane compartment that is linked to actin cytoskeleton. Lipid raft localization enhances the activation of initiator caspases in the TRAIL-DISC, which promotes apoptotic signaling. An activation platform for non-apoptotic TRAIL-signaling has been reported to form downstream of DISC assembly. This complex II consists of the adaptors FADD or TRADD, caspase-8/10, RIP-1, TRAF2 and NEMO/IKKγ, and can promote survival signaling via Akt, MAPK and NF-κB pathways. c-FLIP proteins are able to inhibit caspase activation at the DISC, but also to mediate NF-κB activation. Although the TRAIL–DISC has been shown to undergo clathrin-mediated endocytosis, it is not yet known, how internalization influences TRAIL-induced signaling. Adapted from (Gonzalvez & Ashkenazi 2010).

Besides caspase activation via FADD, DR4 and DR5 are able to recruit TRADD and RIP1 to activate the transcription factor NF-κB (Chaudhary *et al.* 1997, Schneider *et al.* 1997, Sheridan *et al.* 1997, Lin *et al.* 2000) (Figure 9). NF-κB activation by TRAIL is less efficient than the activation of NF-κB by TNFα (Sheridan *et al.* 1997), but it involves a similar activation platform downstream of DISC formation, also called complex II,

consisting of RIP1, TRAF2 and NEMO/IKKγ (Varfolomeev *et al.* 2005). TRAIL-induced NF-κB activity is likely to protect cells from apoptosis by inducing transcription of antiapoptotic proteins, but it has also been suggested to upregulate chemokines that mediate phagocytosis of the dying cell (Varfolomeev *et al.* 2005). In addition, TRAIL-induced NF-κB activity was reported to account for increased proliferation, survival and invasiveness in cancer cells that are resistant to TRAIL-induced apoptosis (Ehrhardt *et al.* 2003, Ishimura *et al.* 2006). It is very important for the use of TRAIL in cancer therapy that the physiological role of TRAIL-induced activation of the NF-κB signaling pathway is clarified.

DR4 and DR5 have also been shown to activate MAPK and Akt kinase pathways. Although the TRAIL-induced signaling cascades to activation of the MAP kinases JNK and p38 seem to have cell type specific features, they have been reported to involve TRAF2 and RIP1 as well as caspase activity (Mühlenbeck *et al.* 1998, Hu *et al.* 1999, Lin *et al.* 2000, MacFarlane *et al.* 2000, Varfolomeev *et al.* 2005). The mechanisms that mediate activation of Akt or ERK1/2 signaling in response to TRAIL stimulation are less clear (Tran *et al.* 2001, Secchiero *et al.* 2003, Zhang *et al.* 2003), but may require similar complexes as JNK, p38 and NF- κB activation (Figure 9).

Kinase-mediated phosphorylation events can, in turn, modulate the components of the TRAIL signaling pathway (reviewed by Tran *et al.* 2004). Transcription-independent effects of ERK1/2 signaling have been shown to inhibit caspase-8 activation upon TRAIL stimulation, but the molecular targets of ERK1/2 within TRAIL–DISC have not yet been defined (Tran *et al.* 2001, Söderström *et al.* 2002). Protein kinase C (PKC) was shown to phosphorylate c-FLIP and increase the stability of the short c-FLIP isoforms, thereby inhibiting caspase-8 activation in the TRAIL–DISC (Kaunisto *et al.* 2009), whereas recruitment of FADD to TRAIL–DISC can also be reduced by PKC (Harper *et al.* 2003). Although the antiapoptotic activity of Akt in TRAIL-induced apoptosis has often been attributed to the promotion of c-FLIP expression (Panka *et al.* 2001), several studies suggest that Akt is able to inhibits TRAIL signaling downstream of caspase-8 activation at the level of Bid cleavage (Chen *et al*. 2001, Nesterov *et al.* 2001, Kandasamy & Srivastava 2002, Goncharenko-Khaider *et al.* 2010). Furthermore, Akt has been shown to inhibit apoptosis by phosphorylating other effectors of TRAIL signaling, including Bad (Datta *et al.* 1997), caspase-9 (Cardone *et al.* 1998) and XIAP (Dan *et al.* 2004).

DR4 and DR5 localization to plasma membrane lipid rafs upon TRAIL-stimulation was demonstrated to play an important role also in TRAIL-induced apoptosis (Song *et al.* 2007). Similar to the mechanisms observed for CD95, palmitoylation of DR4 at the cytoplasmic juxtamembrane region promotes localization to lipid rafts and generation of more efficient TRAIL-signaling (Rossin *et al.* 2009). Interestingly, the non-raft localized receptors were able to induce NF-κB and ERK1/2 survival signals upon TRAIL stimulation, even when the cells were treated with cholesterol-depleting agent, methyl-β-cyclodextrin, that disrupts lipid rafts (Song et al. 2007). Clathrin-dependent death receptor internalization follows after DR4 and DR5 activation (Austin *et al.* 2006, Kohlhaas *et al.* 2007), but unlike the TNFα-TNF-R1 and CD95L-CD95 receptor complexes, the TRAIL receptor-ligand complexes need not be internalized for DISC formation and apoptosis to occur (Austin *et al.* 2006, Kohlhaas *et al.* 2007) (Figure 9). In contrast, inhibition of endocytosis by dominant negative dynamin or hyperosmotic sucrose increases TRAIL-induced apoptosis (Austin *et al.* 2006, Kohlhaas *et al.* 2007). Although receptor endocytosis appears not to regulate non-apoptotic and apoptotic signaling through DR4 and

DR5, as it has been shown to do for TNFR-1 and CD95 signaling (Schneider-Brachert *et al.* 2004, Lee et al. 2006), lipid raft localization could be one of the determinants that dictate, whether apoptosis or survival signaling is induced by TRAIL.

The decoy receptors are unable to recruit DISC upon ligand binding, as the intracellular death domain of DcR2 is truncated (Marsters *et al.* 1997), and DcR1 completely lacks the cytoplasmic domain (Pan *et al.* 1997b, Sheridan *et al.* 1997). The GPI-anchored DcR1 acts as a competitor for TRAIL binding, preventing DR5-associated DISC assembly in lipid rafts, while interaction of DcR2 with DR5 allows DISC formation, but prevents DR4 co-recruitment and initiator caspase activation within the DR5 DISC (Mérino *et al.* 2006). Whether DcR2 spontaneously interacts with DR4 and DR5 via the extracellular PLAD (Clancy *et al.* 2005), or whether the interaction is TRAIL-dependent (Mérino *et al.* 2006), remains controversial.

2.2.5.3 TRAIL induces apoptosis selectively in cancer cells

Employment of CD95L in cancer therapy is limited by induction of fatal liver damage by *in vivo* engagement of CD95 (Ogasawara *et al.* 1993). Systemic toxicity limits also the use of TNFα as an anticancer agent, but the harmful effects of TNFα are rather caused by NF-κB -induced inflammatory responses than activation of caspases through TNFR-1 (reviewed by Vassalli 1992). However, substantial amount of preclinical data indicate that TRAIL can *in vitro* and *in vivo* specifically trigger apoptosis in cancer cells without harming non-transformed cells. First, TRAIL was shown to induce apoptosis in a range of tumor cell lines (Wiley *et al.* 1995, Pitti *et al.* 1996). Repeated injections with soluble recombinant TRAIL were found to be safe in experimental animal models, and the injections actively suppressed growth of established human tumor xenografts in immunocompromised mice (Ashkenazi *et al.* 1999, Walczak *et al.* 1999). Since then, a large number of studies have shown that TRAIL and agonistic antibodies against DR4 and DR5 are able to induce apoptosis in wide range of cancer cell lines and primary tumor cells, while being non-toxic to most of normal cells (reviewed by LeBlanc & Ashkenazi 2003, Hylander *et al.* 2005). Interestingly, TRAIL can enhance apoptosis in cancer cells that have lost functional tumor suppressor p53 (Ravi *et al.* 2004).

The initial discrepancy between several studies demonstrating tumor-specific activity of soluble TRAIL (Lawrence *et al.* 2001, Hao *et al.* 2004, Ganten *et al.* 2005) and studies, which reported that human primary hepatocytes are very sensitive to TRAIL-induced apoptosis (Jo *et al.* 2000, Ichikawa *et al.* 2001, Mori *et al.* 2004), was shown to stem from different *in vitro* culture procedures as well as different versions of recombinant TRAIL used in the experiments (Lawrence *et al.* 2001, Ganten *et al.* 2006). *In vitro* cytotoxicity to hepatocytes was observed only when the investigators used recombinant TRAIL that contained an exogenous tag, such as polyhistidine or Flag, as this was shown to cause over-aggregation of TRAIL receptors and apoptosis of hepatocytes (Lawrence *et al.* 2001, Ganten *et al.* 2006). Therefore, an untagged version of trimerized TRAIL has been used in further studies without toxicity normal cells.

The significance of TRAIL in immune surveillance against cancer has been demonstrated in studies with TRAIL-deficient mice or mice treated with TRAIL-neutralizing antibody. These animals displayed increased susceptibility to initiation, growth and metastasis of experimental tumors (Cretney *et al.* 2002, Sedger *et al.* 2002, Takeda *et al.* 2002).

However, some studies could not detect a critical role for TRAIL in tumorigenesis, as loss of mDR5 or TRAIL did not increase the *in vivo* formation of intestinal tumors in adenomatous polyposis coli (APC) mutant mice (Yue *et al.* 2005) or Her2/neu-initiated mammary tumors (Zerafa *et al.* 2005), respectively. An age-related increase in malignancy was, however, observed in the latter study, in which aged TRAIL-/- mice developed lymphoma with higher occurrence than the wild type mice (Zerafa *et al.* 2005). When Grosse-Wilde *et al.* (2008) aimed at resolving the conflicting data with respect to the physiological role of the TRAIL system during tumorigenesis, they observed that mDR5-deficiency did not increase the growth rate or progression of tumors in multistage mouse model of squamous cell carcinoma, but instead significantly enhanced metastasis to lymph nodes (Grosse-Wilde *et al.* 2008). Increased metastasis was also seen in mDR5 -/- mice on lymphoma-prone genetic background, even though the development of lymphomas was not influenced by the loss of mDR5 (Finnberg *et al.* 2008). These results suggest that TRAIL-induced signaling may play a role specifically in the suppression of metastasis.

2.2.5.4 Overcoming TRAIL resistance in cancer cells

Although some TRAIL-sensitive cancer cell lines have been reported to undergo TRAIL-induced apoptosis independent of the mitochondrial pathway (type I signaling) (Özören *et al.* 2000, Walczak *et al.* 2000), most of the tested cancer cells appear to behave in type II manner requiring the mitochondrial pathway to be activated for efficient caspase activation upon TRAIL treatment (Özören & El-Deiry 2002). Consequently, overexpression of Bcl-2 or Bcl-xL inhibits TRAIL-induced apoptosis in a variety of transformed cells (Hinz *et al.* 2000, Sun *et al.* 2001, Fulda *et al.* 2002). The type II behaviour of cancer cells might be caused by higher expression of inhibitor of apoptosis proteins (IAPs) that oppose efficient caspase activation (Srinivasula *et al.* 2000). However, it has become obvious that the TRAIL-sensitive cancer cells are not as common as initially thought. Approximately half of the tumor cell lines tested so far and the large majority of primary tumors are resistant to TRAIL-mediated apoptosis (reviewed by Koschny *et al.* 2007 and Mellier *et al.* 2010, Todaro *et al.* 2008). The mechanisms of TRAIL resistance are diverse and range from activation of antiapoptotic signaling pathways (NF-κB, MAPKs and PI3K/Akt), overexpression of antiapoptotic proteins (FLIP, Bcl-xL, Bcl-2 and IAPs) and deficiency of proapoptotic Bcl-2 proteins (Bax) to increased expression of TRAIL decoy receptors and reduced DR4/DR5 expression (reviewed by Mellier *et al.* 2010). For example, mutated Bax is often the reason for TRAIL resistance in colon cancer (LeBlanc *et al.* 2002), whereas deregulated PI3K/Akt signaling can be an important source of TRAIL resistance in prostate cancer (Chen *et al.* 2001b).

As the initiation of cancer cell apoptosis via the intrinsic pathway can cause DNA damage in the surviving cells, like many chemotherapeutic agents do, sublethal TRAIL treatment has also been reported to provoke mutations (Lovric & Hawkins 2010). Therefore, further studies are required to determine the effects of TRAIL-treatment in TRAIL-resistant cells, which could respond to the treatment with enhanced survival signaling and even DNA damage (Secchiero *et al.* 2003, Lovric & Hawkins 2010), and careful selection of patients that would benefit from TRAIL treatment is needed. The extracellular domains of DR5 and DR4 can be *O*-glycosylated at specific residues by *O*-glycosylation enzymes, such as GALNT14, with the consequence of facilitating ligand-induced clustering of DR4 and DR5, DISC formation and capsase-8 activation (Wagner *et al.* 2007). Gene expression profiles of specific *O*-glycosylation enzymes may serve as important biomarker to predict the sensitivity of cancer cells to TRAIL-based therapy, as comparison of 119 cancer cell

lines revealed a strong correlation between expression of these enzymes and TRAIL-sensitivity (Wagner *et al.* 2007).

Various strategies are employed in preclinical studies to restore cancer cell sensitivity to TRAIL (reviewed by Ashkenazi & Herbst 2008). Many chemotherapeutics upregulate surface expression of TRAIL receptors by increasing their transcription or protein half-life, downregulate expression of antiapoptotic proteins, including IAPs, c-FLIP, Bcl-2, Bcl-xL and Mcl-1, or enhance TRAIL-DISC redistribution into lipid rafts, thereby causing a synergistic apoptotic effect when combined with TRAIL (reviewed by Mellier *et al.* 2010). Downregulation of c-FLIP by compounds, such as proteasome inhibitors and cytotoxic drugs, or by RNA interference is an efficient way to enhance TRAIL-induced signaling at the level of caspase-8 activation (reviewed by Safa *et al.* 2008). Inhibition of IAPs by using small molecule IAP antagonists enhances antitumor activity of TRAIL (Li *et al.* 2004, Voggler *et al.* 2009). ABT-737 is a BH3-mimetic small-molecule inhibitor of the antiapoptotic proteins, Bcl-2, Bcl-xL and Bcl-w, that does not directly induce apoptosis, but enhances the effects of other death signals, such as TRAIL treatment (Oltersdorf *et al.* 2005, Nieminen *et al.* 2007a). Inhibition of cancer cell survival signaling by compounds, like the tyrosine kinase inhibitor sorafenib, also sensitizes cancer cells to TRAIL-induced apoptosis (Rosato *et al.* 2007). The combinatorial treatments can only be of clinical benefit if they do not harm normal human cells. Ganten *et al.* (2006) tested a range of common chemotherapeutics together with TRAIL and found that most combinations were not toxic to primary human hepatocytes. However, the actual safety profile of each combination will not be defined until investigated in clinical phase I studies.

2.2.5.5 TRAIL receptor agonists in clinical trials for cancer therapy

Recombinant human TRAIL (dulanermin) and fully human or humanized monoclonal agonistic antibodies against DR4 (mapatumumab) or DR5 (conatumumab, CS-1008, lexatumumab and PRO95780) are currently investigated in phase Ib/II clinical trials for cancer therapy. Altogether, these agents have been well tolerated at the tested doses and most agents did not reach the maximum tolerated dose (reviewed by Wiezorek *et al.* 2010). Modest single-agent antitumor activity has been observed in phase I trials in two patients with refractory disease; one partial response in non-small cell lung carcinoma treated with conatumumab and one in chondrosarcoma treated with dulanermin (Herbs *et al.* 2006, LoRusso *et al.* 2007). In a single-agent phase II study with mapatumumab, two partial responses and one complete response were observed among 40 patients with pretreated follicular non-Hodgkin lymphoma (Younes *et al.* 2005). As TRAIL receptor agonist monotherapy appears not to be potent enough, several on-going trials combine TRAIL-based treatments with chemotherapy (Wiezorek *et al.* 2010). Significant increase in TRAIL antitumor activity has been observed in preclinical studies when the intrinsic apoptosis pathway is activated simultaneously with cytotoxic drugs or by sensitizing pretreatments that target the key mediators of TRAIL resistance. As these advances are anticipated to translate into improved results in clinical trials, various cytotoxic drugs and targeted therapeutics are investigated in different combinations together with TRAIL receptor agonists. These clinical studies did not reveal increased toxicity in phase Ib, and the ongoing randomized phase II studies will soon provide further information about the safety and antitumor efficacy of the TRAIL receptor agonists in wide range of combinations and tumor types (Wiezorek *et al.* 2010).

3. TARGETED APPROACHES FOR CANCER ELIMINATION

3.1 Lignans - Natural polyphenols with anticancer effects

Lignans are plant polyphenols traditionally classified into two types, classical lignans and neolignans. Classical lignans are formed from two phenylpropanes linked in a β-β' (8–8') fashion, while neolignans are those dimers whose coupling patterns differ from β-β' linkage (reviewed by Ayres & Loike 1990). Lignans are formed as secondary metabolites throughout the plant kingdom, while coniferous trees present one of the richest sources of lignans (reviewed by Holmbom *et al.* 2003). There is great diversity in lignan structures with several hundred naturally occurring lignans found in different plant parts, and the number of identified lignans is still increasing (reviewed by Pan *et al.* 2009, Ayres & Loike 1990). Some lignans and neolignans participate in the synthesis of lignin, an important component of the plant cell walls, while others act as antioxidants, biocides and perhaps even as plant hormones (Ayres & Loike 1990). The reported antimicrobial, antifungal, antiviral, antifeedant, nematocidal and insecticidal properties suggest an important role for lignans in the plant defense system against various pathogens and pests (reviewed by Lewis *et al.* 1995).

In human diet, lignans are enriched in whole-grain products, vegetables, berries, tea and fruits (reviewed by Adlercreutz 2007). Some of the best dietary sources of lignans are flaxseed and other oilseeds, such as sesame seeds. Plant lignan precursors can be converted into enterolignans, enterodiol (END) and enterolactone (ENL), by the activity of the microflora in the proximal colon of mammals, and therefore END and ENL are termed mammalian lignans (Setchell *et al.* 1981, Axelson *et al.* 1982, Boriello *et al.* 1985). Currently, at least secoisolariciresinol (SECO), matairesinol (MAT), pinoresinol, sesamin, lariciresinol, syringaresinol, 7-hydroxymatairesinol (HMR) and arctigenin are known to function as plant lignan precursors for the mammalian lignans (Axelson *et al.* 1982, Boriello *et al.* 1985, Saarinen *et al.* 2000, Heinonen *et al.* 2001, Peñalvo *et al.* 2005). For example, trachelogenin, isolariciresinol and nortrachelogenin (NTG) seem not to be converted to enterolactone or enterodiol in the gut, but are instead absorbed from the intestine and secreted as such or as metabolites to urine (Heinonen *et al.* 2001, Kitamura *et al.* 2003, Saarinen *et al.* 2005). Figure 10 shows the molecular structures of most lignans discussed in this thesis.

Various lignans also exist as glycosides that are transformed into the aglycones in the gastrointestinal track (Setchell *et al.* 1981). The major lignan component (1% of dry weight) in flax seed is secoisolariciresinol diglucoside (SDG), although smaller amounts of MAT, pinoresinol, lariciresinol, isolariciresinol and SECO can also be found in flax seed (reviewed by Adolphe *et al.* 2010). The bioactivity of dietary SDG and its metabolites, SECO, ENL and END, has been reported to protect against cardiovascular disease, diabetes and cancer (Adolphe *et al.* 2010). Like many other lignans, SECO, ENL and END possess antioxidative activity (Prasad 2000), which can reduce oxidative stress and thereby mediate the observed health effects.

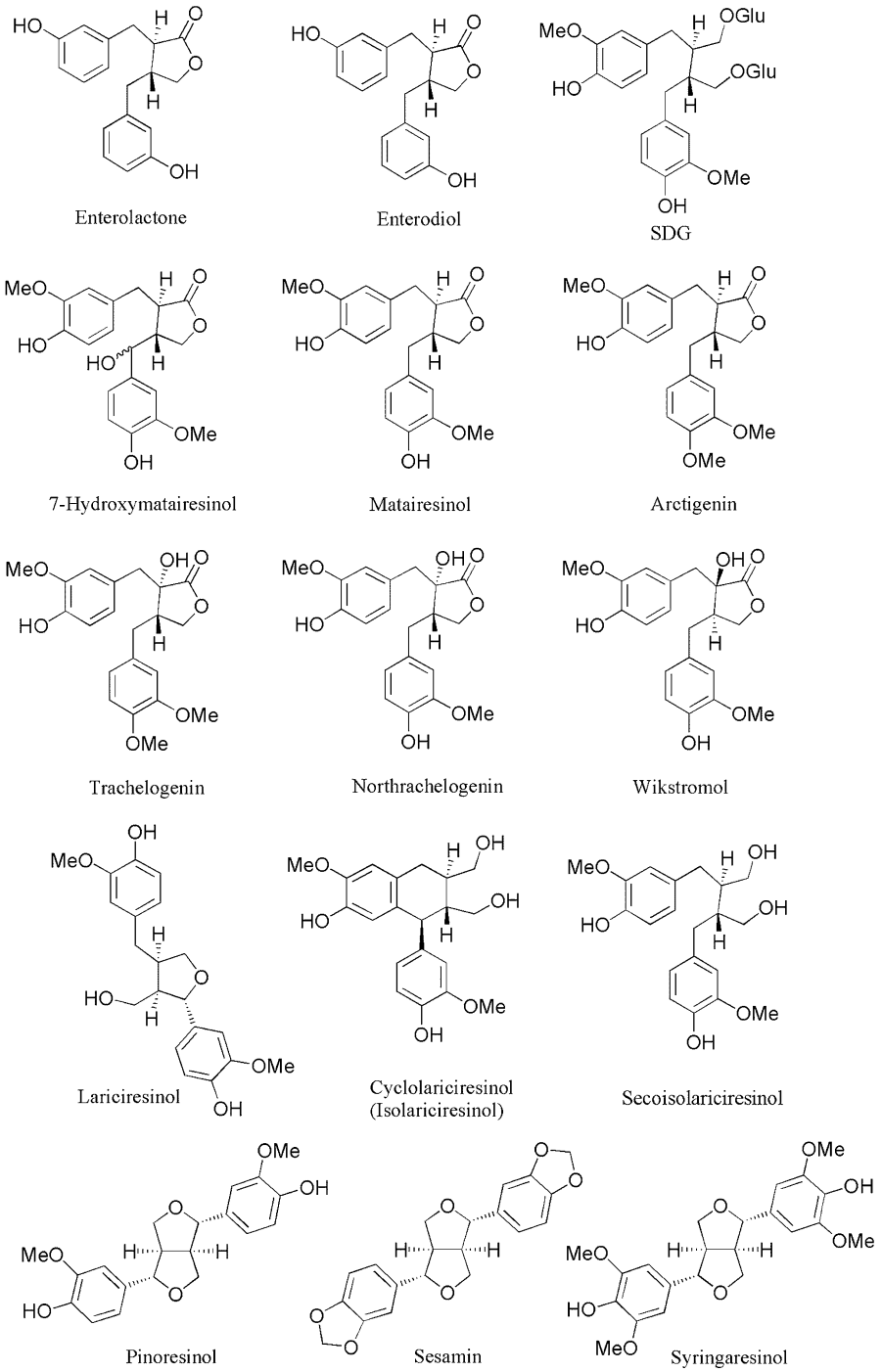


Figure 10. Molecular structures of dibenzylbutyrolactone lignans and some other precursors for mammalian lignans.

The ability of lignans to inhibit inflammatory responses has also been investigated with interest of potential pharmacological benefit. For example arctigenin and MAT were shown to display antiasthmatic activity in guinea pigs in a dose-dependent manner (Lee *et al.* 2010) and inhibition of the inflammatory cytokine TNFα production by arctigenin or HMR was reported to occur in lipopolysaccharide (LPS)-stimulated macrophages or monocytes, respectively (Cho *et al.* 2001, Cosentino *et al.* 2010). Lignans share a structural resemblance with 17β-estradiol and can be considered as phytoestrogens with especially ENL and END displaying the ability to modulate estrogen bioactivity (reviewed by Adlercreutz 2002). ENL is able to inhibit the enzyme aromatase, which converts androgens to estrogens, (Adlercreutz *et al.* 1993) and to bind to estrogen receptors with relatively weak affinity (Mueller *et al.* 2003). The effects of lignans on estrogen receptor signaling are likely to depend on the endogenous estrogen levels, as lignans are capable of exerting both estrogenic and antiestrogenic activities (Adlercreutz 2007).

3.1.1 Anticancer properties of lignans

Various structurally different lignans have demonstrated the ability to decrease cancer cell viability, growth and metastasis (reviewed by Saleem *et al.* 2005). The studies reviewed in this chapter have been conducted with the mammalian lignan ENL, other dibenzylburtyrolactone type lignans or the plant lignan precursors of ENL. *In vitro* studies with cultured cancer cell lines have demonstrated lignans have cytotoxic and antiproliferative characteristics. MAT and arctigenin have been reported to suppress growth of leukemic HL-60 cells (Hirano *et al.* 1994), whereas ENL inhibits the growth of prostate cancer cells (Lin *et al.* 2001, McCann *et al.* 2008). MAT, arctiin and trachelogenin, and most efficiently arctigenin, reduce viability in human gastric adenocarcinoma AGS cells (Kang *et al.* 2007), and wikstromol has demonstrated antileukemic properties (Lee *et al.* 1981). Additionally, ENL and END have been shown to reduce adhesion and the invasive potential of breast cancer cells (Chen & Thompson 2003).

Due to their phytoestrogenic nature, lignans have been extensively studied *in vivo* in hormone-responsive breast cancer models (reviewed by Saarinen *et al.* 2007). Recent studies have shown that lignans are indeed accessible to human breast cancer xenografts in athymic mice, but also display sex-related differences in tissue distribution (Saarinen *et al.* 2008, Saarinen & Thompson 2010). Dietary flax seed or purified SDG inhibit formation and growth of carcinogen-induced mammary tumors in rat (Thompson *et al.* 1996), while HMR can decrease the number of growing tumors and increase the proportion of regressing and stabilized tumors in the same experimental model (Saarinen *et al.* 2000). The enterolignans and their precursors lariciresinol, arctiin and sesamin have also been found to reduce mammary tumor multiplicity or growth *in vivo* (Saarinen *et al.* 2007). In contrast, NTG and tracheloside, both of which are lignans that are not converted to ENL or END, were not able to inhibit carcinogen-induced mammary tumorigenesis (Saarinen *et al.* 2002, Kitamura *et al.* 2003). Hence, breast cancer research on dietary lignans has become more focused on those compounds that can be metabolized to enterolignans with reported estrogen modulating activity (Saarinen *et al.* 2007).

Lignans have been reported to exert antitumorigenic effects also on other forms of cancer. Dietary HMR extract can inhibit the appearance of intestinal adenomas in the adenomatous polyposis colimultiple ApcMin mouse model for intestinal neoplasia (Oikarinen *et al.*

2000), and is able to suppress the growth of hormone-dependent human prostate cancer xenografts in atymic mice (Bylund *et al.* 2005). Intraperitoneal administration of a plant extract containing wikstromol, MAT and dibenzylbutyrolactol inhibits the growth of intramuscular xenografts of Ehrlich ascites carcinoma and colon carcinoma cells in mice (Singh *et al.* 2007), whereas arctigenin and arctiin exhibited inhibitory effects on skin carcinogenesis as well as formation of pulmonary tumors (Takasaki *et al.* 2000).

Tumor metastasis has also been observed to reduce upon lignan exposure. HMR and ENL inhibited the growth and metastasis of subcutaneous hepatomas in rats (Miura et al. 2007), while SDG reduced pulmonary metastasis of injected murine melanoma cells (Li *et al.* 1999) and prevented lymph node and lung metastasis of ortotopical breast cancer xenografts in nude mice (Chen *et al.* 2006). Flax seed, lariciresinol, ENL and END were able to significantly decrease VEGF secretion and tumor microvessel density in human breast cancer xenografts or carcinogen-induced mammary tumors *in vivo* (Bergman Jungeström *et al.* 2007, Saarinen *et al.* 2008). This suggested inhibition of tumor angiogenesis might play a role in the antimetastatic activity of lignans.

The incidence of breast, colorectal and prostate cancer is higher in the Western world compared to countries in Asia, and dietary factors are likely to play an important role in risk of these malignancies (reviewed by Adlercreutz & Mazur 1997). As high levels of circulating estrogens have been associated with increased breast cancer risk (Hankinson & Eliassen 2007), modulation of estrogen bioactivity by lignans can be beneficial. Epidemiological studies concerning the association between plasma levels of ENL and risk of breast cancer have been conflicting, and the association might be different depending on whether premenopausal or postmenopausal women are studied (Pietinen *et al.* 2001, Hultén *et al.* 2002, McCann et al. 2002, Kilkkinen *et al.* 2004). A case-control study nested within a cohort of more than 15 000 Finnish females by Kilkkinen *et al.* (2004) did not support the hypothesis that high serum ENL concentration would be associated with reduced risk of breast cancer. Nevertheless, a recent meta-analysis of 23 individual studies revealed that plant lignan intake might be associated with a 15% reduction in post-menopausal breast cancer risk (Velentzis *et al.* 2009). Further studies are required to confirm the results of this analysis and to decipher whether ENL is protective against breast cancer, or simply a biomarker of a healthy fiber-rich diet. One aspect that is likely to have a significant impact on lignan intake is the use of oral antibiotics that decrease the intestinal microflora and thereby also serum ENL concentration (Kilkkinen *et al.* 2002).

Most epidemiological studies do not support linear correlation between serum enterolactone concentration and prostate cancer risk (Kilkkinen *et al.* 2003, Stattin *et al.* 2004, Hedelin *et al.* 2006, Park *et al.* 2009a). Only one case–control study found a 60% reduction in prostate cancer risk associated with a high concentration of serum ENL (Heald *et al.* 2007). The failure of other studies to detect similar correlations might be due to too low ENL plasma concentrations in the studied population (Park *et al.* 2009a). Interestingly, dietary flaxseed lignan extract can improve lower urinary tract symptoms in patients with benign prostate hyperplasia (Zhang *et al.* 2008). The possibility that dietary lignans influence prostate cancer formation or progression in men needs to be studied further before any definitive conclusions can be made.

3.1.2 Cellular effects of lignan activity

The reported molecular mechanisms for lignan-induced cellular effects are diverse and vary between cell types and lignan compounds assayed. Many lignans function as antioxidants preventing DNA damage induced by reactive oxygen species (ROS), which might be one of the general mechanisms behind the anticarcinogenic activity of lignans (Saleem *et al.* 2005). For example, MAT, NTG, SDG, SECO, ENL and END are all antioxidants *in vitro* (Kitts *et al.* 1999, Prasad 2000, Willför *et al.* 2003). Arctigenin treatment has been neuroprotective and hepatoprotective in primary cultures of rat cells possibly due to its antioxidative activity (Jang *et al.* 2001, Kim *et al.* 2003). Also hinokinin has significant antioxidative effects and the ability to reduce doxorubicin-induced chromosome damage in *in vivo* mouse model (Medola *et al.* 2007). However, the *in vivo* antioxidative effects of lignans require further investigation, as enhanced ENL plasma concentration in humans was not associated with reduced genetic damage in peripheral blood lymphocytes (Pool-Zobel *et al.* 2000).

Cancer cell proliferation relies on growth-stimulatory signaling pathways and lignans have been associated with suppression of several critical signaling pathways. HMR was shown to normalize deregulated signaling through the Wnt/β-catenin pathway in the intestinal adenoma tissue of the ApcMin mice (Oikarinen *et al.* 2000). Also arctigenin inhibited growth of human colon cancer cells via inhibition of Wnt/β-catenin signaling (Yoo *et al.* 2010). Interestingly, a study by Sharma *et al.* (2008) suggests that treatment with a plant extract containing wikstromol, MAT and dibenzylbutyrolactol may also inhibit the Wnt/Wg pathway in the fruit fly. Arctigenin can inhibit LPS-induced activation of MAPKs including ERK1/2, p38 kinase and JNK, possibly through inhibition of the upstream MAPK kinase activity in murine macrophages (Cho *et al.* 2004). While arresting growth of hormone-responsive breast cancer xenografts, SDG inhibits MAPK activity and expression of growth factor receptors, IGF-IR and EGFR (Saggar *et al.* 2010). Lignans have also been shown to modulate the signaling via growth factor receptors and the PI3K/Akt pathway (Vasilcanu *et al.* 2004, Lee *et al.* 2009, Chen *et al.* 2009a). Furthermore, the NF-κB pathway for survival and inflammatory signaling can be suppressed by lignans, such as arctigenin and sesamin, as both compounds have been shown to inhibit IκB degradation and NF-κB-p65 nuclear translocation (Cho *et al.* 2002, Harikumar *et al.* 2010).

Induction of apoptotic cell death or sensitization to apoptosis-inducing signals can lead to the cytotoxic effects that lignans have been reported to produce. This might be accomplished by p53 stabilization, which in turn can induce cell cycle arrest and initiation of the mitochondrial apoptosis pathway (Chen *et al.* 2007, Fini *et al.* 2008). The unfolded protein response (UPR) in glucose-deprived conditions supports survival of cancer cells in stressful environment, whereas arctigenin is able to inhibit this response and to induce apoptosis through the mitochondrial pathway in colon cancer cells (Kim *et al.* 2010, Sun *et al.* 2010). Lignan compounds have been reported to downregulate the expression of the antiapoptotic Bcl-2 proteins that counteract signals that promote mitochondrial permeabilization (Giridharan *et al.* 2002, Hausott *et al.* 2003, Saggar *et al.* 2010) and to reduce expression of c-FLIP, a protein which protects cells from the extrinsic death pathway (Raja *et al.* 2008), but also other molecular mechanims for enhanced induction of apoptosis are likely to be employed by lignans.

3.2 Mesoporous silica nanoparticles as targeted drug delivery vectors for cancer therapy

The efficacy of a drug compared to its toxicity to normal tissues, also termed the therapeutic index, is often a critical issue for cancer treatment, particularly when several different therapeutic agents are used in combination. To have a pharmacological response, clinicians generally have to administer far more drug molecules than would be needed if the drug was concentrated at the target site. Drug molecules may become degraded in the blood stream and tissues, and active detoxification can chemically modify the compound by conjugating reactions (reviewed by Meijerman *et al.* 2008). Another significant challenge in cancer therapy stems from the fact that many effective anticancer compounds are hydrophobic, and due to their poor solubility cannot be administrated properly in significant concentrations. More soluble derivatives from these compounds might be easier to administer, but can have problems to cross the plasma membrane to have the required effects in cells. Both increased therapeutic index and more efficient drug delivery can be accomplished by targeting the therapeutic agents specifically into cancer cells.

Nanoparticle carriers could meet many of the critical challenges in drug delivery (reviwed by Farokhzad & Langer 2009). Firstly, due to their small size (up to few hundred nanometers), nanoparticles can bypass biological barriers, be internalized by cells, and thereby allow efficient drug accumulation at the target sites. Secondly, sustained drug release at the target site can be obtained for a prolonged period of time to achieve optimal therapeutic efficacy. Thirdly, the particle surface can be engineered so that desired biodistribution occurs or conjugated to biospecific ligands, which direct them to the target tissues. Currently over twenty therapeutic products employing nanotechnology have been approved for clinical use, the majority of which improve the pharmaceutical efficacy or dosing of clinically approved drugs (Wagner *et al.* 2006). Research efforts are being placed on generating delivery systems that target drugs to specific tissues and cells of the body, including tumors.

Various different nanoparticles of both inorganic (ceramic) and organic (polymeric) materials have been developed for drug delivery purposes. Organic nanoparticles include poly(lactic acid) (PLA) and poly(lactic-co-glycolic acid) (PLGA) polyester-based particles, polymeric micelles, liposomes, dendrimers and protein cages. Inorganic nanocarriers are made of materials, such as metal or metal oxides. The developed nanoparticle systems are different in terms of parameters, including the size and hydrophobicity of the particle core, particle size distribution, biocompatibility, charge, mechanical strength and stability. Surface coating of nanoparticles with polymers like poly(ethylene glycol) (PEG) can create a ‘stealth’ layer that reduces protein adsorption as well as inhibits the particle from being recognized as foreign by the immune system (reviewed by Moghimi *et al.* 2001). Furthermore, the particle surface may be functionalized with specific targeting ligands or tracers, such as fluorophores for light microscopy and magnetic components for magnetic resonance imaging (MRI).

3.2.1 Targeting strategies for specific delivery of nanoparticles to cancer cells

Nanoparticles have been observed to concentrate at the tumor site through the fenestrated and leaky blood vessels that are typical for tumor vasculature (Matsumura & Maeda 1986). Although this enhanced permeability and retention (EPR) effect allows passive

accumulation of optimally sized nanoparticles to the tumor, further targeting mechanisms may be needed for efficient and cancer-specific delivery of cytotoxic drugs (Figure 11). Such active targeting systems can be obtained by conjugation of nanocarriers with cancer-specific targeting ligands that enhance the internalization of the particles into the tumor cells, while accumulation to normal cells is minimized (Table 1.). Increased cellular uptake may further promote the passive targeting mechanisms, as the nanocarrier concentration at the tumor interstitial compartment becomes lower creating a stronger diffusion gradient across the vasculature (Paulos *et al.* 2004).

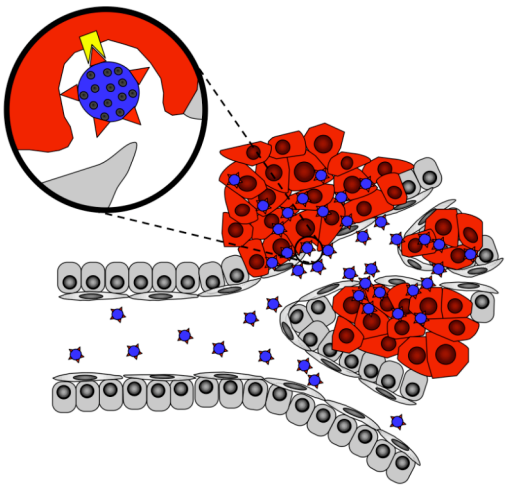


Figure 11. Passive and active targeting of nanoparticles. The EPR effect plays a major role in the passive accumulation of nanoparticles from the blood stream to the tumor site. Targeting ligands conjugated on the surface of the nanoparticle are recognized by cancer cell-specific receptors, which mediates the enhanced cellular uptake of the particles by endocytosis (magnification).

Indeed, tumor cells and tumor-associated endothelial cells have been shown to display cell surface receptors that are associated with the malignant phenotype, and which are rare or absent on the surfaces of healthy cells. Targeted delivery via these specific receptors may result in higher bioavailability of the therapeutic agent at the tumor site and simultaneously reduce the side effects (reviewed by Brannon-Peppas & Blanchette 2004). Cancer-targeting ligands that specifically bind to these surface receptors can be antibodies, antibody fragments (FAb), protein ligands, small molecular enzyme inhibitors, receptor agonists/antagonists, peptides, hormones, venoms, or DNA/RNA aptamers (Table 1.). The attached ligand must preserve the active conformation when conjugated to the nanoparticle, exhibit high affinity for the corresponding receptors and induce high rate of receptor-mediated endocytosis. In addition to the physicochemical properties of the nanoparticle system, the efficiency of cellular uptake depends on the surface expression level of the target receptor. High affinity peptides for designated tumor specific receptors can be identified for example by phage display screening (reviewed by Brown 2010). In addition to the ligands listed in Table 1, various peptides that bind to endothelial cells at angiogenic vasculature have been investigated as potential tumor-targeting moieties. These peptides have been reported to specifically bind the platelet-derived growth factor receptor β (Joyce *et al.* 2003), the angiopoietin receptor Tie2 (Mai *et al.* 2009) or adhesion molecules, such as E-selektin (Shamay *et al.* 2009) and integrins (Arap *et al.* 1998, Hood *et*

al. 2002). For many other peptides with tumor specific affinity the receptors remain to be identified (Brown *et al.* 2010). Some ligands have been investigated for targeted drug delivery to intracellular organs, such as the nucleus and the mitochondria. For example, the nuclear localization signal (NLS) has been coated onto gold nanoparticles (Tkachenko *et al.* 2003).

Table 1. Examples of cancer cell targeting ligands.

Targeting ligand	Receptor	Nanocarrier	Reference
Monoclonal antibodies			
HER2/Neu mAb	ErbB2	Liposome	Nielsen *et al.* 2002
Proteins			
Epidermal growth factor (EGF)	EGFR	Peptide-lipid nanocarrier	Zhang *et al.* 2010
Transferrin	Transferrin receptor	Liposome	Ishida *et al.* 2001
Peptides			
Fibronectin mimetic peptide	$\alpha 5\beta 1$ Integrin	Liposome	Garg *et al.* 2009
Urokinase plasminogen activator (uPA) peptide	uPA receptor	Iron oxide	Yang *et al.* 2009
SDFα derived peptides	CXCR4	-	Egorova *et al.* 2009
Chlorotoxin	Matrix metalloproteinase-2	Iron oxide	Veiseh *et al.* 2009
Glucose-regulated protein-78 kDa (GRP-78) targeted peptide	GRP-78	Hybrid polymer	Wood *et al.* 2008
Nucleic acids			
RNA aptamer A10	Prostate-specific membrane antigen	PLGA-b-PEG copolymer	Farokhzad *et al.* 2006
DNA aptamer sgc8	-	MSN	Zhu *et al.* 2009
Small molecules			
Folic acid (FA)	FA receptor, FA carrier	Liposome	Goren *et al.* 2000
Riboflavin (RF)	RF binding protein, RF carrier, RF transporter	Dendrimer	Thomas *et al.* 2010
Testosterone	Androgen receptor	Liposome	Mishra *et al.* 2009
Galactose	asialoglycoprotein receptors	Solid lipid nanoparticles	Xu *et al.* 2009
Anisamide	Sigma receptor	Liposome	Banerjee *et al.* 2004

3.2.2 Cellular uptake mechanisms and intracellular transport of nanoparticles

Both non-specific and receptor-mediated cellular uptake mechanisms function in nanoparticle internalization (Figure 12). The physicochemical properties of the particle play an important role in the process, but for ligand-targeted particles also the receptor in question determines, which endocytic pathway is utilized. Internalization of engineered nanoparticles occurs via pathways that are employed by cells to take up extracellular material, but also viruses use these gateways to enter cells (reviewed by Mercer *et al.* 2010). In addition to being internalized by the cells of the target tissue, nanoparticles are often recognized as foreign objects in the blood stream and taken up by the professional

phagocytes of the reticuloendothelial system (RES) (reviewed by Dobrovolskaia & McNeil 2007). These immune cells reside in the liver and spleen, where nanoparticles may accumulate in case particle opsonization by immunoglobulins, complement and other serum proteins occurs in the bloodstream. Whether or not opsonization and subsequent phagocytosis are induced, depends mostly on the size of the particle as well as the stealth properties of the particle surface (reviewed by Hillaireau & Couvreur 2009). Drug delivery to RES organs can also be preferential, for example in the case of hepatocarcinoma or liver metastasis (Hillaireau & Couvreur 2009). However, in most cases avoidance of phagocytosis is considered desirable. Targeting-ligands have been observed to inhibit RES phagocytosis (reviewed by Wang & Thanou 2010), but too high coverage of the particle with the ligand should be avoided, as this might reduce the stealth properties and increase clearance by RES (Gu *et al.* 2008, Shmeeda *et al.* 2009).

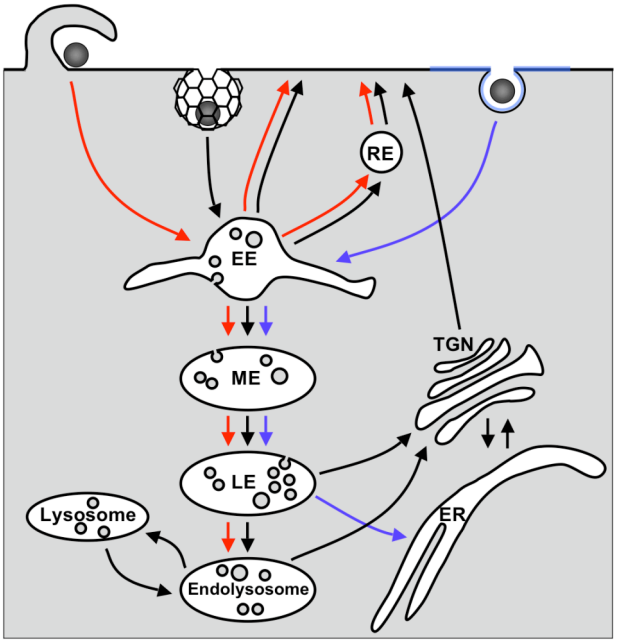


Figure 12. Endocytosis and intracellular trafficking of nanoparticles. Nanoparticles can be internalized via various routes, including macropinocytosis (red), clathrin-mediated endocytosis (black) and caveolar/raft-dependent endocytosis (blue). Vesicles containing the particles fuse with the endosomes characterized by gradually decreasing pH as the particles travel towards lysosomal compartment. The endosomal pathway from caveolar/raft-dependent endocytosis is directed to endoplasmic reticulum (ER) instead of leading to the acidic environment of the endolysosomes. RE, recycling endosomes; EE, early endosomes; ME, maturing endosomes; LE, late endosome; TGN, trans-golgi network. Adapted from (Mercer *et al.* 2010).

Clathrin-mediated endocytosis (CME) is a process that is taking place in all mammalian cells (reviewed by Conner & Schmid 2003). It is involved in nutrient uptake and intracellular communication, but also in uptake of nanocarriers (Hillaireau & Couvreur 2009). The pathway comprises the assembly of clathrin-coated pits that develop into deep invaginations and the fission of the vesicles by dynamin GTPase activity (Conner & Schmid 2003) (Figure 12). The clathrin-coated vesicles will thereafter become uncoated and deliver the contents into the endosomal compartment. The mildly acidified (pH~6)

"early" and "maturing" endosomes will transform into "late" endosomes also known as multivesicular bodies (pH~5), that subsequently fuse with prelysosomal vesicles containing acid hydrolases. Thus, CME causes the endocytosed material to end up in the degradative conditions in the lysomes, which can be utilized as a drug release mechanism in the cell interior (Hillaireau & Couvreur 2009). An alternative pathway from early endosomes leads to recycling endosomes and back to the plasma membrane after dissociation of the ligands forms their receptors.

The caveolar/raft-dependent endocytosis pathway occurs in membrane invaginations enriched in caveolin, cholesterol and/or sphingolipids (Conner & Schmid 2003) (Figure 12). Dynamin is required also for the generation of cytosolic caveolar vesicles that join the endosomal pathway. However, instead of being acidified in endolysosomes, the cargo from this uptake pathway is typically directed to endoplasmic reticulum. The caveolar/raft-dependent endocytosis occurs at slower rate than CME and involves more complex signaling and regulatory mechanisms. For delivery of pH-sensitive molecules, such as nucleic acids, proteins or peptides, which cannot tolerate the lysosomal environment, targeting via the caveolar/raft-dependent endocytosis pathway may turn out advantageous (Hillaireau & Couvreur 2009). Also macropinocytosis as well as clathrin- and caveolin-independent endocytic pathways have been described as means of nanoparticle uptake (Hillaireau & Couvreur 2009). Nanoparticle size has been shown to influence the mode of endocytosis. Particles with diameter less than 200 nm were found to internalize via CME, whereas the larger particles (500 nm) might favor the caveolar/raft-dependent pathway (Rejman *et al.* 2003). Again, the surface properties play a major role in determining how effectively particle uptake occurs. Because of the negatively charged cell plasma membrane, nanocarriers with positive surface charge generally demonstrate increased adherence and internalization (Harush-Frenkel *et al.* 2007). It should be noted that multiple endocytic pathways might be simultaneously functional in nanoparticle internalization.

Nanoparticle systems can be made multifunctional by combininig cell-specific targeting ligands with other features that allow for more efficient internalization into cancer cells, escape from the endosomal compartment and even organelle targeting. This can be accomplished for example through pH-sensitive systems that enable new features to emerge on the particle surface in acidic environment of the endo/lysosomes (Lee *et al.* 2005). Furthermore, the shape of the particle may influence intracellular compartmentalization, which could be exploited in organelle targeting (Xu *et al.* 2008). It is important that the nanocarrier or at least its cargo makes a successful escape from the endosomal compartment into the cytoplasm or nucleus, where the drug targets typically reside. Molecules that respond to the pH lowering, such as poly(ethylene imine) (PEI), can be used for destabilization of endosomes (reviewed by Demeneix *et al.* 2004). PEI has been reported to function as a 'proton-sponge' because of its the high buffering capacity that upon decrease of the pH causes PEI to become highly protonated (Boussif *et al.* 1995). This leads to osmotic swelling due to water entry into the vesicle and finally disruption of the vesicle, allowing the release of endosomal content to the cytoplasm. Another suggested mechanism for PEI-induced endosomal escape involves swelling of the polymer network due to increasing repulsion of the protonated groups (Demeneix *et al.* 2004).

3.2.3 Mesoporous silica nanoparticles as cancer drug delivery vectors

Inorganic nanoparticles based on silica have been receiving increasing attention as sufficiently stable vehicles for drug delivery (reviewed by Tan *et al.* 2004). As an inherent component of cells, silica is non-toxic, biocompatible, becomes degraded in biological systems and ultimately gets excreted in urine. Preparation of porous silica nanoparticles by the sol-gel method allows control of the particle and pore size on a nanometric scale. Co-condensation approach enables covalent incorporation of functional groups to the pore walls during particle synthesis and subsequently facilitates further funtionalization with molecules, like fluorophores and bioactive ligands (reviewed by Rosenholm *et al.* 2010). Mesoporous silica nanoparticles (MSNs) have pore sizes in the molecular size range (typically 2-10nm), high pore volume and surface area, 0.6–1cm^3/g and 700–1000 m^2/g respectively, which permits efficient accommodation of small molecules (Rosenholm *et al.* 2010). Drug-loading capacities of up to 30 w% has been reported for mesoporous silica (Vallet-Regi *et al.* 2001). MSN loading with drug molecules can reduce the access of water into the pores, and thereby increase the hydrolytic stability of initially well-soluble particles (Andersson *et al.* 2004).

Introducing organic functions, such as the amino groups of PEI, on the MSN surface can significantly reduce particle aggregation that might pose a threat in biological applications (Rosenholm *et al.* 2006). Also avoiding serum protein adsorption to the particle surface is important, as this could lead to decreased targeting of the particles and increase the clearance of the particles from circulation by RES. Covering the particles with optimized PEG layer can create a 'shield' around the particle inhibiting adsorption of serum proteins (He *et al.* 2010). Furthermore, MSNs have been conjugated to cancer specific targeting-ligands, such as folic acid (Slowing *et al.* 2006, Gu *et al.* 2007, Liong *et al.* 2008), DNA aptamers (Zhu *et al.* 2009), sugar moieties (Gu *et al.* 2007, Park *et al.* 2008, Brevet *et al.* 2009) and HER2/Neu antibodies (Tsai *et al.* 2009).

The rate of MSN degradation in a biological setting needs to be carefully engineered to meet the needs of effective drug delivery. The hydrolytic stability of MSNs determines the ability of the particle to endure until it reaches the target site from the blood circulation and the rate of degradation in the intracellular compartment. Optimization of such particle behavior is complicated by the fact that intracellular and extracellular decomposition kinetics are likely to be different. For *in vivo* targeting of MSNs also the particle size, charge and polarity of the particle coating (stealth layer) are important parameters (Rosenholm *et al.* 2010). For example, the size of the nanoparticle is critical for permeability through biological membranes, but also influences internalization by target cells and the phagocytes of the RES (Hillaireau & Couvreur 2009). In the case of MSNs, particle diameter between 50-200 nm is considered suitable for cellular targeting *in vivo*, as particles larger than 200 nm cannot easily cross biological membranes, whereas smaller mesoporous particles are difficult to synthesize (Rosenholm *et al.* 2010).

Loading of the cargo drug into MSNs can be done by adsorption from organic solvent or pH-matched aqueous solvent (Rosenholm & Lindén 2008). Drug molecules can also be covalently linked to the functional groups of the pore walls of the MSNs (Tournée-Péeteilh *et al.* 2003). In this case, the drug molecule must be able to reacquire the active conformation upon release from the MSN. MSNs have been employed for intracellular delivery of hydrophobic anticancer agents, such as camptothecin (Lu *et al.* 2007a, Liong *et al.* 2008) and paclitaxel (Lu *et al.* 2007b, Xia *et al.* 2009, Vivero-Escoto *et al.* 2009), as

well as the hydrophilic cancer drug doxorubicin (Chen *et al.* 2009b, Zhu *et al.* 2009). Other membrane-impermeable agents, including propidium iodide (Lu *et al.* 2008), calcein (Liu *et al.* 2009), cytochrome c (Slowing *et al.* 2007) and nucleic acids (Radu *et al.* 2004, Park *et al.* 2008, Xia *et al.* 2009, Torney *et al.* 2007, Zhu *et al.* 2009) have been loaded in MSNs and delivered into cultured cancer cells. The high drug-loading capacity of MSNs could also allow multidrug delivery (Chen *et al.* 2009b), which is the likely scenario for successful cancer treatment.

An optimal drug delivery system releases the cargo in the right concentration at the target site within a predetermined amount of time. Although drug release rates can be easily measured in test tube conditions, the actual biological effect is the most important readout of successful drug delivery and release. Depending on the delivered substance, the outcome can vary from inhibited kinase activity or cell proliferation to induced gene expression, gene silencing or programmed cell death. MSNs allow versatile design of gate-keeping functions for minimal premature drug release and controlled release at the target site. The release of the drug from its carrier may be designed to occur in response to specific exogenous cues, such as heat or magnetic field (reviewed by Cotí *et al.* 2009). Alternatively, the nanoparticle can be engineered to disassemble in the intracellular milieu, where differences in pH, redox state and enzymatic activity may activate the releasing mechanism (Cotí *et al.* 2009). The gate-keeping properties at the particle surface are in a key role regulating the release dynamics.

AIMS OF THE STUDY

The aim of this thesis work was to discover and validate new methods for cancer cell-specific apoptosis induction. Previous studies in the laboratory had addressed the mechanisms of death receptor-mediated apoptosis and the role of kinases as regulators of death receptor signaling. I aimed at finding novel combinatorial approaches to sensitize resistant cancer cells to death ligand stimulation and apoptosis. Prior to the work described in this thesis, relatively little was known about cellular activities mediating the anticancer effects that had been attributed to lignan polyphenols. The goal of my study was to examine if lignans could be employed to sensitize androgen-dependent prostate cancer cells to TRAIL, a death ligand that has been reported to initiate tumor-specific apoptosis. During this work it became evident that lignans are powerful sensitizers to death receptor-mediated apoptosis, and my further studies focused on elucidating the cellular mechanisms and molecular determinants of lignan structure that bring about the observed effects, the latter being conducted in collaboration with the Department of Organic Chemistry in Åbo Akademi University.

Furthermore, I aimed at testing a novel nanoparticle drug carrier system under *in vitro* conditions for cancer cell targeting. A laboratory at the Department of Physical Chemistry in Åbo Akademi University had previously developed this drug delivery system with several advantages over conventional nanoparticle systems. The goal of this thesis work was to demonstrate cancer cell-specific nanoparticle internalization, drug delivery and apoptosis induction by the functionalized mesoporous hybrid silica nanoparticles.

Briefly, the aims of this thesis were

1) To investigate lignans as sensitizers for TRAIL-induced apoptosis in androgen-dependent prostate cancer cells

2) To test if mesoporous hybrid silica nanoparticles could be employed in development of targeted cancer therapies.

EXPERIMENTAL PROCEDURES

1. Cell culture (I-V)

HeLa cervical carcinoma cells and human embryonic kidney (HEK) 293 cells were purchased from ATCC and cultured on 12-well plates in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% fetal calf serum (BioClear), 2 mM L-glutamin, 100 U/ml penicillin, 100 µg/mL streptomycin in 37°C, 5% CO_2. The LNCaP prostate cancer cells (clone FGC; EACC) were cultured in RPMI-1640 medium (Sigma) supplemented with 10% fetal calf serum (BioClear), 2 mM L-glutamin, 100 U/ml penicillin, 100 µg/ml streptomycin and 1mM sodium pyruvate (Sigma). As the androgen-dependence of LNCaP cells can be maintained when the passage number is restricted, we cultivated the cells only up to 20 passages. Before growth factor stimulation, the cells were serum-starved (1 % serum) overnight. When the role of androgen-stimulation was investigated, the medium was changed to phenol red free RPMI-1640 (Gibco) with or without 1 nM synthetic androgen (Mibolerone) one day before the experiment. This medium was supplemented with 10% charcoal/dextran treated fetal bovine serum (Hyclone), L-glutamine, penicillin and streptomycin. SV40 transformed prostate epithelial RWPE-1 cell line was kindly provided by Professor Olli Kallioniemi. RWPE-1 cells were cultivated in Keratinocyte medium (Gibco) with 100 U/ml penicillin, 100 µg/ml streptomycin, 1% fetal calf serum (BioClear), 5 ng/ml human recombinant epidermal growth factor (EGF; Gibco) and 50 µg/ml Bovine Pituitary Extract (Gibco).

2. Reagents and treatments (I-II, IV)

Lignan synthesis was performed at the Department of Organic chemistry, Åbo Akademi University. A 100 mM stock solution of each lignan compound was prepared in EtOH or DMSO and stored at +4°C or room temperature. The cells were treated with 0–100 µM lignans or solvent control for indicated times. Human recombinant isoleucin-zipper TRAIL (izTRAIL; kindly provided by Professor Henning Walczak) was used at 0–1 µg/ml with incubation time of 20 hours. Alternatively, doxorubicin (Sigma) was used at 0.2–1 µM for 20 h. Insulin receptor (IR) and IGF-IR were stimulated with 10 nM IGF-1 (Millipore) or 10 nM insulin (Sigma) for 10 minutes. EGFR activation was performed with 20 ng/ml EGF for 5 minutes (a kind gift from Professor Klaus Elenius laboratory). Treatment with 1 µM staurosporine for 24 h was used as a positive control in an apoptosis assay. Inhibition of actin polymerization was done by pretreating the cells with 10 µM cytochalasin D (CytD) for 30 min and incubating in 5 µM CytD during nanoparticle uptake. Dose-response to methotrexate (MTX) was measured after treatment with 0.2 ng–2 µg/ml MTX for 72 h.

3. Particle synthesis (III-IV)

Fluorescent MSNs with diameter of approximately 400 nm were synthesized according to the procedure described by Nakamura et al. (2007), but with the thiol-silane replaced by 3-aminopropyltrimethoxysilane together with fluorescein isothiocyanate (FITC) in order to create inherently fluorescent particles. Functionalization of the particles was performed as explained by Rosenholm et al. (2006) and Rosenholm & Lindén (2007). Folic acid (FA) conjugation was performed according to standard bioconjugation protocols leading to 0.2 or 2 weight percent (wt %) of FA or methotrexate (MTX) on the particle. The 2 wt% MTX conjugation in 1 µg/ml particle concentration and the 0.2 wt% MTX conjgation in 10

µg/ml particle concentration corresponded approximately to 20 ng/ml free MTX. DiI (1,1′-dioctadecyl-3,3,3′,3′-tetramethindocarbocyanine perchlorate) or DiO (3,3-dioctadecyl-oxacarbocyanine perchlorate) dissolved in cyclohexane were loaded to 1 wt % in the MSNs. After vacuum drying overnight to remove any excess solvent, the particles were washed in a HEPES buffer solution (25 mM, pH 7.2). All particles were resuspended in dimethyl sulfoxide (DMSO) or HEPES buffer at a concentration of 1 mg/ml.

4. Particle preparation, application to cells and detection of intracellular particles by flow cytometry (III-V)

FITC-, FITC/PEI-, FITC/PEI/FA- or FITC/PEI/MTX-functionalized MSNs with or without DiI/DiO loading were suspended in growth medium at a concentration of 1–10 µg/mL. After 20–30 min of sonication in a water bath, the medium with particles or control medium was added to the 50–70% confluent cells and incubated for 3–72 h at 37°C depending on the experimental setup. The cells were cultured in folate-free RPMI medium (Gibco) with supplements for 24 h before incubation with MTX-functionalized nanoparticles or the FA-functionalized reference particles. For FA competition experiments, the cells were cultured overnight with 0–3 mM FA (Sigma) prior to addition of the particles. After incubation with MSNs, the cells were trypsinized and the extracellular fluorescence was quenched by resuspension in trypan blue (200 mg/ml; Fluka) for 5–10 min at room temperature. The cells were washed once and resuspended in phosphate-buffered saline (PBS). Intracellular FITC fluorescence was used as a measure of the number of endocytosed particles inside the cells, and it was analyzed by using a FacsCalibur or an LSRII flow cytometer (BD Pharmingen). The MFI of the cells at the FITC channel (FL-1 or FITC-A) was measured, and the data were analyzed with BD FacsDiva, Cyflogic and Flowing softwares. The fraction of cells with fluorescent particles was gated above the background fluorescence.

5. Assessment of apoptotic cell death (I-III,V)

5.1 Mitochondrial depolarization (I-II)

Tetramethyl Rhodamine Methyl Ester (TMRM; Invitrogen) was stored as 20 mM DMSO stock and diluted in medium prior to use. Floating and trypsinized cells were incubated in 20 nM TMRM for 10 min in 37°C waterbath. The cells were placed on ice and analysed immediately by FACSCalibur flow cytometer (FSC; FL-2 channel). When the labeling was done in a 96-well format, the plate was centrifuged with a culture plate rotor (1000 rpm, 3 minutes), the cells were trypsinized and prewarmed medium containing 20 nM TMRM was incubated with the cells for 10 minutes. The plate was analyzed with LSRII flow cytometer equipped with HTS platform (FSC, PE-A channel). The cells dsiplaying decreased labeling with TMRM that accumulates into active mitochondria, were considered to have undergone mitochondrial membrane depolarization.

5.2 Caspase-3 activation (I-II)

Activated caspase-3 in cells was labeled with phycerythrin (PE)-conjugated antibody according to manufacturer's protocol (BD Pharmingen). Briefly, the floating and attached cells were collected by trypsinization, and placed on ice. After washing once with cold PBS the cells were fixed in Fix/Perm buffer for 20 minutes on ice. The cells were subsequently washed twice with Perm/Wash buffer and resuspended to the same buffer containing 10 µl antibody. After 30 minutes incubation at room temperature, the cells were washed and resuspended to Perm/Wash buffer. The samples were analyzed by FacsCalibur

flow cytometry (FL-2, FSC channel). Cells with high PE labeling were considered apoptotic cells with activated caspase-3. For flow cytometric analysis of transfected LNCaP cells, GFP-positive cells were gated (FL-1) and measured for caspase-3 activation (FL-2).

5.3 Nuclear fragmentation (I-III,V)

The cells were collected by trypsinization and resuspended in propidium iodide (PI) buffer (40mM Na citrate, 0.3% Triton X-100, 50 mg/ml PI; Sigma). After 10 min of incubation at room temperature the samples were analyzed for nuclear fragmentation with a FacsCalibur flow cytometer (FL-2, BD Pharmingen). When the cells were grown on 96-well plates, the culture plate was centrifuged with a culture plate rotor (1000rpm, 3 minutes), and PI buffer was added to the wells. After 10 minutes incubation at room temperature, the plate was analyzed with LSRII flow cytometer equipped with HTS platform (PE-A channel). The fraction of sub-G0/G1 events (nuclear fragmentation) was gated as a measure of apoptotic cell death.

5.3 Nuclear morphology (I,III-V)

For examination of nuclear fragmentation, the cells were collected, washed once in PBS and fixed for 15 minutes in 3% paraformaldehyde at room temperature. Cytospin (Thermo Shandon) preparations were made and mounted in 40,6-diamidino-2-phenylindole (DAPI) Vectashield (Vector Laboratories). The nuclei were viewed with Leica DMRE fluorescence microscope (40x objective) and the images collected with Wasabi (1.4) software (I, Figure 2C). Alternatively, when nanoparticles were applied to cells, the samples were fixed on glass-bottom culture plates (MatTek Corp), labeled with DAPI Vectashield and viewed by using Zeiss LSM 510 META laser-scanning confocal microscope (40x or 63x oil objective, 405/488 nm excitation) (III-V).

6. Plasmid construction and transfection (I)

The coding sequence for a constitutively active gag-Akt (kindly provided by Julian Downward) was amplified from the pSG5-PKBGAG vector by PCR and cloned in-frame into pEGFP-N1 vector (Clontech) in EcoRI and KpnI sites. LNCaP cells were seeded on 24-well plates and grown to 30–50% confluency. The JetPEI™ transfection complexes (PolyPlus-transfection) were prepared and transfected according to the manufacturer's protocol. Empty pEGFP-N1 vector was transfected to control cells. The culture medium was replaced by medium with or without androgen 24 h post-transfection, and the cells were cultured for further 24 hours.

7. Insulin receptor and IGF-I receptor immunoprecipitation (II)

The LNCaP cells cultured on 10ml plates were placed on ice, rinsed with cold PBS and lysed in 1 ml of cold lysis buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA, 1.25% CHAPS, 1 mM Na3OV4, 2 mM NaF, 10 mM sodium pyrophosphate and complete protease inhibitor cocktail [Roche Applied Science]) for 10 minutes. After centrifugation at 15 000 x g for 10 minutes, the supernatant was collected and subjected to preclearing with Protein A sepharose beads (Sigma) and Protein G sepharose beads (Amersham Biosciences) for 1 hour at +4°C. After preclearing, the lysate sample was collected and rest of the sample was divided in two for immunoprecipitation with 5 µg anti-Insulin receptor ß or 5 µg anti -IGF-I receptor ß antibody (Santa Cruz Biotechnology). After overnight incubation at + 4°C, Protein A and Protein G sepharose beads were added to samples and

incubated for 4 hours. The beads were washed 4 times with lysis buffer, resuspended to 50 µl of Laemmli sample buffer (LSB) and boiled for 10 minutes.

8. DISC-immunoprecipitation (I)

To stimulate TRAIL receptors LNCaP cells were detached, pelleted by centrifugation and resuspended in 1 ml of the collected medium. Thereafter 1 µg izTRAIL was added to the cell suspension. The cells were incubated at 37 °C for 15–60 minutes after which the reaction was stopped by adding 10 ml of ice-cold PBS. Control cells were incubated without TRAIL at 37 °C for 60 min. After washing, the cells were lysed in 1 ml of lysis buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10% glycerol, 0.2% Nonidet P40, and Complete protease inhibitor mixture [Roche Applied Science]) for 30 min on ice. The cell debris was removed by centrifugation at 15 000 x g for 15 min at 4 °C. Equal amount of protein from each sample was precleared with Sepharose CL-4B for 1,5 h at 4 °C. Monoclonal anti-DR4 (Clone HS101; Alexis) and anti-DR5 (Clone HS201, Alexis) were added 2.5 µg each to the samples and immunoprecipitated with 15 µl of protein G beads (Amersham Biosciences) for 2.5 h at 4 °C. The beads were washed 6 times with lysis buffer, resuspended in LSB, and finally boiled for 5 minutes.

9. Western blotting (I-II)

Whole cell lysates were prepared by lysing floating and attached cells in LSB and boiling the samples for 10 minutes after which proteins were separated with sodium dodecyl sulphate (SDS)-PAGE. The DISC-immunoprecipitated samples and corresponding cell lysates (input) were analyzed by 10% Criterion SDS-PAGE (BioRad). The proteins were transferred onto nitrocellulose (Schleicher & Schuell) or PVDF membrane (Millipore), which was then blocked with 5% non-fat milk or 5% bovine serum albumin. Western blotting was performed using antibodies against Akt, phospho(Ser473)-Akt, Bid, Bax, Bim, GSK-3ß, phospho(Ser9)-GSK-3ß and phospho-Tyr(1068) EGF receptor (Cell Signalling Technology), Bcl-xL, DR5, EGF receptor, Insulin receptor ß and IGF-I receptor ß (Santa Cruz Biotechnology), FADD (Transduction Laboratories), poly (ADP-ribose) polymerase (PARP, clone C-2-10; Sigma-Aldrich), Actin (clone AC-40; Sigma-Aldrich), caspase-8 (clone C15; Alexis) and c-FLIP (clone NF6; Alexis), PI3K p85α (clone 4/PI3-Kinase; BD Pharmingen) and phospho-tyrosine (clone 4G10; Millipore). HRP-conjugated secondary antibodies were from Southern Biotechnology Associates, Promega, and Amersham Biosciences. The results were visualized using the enhanced chemiluminescence (ECL) method (Amersham Biosciences) on x-ray film. For densitometric analysis of Western blots, the x-ray films were scanned and the analysis was done with the MCID M5+ software. The values were normalized to the untreated control sample, which was given the value 1. The histograms present a relative change in ratio of phosphorylated vs. non-phosphorylated protein in treated samples as compared to control samples.

10. Receptor surface expression (I,III)

Surface expression of TRAIL receptors was evaluated by indirect immunostaining using the anti-DR4 (clone HS101), anti-DR5 (clone HS201), anti-DcR1 (clone HS301) and anti-DcR2 (clone HS402) primary antibodies (5 µg/ml). Surface expression of the folate receptor was detected by labeling with anti-folate receptor α primary antibody (2 µg/ml, clone Mov18/ZEL, Alexis). Alexa 488 -conjugated anti-mouse secondary antibody (Alexis Biochemicals) was used for labeling of all primary antibodies. Nonspecific fluorescence was assessed using the secondary antibody only. Flow cytometric analyses of the mean

fluorescence intensity (MFI) were performed using a FACSCalibur (FL-1) or an LSRII flow cytometer (FITC-A).

11. Immunofluorescence (II)

After treatments, the cells cultured on coverslips were washed with PBS and fixed with 3% paraformaldehyde for 15 minutes at room temperature. The samples were permeabilized and blocked for one hour in 3% BSA 0.1% Tween in PBS. Akt was labeled with rabbit anti-Akt antibody (Cell signaling) and Alexa 546 -conjugated anti-rabbit secondary antibody (Molecular Probes). After washing with PBS, the samples were mounted with DAPI Vectashield (Vector Laboratories) and viewed by Zeiss LSM 510 META laser-scanning confocal microscope (63x oil objective, 543 nm excitation).

12. Live cell microscopy (II-V)

For microscopic assessment of nanoparticle endocytosis, cells were plated on glass-bottomed culture dishes (MatTek Corp). In some studies, the cells were labeled with 20 µM CMAC CellTracker (Invitrogen) or 5 µM CellTracker Red (Invitrogen) in medium without additives for 30 min, after which the medium was replaced with normal culture medium. For assessment of lysosomal compartmentalization of particles, the cells were labeled with Lysotracker Red (Molecular Probes) according to the manufacturer's instructions. Cells were incubated with nanoparticles (10 µg/ml) for up to 72 h and viewed with Leica DM Ibre inverted fluorescence microscope (16x, 20x objective) or Zeiss LSM 510 META laser-scanning confocal microscope (63x oil objective, 543 nm excitation) at indicated time points.

13. Statistical analysis (I-II)

The statistical significance of differences in the data were calculated with a two-way Student's t-test by using the GraphPad Prism software. The p-value < 0.05 was considered to indicate significant differences. The graphs in the figures represent mean values±standard error of mean (SEM) and numbers of independent experiments is indicated in the figure legends.

RESULTS AND DISCUSSION

1. SENSITIZATION OF PROSTATE CANCER CELLS TO TRAIL-INDUCED APOPTOSIS BY LIGNANS

1.1 Dibenzylbutyrolactone lignans enhance the death receptor-mediated apoptosis pathway in androgen-dependent prostate cancer cells (I)

Activation of death receptor-mediated apoptosis by TRAIL has been shown to play an important role in immune surveillance against tumors *in vivo* (reviewed by Gonzalvez & Ashkenazi 2010). Because TRAIL is able to induce apoptosis selectively in cancer cells, different forms of TRAIL receptor agonists are investigated in clinical trials. However, TRAIL resistance has been reported in many primary tumors and combinatorial treatment approaches will be needed for successful TRAIL-based anticancer treatment. The initial aim of the study was to discover new combinatorial treatments that would be non-toxic *per se*, but able to selectively promote TRAIL-induced apoptosis in prostate cancer. Lignans had previously been reported to inhibit growth of prostate cancer cells (Lin et al. 2001), induce anticancer effects in prostate cancer (Bylund *et al.* 2005), and potentially reduce the risk of prostate cancer in humans (Heald *et al.* 2007). As these compounds have been extensively studied at the Department of Organic chemistry at Åbo Akademi University, we began to examine the possibilities to employ lignans as sensitizers to TRAIL.

The group of polyphenols, termed lignans, comprises compounds that are structurally very different from each other, and the existing information about the effects of lignans at the cellular level also reflects this variability. When this thesis project was initiated, the lignans arctigenin, SDG, SECO, ENL and END had been demonstrated to induce antioxidative effects in cultured cells (Prasad 2000, Jang *et al.* 2001, Kim *et al.* 2003), and some lignans, such as arctigenin, HMR and picropodophyllin had been investigated for their role in cellular signaling (Oikarinen *et al.* 2000, Cho *et al.* 2002, Cho *et al.* 2004, Vasilcanu *et al.* 2004). These studies showed that lignans are able to inhibit various important survival signaling pathways, including the Wnt/β-catenin, NF-κB, MAPK and PI3K pathways. Later ENL was shown to inhibit Akt signaling and to induce p53 stabilization, cell cycle arrest and apoptosis in prostate cancer cells (Chen et al. 2007). As the mechanisms of lignan activity in cells had been investigated mainly with single compounds or with extracts of several lignan components, the understanding the structure-activity relationship of lignan effects in cells was poor. Thus, more systematic approach was taken in order to elucidate the effects of lignans in prostate cancer cells, and the focus of the study was placed on dibenzylbutyrolactone (DBL) lignans, like the mammalian lignan ENL, and their derivatives (Figure 13).

The androgen-dependent LNCaP cell line that was used as a prostate cancer model in this thesis originates from a lymph node metastasis of human prostatic adenocarcinoma (Horoszewicz *et al.* 1983). A single point mutation in the ligand-binding domain of the androgen receptor (AR) broadens the steroid binding specificity of the AR expressed in LNCaP cells (Veldscholte *et al.* 1992). The model systems for human prostate cancer contain around 20 established cell lines and derivate sublines, only minority of which display androgen-sensitivity (reviewed by Russell & Kingsley 2003). The androgen-

dependent cell lines can be employed for developing new therapeutic strategies to eradicate locally advanced prostate cancer that is currently treated by ADT. In our studies, the LNCaP cells were grown the presence or absence of androgen in order to study the effects of androgen-deprivation in combination with the new treatment protocol. Unlike the commonly studied androgen-independent prostate cancer cell lines, PC3 and DU-145, LNCaP cells are resistant to TRAIL-induced apoptosis (Nesterov *et al.* 2001, Bucur *et al.* 2006), even though they express the TRAIL receptors DR4 and DR5 as well as only low levels of the decoy receptor DcR2 on the cell surface (I, Figure 3A). Like various other prostate cancer cells, the LNCaP cells have lost the tumor suppressing function of PTEN that negatively regulates PI3K activity (Li *et al.* 1997, Vlietstra *et al.* 1998). This feature of LNCaP cells is linked to the mechanisms of TRAIL-resistance, which have been been shown depend on the constitutive Akt activity downstream of PI3K and to result from impaired Bid cleavage upon TRAIL-stimulation leading to less efficient mitochondrial amplification of the signal (Chen *et al.* 2001b, Nesterov *et al.* 2001).

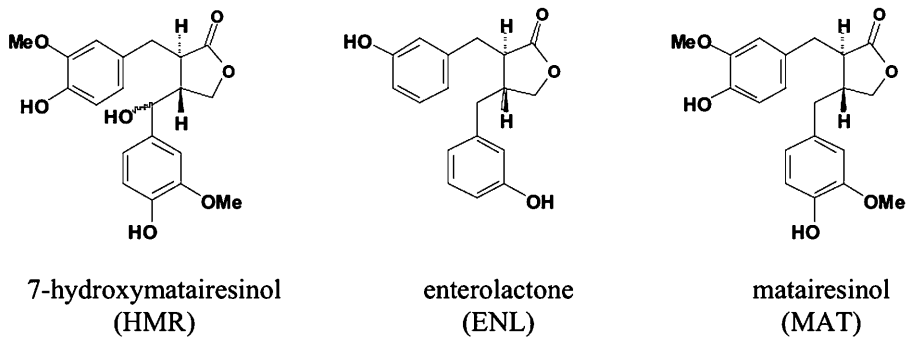


Figure 13. Molecular structures of 7-hydroxymatairesinol, enterolactone and matairesinol.

To examine if lignans can sensitize LNCaP cells to TRAIL-induced apoptosis, we first tested three lignans, the mammalian lignan ENL and two plant lignans, MAT and HMR, which can be converted to mammalian lignans by the gut microflora. All three compounds were non-toxic at the tested concentrations, whereas only MAT was found to be efficient in sensitization of the cells to TRAIL (I, Figure 1B and 2A). When the LNCaP cells were pretreated with 0–100 µM MAT for 1 hour prior to addition of trimeric human recombinant izTRAIL for 20 hours, both the androgen-deprived and -stimulated LNCaP cells became significantly sensitized to izTRAIL-induced apoptosis in a dose-dependent manner (I, Figure 2AB). When 40 µM MAT was combined to izTRAIL treatment, much lower izTRAIL concentrations were able to induce caspase-3 activation and the maximum percentage of apoptotic cells was clearly higher. The observed sensitization was more pronounced in the androgen-deprived LNCaP cells suggesting that androgen is able to promote the mechanisms of TRAIL resistance, and combination of ADT to lignan and TRAIL treatment could be considered as an attractive therapeutic approach (I, Figure 2B). MAT pretreatment did not increase apoptotic cell death of LNCaP cells in response to doxorubicin, a commonly used chemotherapeutic agent that activates mitochondrial apoptosis pathway primarily by inhibition of topoisomerase II enzyme (I, Figure 2D). These results imply that lignans can have synergistic effects with the TRAIL-induced extrinsic apoptosis pathway, whereas the cytotoxic action of agents that promote the intrinsic mitochondrial pathway is probably not equally enhanced.

The rate of apoptosis in TRAIL-sensitive PC3 prostate cancer cells was not significantly enhanced by identical MAT pretreatment (I, Supplementary Figure 3), and longer MAT pretreatment (24 h) augmented TRAIL-induced apoptosis only to some extent in TRAIL-sensitive leukemic Jurkat T cells and HeLa cervical carcinoma cells (I, Supplementary Figure 1). No further increase in the TRAIL treatment efficacy could therefore be seen in already TRAIL-sensitive cells. However, both ENL and MAT were capable of sensitizing HeLa cells to CD95-induced apoptosis (I, Supplementary Figure 2), which implies that common mechanisms of resistance in death receptor-mediated apoptosis can be overcome by lignan treatment. These mechanisms might be linked to the inhibitory effects that DBL lignans have previously been shown to exert on survival signaling pathways (Oikarinen *et al.* 2000, Cho *et al.* 2002). Further studies on lignan influence should be performed in cell types that are involved primarily in CD95-signaling, including various immune cells, in order to elucidate this matter. In addition, the potential effects of lignans on CD95-mediated processes need to be taken into account when *in vivo* effects of TRAIL and lignan treatment are evaluated.

1.2 Structure-activity relationship analysis identifies (-)-nortrachelogenin lignan as an effective compound for sensitization to TRAIL-induced apoptosis (II)

The results obtained with MAT, ENL and HMR lignans prompted us to test 27 naturally occurring lignans and lignan derivatives in a structure-activity relationship (SAR) analysis (II, Table 1). A panel of 18 lignans (II, Figure 1) and 9 norlignans (II, Figure 2), including several ENL precursors and DBL lignans, was assayed for the ability of the compounds to sensitize androgen-deprived LNCaP prostate cancer cells to TRAIL-induced mitochondrial depolarization and nuclear fragmentation (II, Figure 3). Our results demonstrate that the DBL skeleton in its non-cyclic form (MAT, ENL, NTG, Me-MR, demetyl-MR) is the most active lignan structure in sensitizing to TRAIL-induced apoptosis. The substitution pattern of the aromatic rings only has a small effect on the activity, while addition of methyl, hydroxyl or oxo groups at position C-7 completely abolishes the active conformation. However, we discovered that an aliphatic OH-group in the lactone ring at position C-8 significantly enhances the lignan activity (II, Figure 1). The (-)-nortrachelogenin (NTG) lignan containing this structure sensitizes androgen-deprived LNCaP cells to TRAIL-induced apoptosis more efficiently than MAT (II, Figure 3).

NTG, also called (-)-wikstromol or pinopalustrin, is not found in human nutrition, but it can be isolated in relatively large amounts from soft wood species such as *Pinus sylvestris* (Ekman et al. 2002). Early reports on the NTG enantiomer, (+)-wikstromol, suggest that wikstromol has antileukemic activity (Torrance *et al.* 1979, Lee *et al.* 1981), and both NTG and wikstromol have been shown to function as antioxidants (Tiwari *et al.* 2001, Willför *et al.* 2003). However, there is very limited experimental data on the *in vitro* and *in vivo* effects of NTG. In the only available *in vivo* study by Saarinen *et al.* (2005), NTG failed to inhibit the growth of carcinogen-induced mammary tumors, while HMR was able to increase the number of regressing and stabilized tumors in the same experimental setting (Saarinen *et al.* 2000). Unlike HMR, NTG is not converted into the mammalian lignans, ENL or END, in the colon, but is excreted in urine as such or as metabolites (Saarinen *et al.* 2005). Therefore, the effects of NTG are likely to differ from those obtained with HMR. Daily exposure to NTG beginning from the pregnancy showed weak endocrine-modulatory effects in adult rats, whereas the influence of HMR with same dosing was not statistically significant (Saarinen *et al.* 2005).

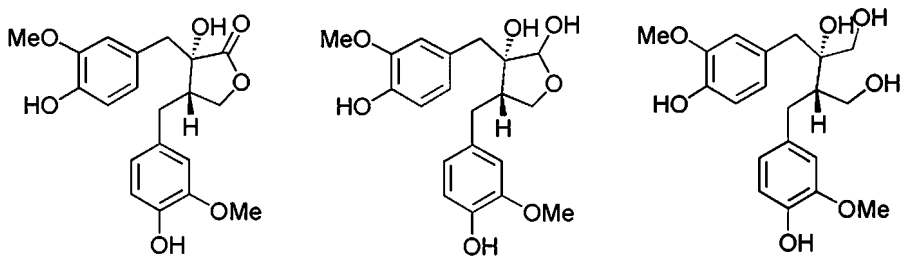
Arctigenin and wikstromol are examples of the interesting DBL lignans that could not be tested in our screening study due to availability problems, but have the structural determinants for activity in TRAIL-induced apoptosis. Also lignans that have methylenedioxy ring(s) (eg. savinin) or more than two substituent groups in the aromatic rings (eg. traxillagenin), would be attractive compounds for further SAR analysis of lignans as sensitizers to TRAIL-induced apoptosis. The enhanced TRAIL-induced apoptosis with NTG treatment suggests that the possible antitumorigenic effects of NTG might yet be revealed in other types of animal models for cancer. As TRAIL has demonstrated antimetastatic functions *in vivo* (Finnberg *et al.* 2008, Grosse-Wilde *et al.* 2008), the influence of NTG on tumor metastasis alone or in combination to TRAIL treatment deserves to be studied *in vivo*.

Interestingly, NTG did not sensitize non-malignant human epithelial prostate cells (RWPE-1) to TRAIL, indicating that the combination of lignans and TRAIL may leave normal cells unharmed (II, Figure 4C). Due to the potential hepatotoxicity of the combinatorial treatments involving TRAIL (Ganten *et al.* 2006), the influence of TRAIL and NTG should also be assayed in primary human hepatocytes. We are still on our way to understand what kind of molecular mechanisms determine that tumor cells selectively become victims of TRAIL-induced apoptosis while normal cells remain unharmed. It has been suggested that the mechanisms for tumor-selective apoptosis-induction by TRAIL would lie downstream of receptor activation and DISC formation, as inhibited FADD and caspase-8 recruitment would abrogate also TRAIL-induced survival signaling in normal cells (reviewed by Nieminen et al. 2007a, Varfolomeev et al. 2005). Concequently, it has been proposed that activation of oncogenes, such as c-Myc, might predispose cancer cells to TRAIL-induced apoptosis by enhancing mitochondrial permeabilization via proapoptotic Bcl-2 proteins and inhibiting NF-κB survival signaling and c-FLIP transcription upon TRAIL-treatment (Nieminen et al. 2007b).

1.3 The γ-butyrolactone ring is essential for the TRAIL-sensitizing activity of lignans (II)

We had observed that MAT was an active lignan and SECO could not sensitize the LNCaP cells to TRAIL, while the only structural difference between these two molecules is that the γ-butyrolactone ring is not present in SECO (II, Figure 1). As only the DBL lignans, including MAT, ENL and NTG, were able to enhance TRAIL-induced apoptosis, the γ-butyrolactone ring was considered likely to play an important role. To further study the importance of the γ-butyrolactone ring for sensitization to TRAIL-induced apoptosis, we synthesized two NTG derivatives lacking this functional group. To this end, NTG was reduced to corresponding lactol (CARS) and diol (CAR) derivatives (Figure 14). These compounds were tested and found inactive, while NTG could potently sensitize LNCaP cells to TRAIL stimulation (II, Figure 4D). The lactone ring has been mentioned as a critical structural determinant also in other SAR analyses of DBL lignans. Muta *et al.* (2004) observed *in vitro* that lignans with DBL structure could inhibit the enzymatic activity of matrix metalloproteinase-7, an extracellular protease that may play a critical role in tumor invasion and metastasis. They postulated that the lignan lactone ring may be involved in the binding to the active site of the enzyme, whereas the introduction of methylenedioxy ring(s) and hydroxyl group(s) to the aromatic rings was shown to contribute only to enhancement of the inhibitory activity (Muta *et al.* 2004). The lactone

ring and its polar position may also play an important role in inhibition of TNFα production in macrophages, but the underlying mechanisms remain to be determined in further studies (Cho *et al.* 2001). The carbonyl group of the DBL lignans can serve as a hydrogen bond acceptor in intermolecular interactions, while the additional hydroxyl group in NTG may enable enhanced hydrogen bonding between lignans and their target molecules (Figure 14). In this respect, the stereochemistry of NTG is likely to be important for the lignan activity, but this remains to be shown experimentally. The results of this study suggest that lignan derivatives with further increased activity in sensitization to TRAIL-induced apoptosis may possibly be synthesized and employed as anticancer agents.



nortrachelogenin (NTG) carissanol (CARS) carinol (CAR)

Figure 14. Molecular structures of nortracelogenin and the inactive derivatives, carissanol and carinol.

1.4 Dibenzylbutyrolactone lignans increase TRAIL-induced Bid cleavage and signal amplification through the mitochondrial apoptosis pathway (I,II)

To determine the molecular mechanisms by which DBL lignans permit the increased caspase activation and apoptosis upon TRAIL stimulation, we studied the effect of MAT on the main components of TRAIL receptor signaling: 1. Cell surface expression of the TRAIL receptors, 2. Formation of the TRAIL–DISC at the cytoplasmic death domain, 3. Depolarization of the mitochondrial membrane, and 4. Activation of the initiator and effector caspases. Lignans without DBL structure, namely nordihydroguaiaretic acid (NDGA), silibinin and honokiol, have recently been shown to facilitate TRAIL-induced apoptosis by induction of DR5 expression and downregulation of the apoptosis inhibitors c-FLIP and survivin (Son *et al.* 2007, Yoshida *et al.* 2007, Raja *et al.* 2008). Our results show that the membrane proximal events in TRAIL-induced signaling, including TRAIL receptor surface expression, TRAIL-DISC formation, and caspase-8 activation in the DISC, are not amplified by MAT treatment in androgen-stimulated or androgen-deprived LNCaP cells (I, Figure 3). These findings suggest that MAT and other DBL lignans may exert their sensitizing effect downstream of DISC formation. We noticed that androgen stimulation increases DR5 cell surface expression, enhances TRAIL-DISC formation and accelerates caspase-8 activation, although these proapoptotic events do not correlate with the apoptosis sensitivity of the cells. Our data, however, support the reported role of androgens as enhancers of TRAIL–DISC formation in LNCaP cells (Rokhlin *et al.* 2002).

Like most cancerous cells, the LNCaP prostate cancer cells have been shown to require mitochondrial amplification of the death receptor-initiated signals for efficient apoptosis induction. This has been demonstrated in experiments where the inhibition or downregulation of antiapoptotic Bcl-2 proteins has resulted in enhanced apoptosis in response to TRAIL receptor activation (Kim *et al.* 2005, Ray *et al.* 2005). This type II behavior in death receptor-mediated apoptosis often plays an important role in cancer cell TRAIL resistance, and involves high expression of antiapoptotic proteins, such as c-FLIP, XIAP, Bcl-2, Bcl-xL, survivin or Mcl-1, able to inhibit caspase activation at different steps of the cascade (reviewed by Mellier *et al.* 2010). Thus, overcoming these mechanisms of resistance upon TRAIL receptor engagement is likely to promote the execution of cancer cell death. Our results demonstrate that 40 µM DBL lignan pretreatment is able to increase TRAIL-induced mitochondrial depolarization and caspase-9 cleavage in LNCaP cells (I, Figure 4A; II, Supplementary Figure 2). The enhanced MOMP downstream of TRAIL-DISC formation is a probable source for the boosted caspase activation and TRAIL-induced apoptosis, when DBL lignans are combined with TRAIL.

Although some lignans have been reported to downregulate the expression of the antiapoptotic Bcl-2 proteins (Giridharan *et al.* 2002, Hausott *et al.* 2003, Saggar *et al.* 2010), we did not detect any MAT-induced changes in the expression of the Bcl-2 proteins known to regulate mitochondrial apoptosis pathway upstream of mitochondrial permeabilization (I, Figure 4C). In addition, the expression of the downstream caspase-inhibitor XIAP remained uninfluenced by MAT treatment (unpublished results). ENL has been reported to induce p53 stabilization (Chen *et al.* 2007) and to inhibit the proliferation of the LNCaP cells (McCann *et al.* 2008). Chen *et al.* (2007) also showed that a 48–72h incubation with 50–100 µM ENL may induce apoptosis in LNCaP cells by initiation of the mitochondrial pathway of apoptosis. MAT has also been shown to reduce viability in human gastric adenocarcinoma AGS cells (Kang *et al.* 2007), but these effects were observed at concentrations corresponding to 150–500 µM MAT. We have also noticed that DBL lignans induce growth arrest in LNCaP cells (data not shown), and MAT has a minor effect on mitochondrial polarity (I, Figure 4A), but our data indicate that neither MAT (Figure 15) nor NTG (II, Supplementary Figure 1) is an effective inducer of apoptotic cell death at the concentration of 0–100 µM when incubated up to 72h. Therefore, we conclude that treatment with 40 µM MAT or NTG clearly requires TRAIL -induced signals to provoke apoptosis in LNCaP prostate cancer cells. In addition to the TRAIL-sensitizing influence, the growth suppressing effects of DBL lignans, which may result from inhibition of mitogenic signaling, are likely to contribute to the anticancer effects observed *in vivo* (Bylund et al. 2005).

The caspase-cleaved form of the Bcl-2 BH3-only protein Bid, tBid, has been implicated as an important mediator between the death receptor-induced caspase activity and induction of the mitochondrial outer membrane permeabilization, or MOMP (Li *et al.* 1998, Luo *et al.* 1998). As the TRAIL resistance of LNCaP cells has previously been shown to arise from defective Bid cleavage (Chen *et al.* 2001b, Nesterov *et al.* 2001), we investigated if DBL lignans can amplify this link between the two cell death pathways. Our results show that MAT or NTG pretreatment increases the TRAIL-induced cleavage of the full-size Bid in androgen-deprived LNCaP cells (I, Figure 4B; II, Supplementary Figure 2). We were only able to detect the cleaved tBid after proteasomal inhibition by epoximicin (data not shown), suggesting that the active tBid protein fragment may be subject to fast turn over. Nevertheless, the efficient Bid processing upon TRAIL receptor activation was found caspase-dependent (data not shown), and is a likely reason for the enhanced mitochondrial

depolarization when the androgen-deprived cells are pretreated with DBL lignans. In the presence of androgen, TRAIL–DISC formation and caspase-8 activation are increased, and TRAIL treatment as a single-agent is enough for inducing Bid cleavage (I, Figure 4B; II, Supplementary Figure 2). The cellular processes that prevent the mitochondrial depolarization and apoptosis execution in response to TRAIL in the androgen-stimulated cells are likely to require androgen-induced transcription, but we have not investigated this matter further. How these mechanisms of resistance are influenced by DBL lignans to enable enhanced apoptosis induction in androgen-stimulated cells, remains also to be examined in future studies.

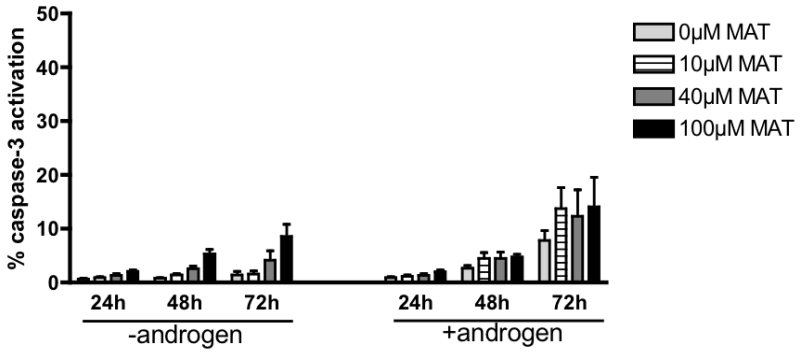


Figure 15. Matairesinol (MAT) is not a potent initiator of apoptosis in LNCaP cells. The cells were cultured in the presence or absence of androgen and incubated with 0–100 μM MAT for 0–72 hours. The percent of apoptotic cells was determined by labeling of activated caspase-3 and analyzing by flow cytometry.

In summary, our data demonstrate that DBL lignans are able to sensitize cancer cells to receptor-mediated apoptosis. In LNCaP cells, DBL lignans facilitate TRAIL-induced Bid cleavage, mitochondrial depolarization and caspase activation, especially when the cells are also deprived of androgen. Nortrachelogenin was identified as the most effective DBL lignan for TRAIL-sensitization in the systematic SAR screening study, which also recognized the γ-butyrolactone ring of DBL lignans as a critical structure in sensitization to TRAIL-induced apoptosis. Our results suggest that sensitization to TRAIL-induced apoptosis may be a key mechanism of DBL lignan anticancer activity *in vivo*, and this ability could be employed in cancer treatment to overcome resistance to therapeutic TRAIL receptor agonists.

2. LIGNANS AS INHIBITORS OF AKT SURVIVAL SIGNALING

2.1 Dibenzylbutyrolactone lignans sensitize to TRAIL-induced apoptosis by downregulating Akt activity (I,II)

Recent studies indicate that inhibition of the PI3K pathway with chemotherapeutic agents is an attractive strategy to treat prostate cancers (reviewed by Sarker *et al.* 2009). The approach is based on the increasing evidence suggesting frequent deregulation of the PI3K survival pathway in prostatic carcinomas (reviewed by Engelman et al. 2009). In LNCaP cells, the loss of PTEN can be observed as constitutive activity of Akt, one of the most

important downstream targets of PI3K (Carson *et al.* 1999). Akt is a serine/threonine kinase that activates or inhibits a range of signaling molecules via phosphorylation, and thereby promotes cell growth, proliferation and survival. For example, GSK-3β, a protein that amongst other functions induces glycogen synthesis and suppresses cell cycle progression, is inhibited by Akt-mediated phosphorylation in order to promote growth and proliferation (Cross *et al.* 1995).

As the tested DBL lignans were found to be non-toxic to LNCaP cells, lignans are likely to exert their anticancer activity by influencing factors that prevent effective TRAIL-induced apoptosis. As ENL was shown to inhibit Akt activity (Chen *et al.* 2007), we decided to investigate if DBL lignans could sensitize LNCaP cells to TRAIL-induced apoptosis by inhibition of Akt activity. Our results show that MAT and NTG are more efficient than ENL in reducing the level of phosphorylated, and therefore active, Akt kinase especially in androgen-deprived LNCaP cells (I, Figure 5A; II, Figure 5). In addition, we observed that NTG treatment downregulates phosphorylation of the Akt target protein GSK-3β (II, Figure 5D). The inhibitory effect on Akt activity reaches the maximal repression at 2h NTG incubation and starts to recover somewhat thereafter (II, Figure 5C). These results suggest that the three investigated DBL lignans, MAT, NTG and ENL, have a common mode of action. MAT has previously been shown to function as a direct inhibitor of kinase activity. Already in 1981, MAT was demonstrated to inhibit cyclic AMP phosphodiesterase (Nikaido *et al.* 1981), and more recently, to prevent casein kinase-I (CKI) activity *in vitro* (Yokoyama *et al.* 2003). It is interesting to speculate that these inhibitory activities might be connected to the observed downregulation of Akt activity. Furthermore, several of the previously described DBL lignan effects on cell survival signaling could be linked to the observed reduction of Akt activity, but further studies are needed to investigate these possibilities

The constitutive Akt signaling has been reported to protect hormone-dependent prostate cancer cells from TRAIL-induced apoptosis by inhibition of efficient Bid cleavage (Chen *et al.* 2001b, Nesterov *et al.* 2001). The recent results of Goncharenko-Khaider et al. (2010) also highlight Akt as a critical mediator of cancer cell TRAIL resistance that inhibits cleavage of Bid, prevents accumulation of tBid and also donwregulates Bid expression level. The mechanisms behind Akt inhibited Bid function have only recently been clarified, when Akt was shown to inhibit Bid localization to the mitochondria upon TRAIL stimulation by phosphorylation of PACS-2, a multifunctional sorting protein (Aslan *et al.* 2009). These results suggest that inhibited Akt activity is a likely reason for the enhanced Bid cleavage and apoptosis rate in response to TRAIL when the cells are also treated with DBL lignans. Consistently, synthetic PI3K inhibitors have been demonstrated to sensitize LNCaP cells to TRAIL-induced apoptosis, while overexpression of antiapoptotic Bcl-2 proteins is able to protect the cells from this combinatorial treatment (Chen *et al.* 2001b, Nesterov *et al.* 2001). We also observed that LNCaP cells can be sensitized to TRAIL by the PI3K inhibitor LY294002, and simultaneous treatment with LY294002 and MAT only showed minor additive effects in TRAIL-induced apoptosis, suggesting that these compounds are inhibiting at least partially the same mechanism of TRAIL resistance, the PI3K/Akt pathway (data not shown). To verify the involvement of PI3K/Akt pathway inhibition in DBL lignan action, we introduced a constitutively active form of Akt (ca-Akt) into androgen-deprived LNCaP cells. We found that ca-Akt is able to rescue a large portion of the cell population from MAT-mediated sensitization to TRAIL-induced apoptosis (I, Figure 5C). These results provide evidence for the importance of DBL lignan-mediated inhibition of the PI3K/Akt pathway signaling as a mechanism of

sensitization to TRAIL treatment. Deregulated activation of PI3K/Akt signaling may play a critical role also in allowing prostate tumors to survive and maintain proliferation in androgen-deprived environments (reviewed by Mulholland *et al.* 2006). Although the constitutive Akt phosphorylation did not markedly differ between androgen-deprived and androgen-stimulated LNCaP cells (I, Figure 5A; II, Figure 5), inhibition of Akt by DBL lignans was more pronounced in the androgen-deprived cells. These results suggest that androgen stimulation counteracts the effects of the lignans by promoting Akt activity. Therefore, ADT is likely to enhance the anticancer activity of DBL lignans in treatment prostate cancer.

Various naturally occurring polyphenols, such as flavonoids, stilbenes and phenolic acids, have been shown to sensitize cancer cells to TRAIL, and some of them inhibit Akt (reviewed by Jacquemin *et al.* 2010). For example, quercetin and genistein that belong to plant flavonoids are able to inhibit Akt activity while sensitizing to TRAIL (Kim & Lee 2007, Siegelin *et al.* 2009). Curcumin, the yellow pigment in turmeric, has been shown to inhibit Akt by activation of PP2A and/or some other calyculin A-sensitive protein phosphatase (Yu *et al.* 2008), but also to enhance the apoptosis-inducing potential of TRAIL in prostate cancer cells both *in vitro* (Deeb *et al.* 2007) and *in vivo* (Shankar *et al.* 2008). In addition, the stilbene resveratrol, found enriched in red wine, possesses ability to downregulate Akt activity as well as to sensitize prostate cancer cells to TRAIL-induced apoptosis (Gill *et al.* 2007). However, these studies do not demonstrate a direct link between Akt inhibition and increase in TRAIL-induced apoptosis. Our data show that inhibition of the PI3K/Akt signaling pathway is one of the major mechanisms by which DBL lignans sensitize androgen-deprived prostate cancer cells TRAIL-induced apoptosis.

2.2 The effects of the lignan nortrachelogenin on Akt activity and TRAIL-sensitivity are rapidly reversible (II)

Intriguingly, the effect of NTG on Akt activity and TRAIL-induced apoptosis was found to be rapidly reversible (II, Figure 7). When the medium with lignan pretreatment was removed and replaced by fresh culture medium prior to addition of izTRAIL, the apoptosis-enhancing effect was abrogated completely. Similarly, the inhibited Akt activity rapidly recovered to the phosphorylated state after medium replacement. Even a longer pretreatment (24h) with MAT was ineffective if the culture medium was replaced before addition of izTRAIL (data not shown). These results suggest that NTG may act directly on the Akt signaling pathway or other molecular targets regulating the cellular susceptibility to TRAIL-induced apoptosis, and imply that the cellular effects of lignans are likely to be membrane proximal or even occur outside of the cell. Preliminary results from biochemical studies with artificial membranes suggest that MAT or NTG are unlikely to spontaneously traverse the plasma membrane due to their polar nature. Instead, recent results suggest that both lignans tend to adhere at the lipid interphase (Bjorkbom A, unpublished results). Therefore, the Akt inhibition we observed might result from lignans effecting plasma membrane-tethered molecules involved in PI3K/Akt signaling, such as the receptor tyrosine kinases.

2.3 Nortrachelogenin (NTG) is a broad-spectrum inhibitor of receptor tyrosine kinase activity (II)

In the prostate, the PI3Ks are generally activated upon growth factor stimulation by RTKs, such as IGF-IR, EGFR, FGFR, PDGFR and VEGFR (reviewed by Hellawell & Brewster 2002). Inhibition of the catalytic subunit p110 by the regulatory p85 subunit in the heterodimeric class IA PI3Ks is relieved upon binding of p85 to tyrosine phosphorylated RTKs or RTK-bound adaptor proteins, which leads to enzymatic activation of PI3K (Yu *et al.* 1998). Recently, ENL was shown to suppress IGF-IR signaling and Akt activity in PTEN-deficient prostate cancer cells along with inhibition of cell proliferation and migration (Chen *et al.* 2009a). Prompted by these data and the efficient Akt inhibition we had observed with NTG treatment, we investigated how NTG influences the growth factor signaling of LNCaP cells in serum-starved conditions resembling low androgen environment. According to our results, NTG is not only able to inhibit activation of IGF-IR, but also insulin receptor (IR) and EGFR, by suppressing tyrosine autophosphorylation of the receptors upon ligand binding (II, Figure 6). We also observed that the p85 PI3K subunit is constitutively bound to IR and IGF-IR, and this recruitment is enhanced upon RTK activation. Importantly, NTG treatment reduced the amount of RTK-bound p85 in a dose-dependent manner (II, Figure 6B).

In the PTEN-deficient LNCaP prostate cancer cells, however, basal PI3K activity was recently found not to depend on RTK tyrosine phosphorylation (Jiang *et al.* 2010). The authors postulated that particular p110 subunits of PI3K may have a modest level of constitutive activity in the absence of RTK or GPCR stimulation that would be sufficient for the maintenance of PI3K pathway activation in PTEN-deficient cells. We also discovered that in LNCaP cells Akt activity remains surprisingly unaltered both in low-serum conditions and upon growth factor treatment (II, Figure 6B), suggesting that such RTK-independent mechanisms of PI3K activation may be functional. Nevertheless, we noticed that NTG is able to downregulate Akt phosphorylation regardless of the growth factor supply. Therefore, it remains to be clarified, whether NTG induces the observed Akt inhibition by 1. Interfering with RTK signaling, 2. Suppressing the basal PI3K activity, 3. Inhibiting Akt activity, 4. Activating pathways that promote Akt inactivation, or 4. Employing a mechanism, which is yet to be identified (Figure 16).

As RTK-induced signaling has a critical role in malignancy, various therapeutic approaches have already been developed against GFR families, such as the VEGFRs, EGFRs, PDGFRs and IGF-IR. It would be worth examining if NTG is able to inhibit RTK activity in other cancer cell types, and what the consequences of NTG treatment are in cells that have wild type PTEN. Furthermore, sensitization of LNCaP cells to TRAIL-induced apoptosis by NTG may involve the inhibition of RTK activity. Karasic *et al.* (2010) have previously observed such mode of action for a cyclolignan, picropodophyllin, which was shown to inhibit IGF-IR signaling and to sensitize melanoma cells to TRAIL. The neolignan magnolol has been reported to inhibit EGFR signaling without influencing insulin-induced pathways in prostate cancer cells (Lee *et al.* 2009). In the same study, it was proposed that the binding affinity of magnolol to EGFR may depend on the intracellular redox state, and differences between cancer cells and normal cells with this respect would allow cancer cell-specific effects. Whether or not such mechanisms operate and can be applied to functions of NTG warrants further investigation. As we now observed that the reversibility and dynamics of the TRAIL-sensitizing NTG effect is equal in the presence and in the absence of androgen, the molecular mechanisms of lignan

activity may involve same targets in both conditions despite the differences in apoptotic signaling responses. Although the RTK/PI3K/Akt pathway seems to be one of the major lignan targets that regulate TRAIL sensitivity, other cell surface receptors and their downstream signaling cascades could be influenced by lignans. The Frizzled receptor could be such a target, as lignans have been shown to inhibit activity of the Wnt signaling pathway (Oikarinen *et al.* 2000, Yoo *et al.* 2010), but this possibility has not yet been experimentally addressed.

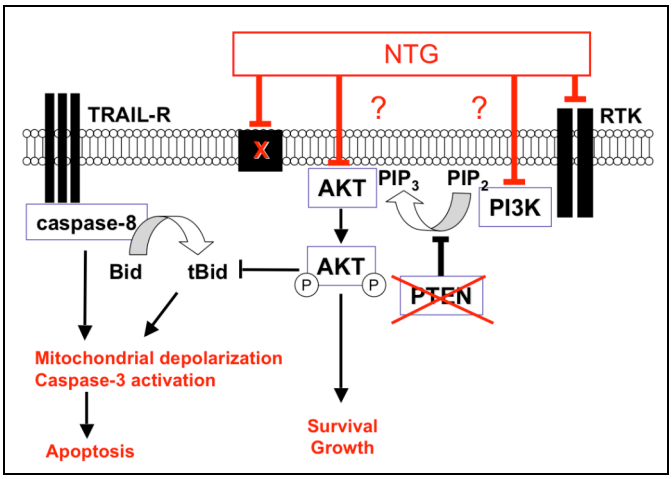


Figure 16. A schematic presentation of the potential DBL lignan targets in the RTK signaling pathway of LNCaP prostate cancer cells. Activated PI3K induces generation of PIP_3 at the plasma membrane, which leads to Akt recruitment and activation by phosphorylation. The lack of the phosphatase PTEN causes constitutive Akt activity and survival signaling in LNCaP cells, which accounts for suppression of TRAIL-induced apoptosis in these cells. The resistance to TRAIL has been shown to occur at the level of impaired Bid cleavage and mitochondrial permeabilization. DBL lignans, such as NTG, were found to inhibit Akt activity and RTK activation by growth factors. Both of these effects can result from inhibition of RTK activity, but independent effects on RTK, PI3K and Akt activity cannot be ruled out in the light of current evidence. Furhermore, other regulatory mechanisms ('X') that influence RTK/PI3K/Akt signaling can be targeted by DBL lignans.

Due to the antioxidative nature of DBL lignans (Jang *et al.* 2001, Kim *et al.* 2003, Willför *et al.* 2003, Cosentino *et al.* 2010), it is tempting to hypothesize that DBL lignans would mediate the inhibitory effects on RTK signaling by reducing intracellular levels of ROS. Several lines of evidence suggest that low concentrations of ROS, such as hydrogen peroxide, may function as essential second messengers in mitogenic signaling pathways (reviewed by Lander 1997). RTK stimulation by insulin or EGF has been demonstrated to induce hydrogen peroxide production that inhibits protein tyrosine phosphatases and thereby further promotes tyrosine phosphorylation of IRβ and EGFR, respectively (Bae *et al.* 1997, Mahadev *et al.* 2001). Subsequently, inhibition of growth factor-induced positive feedback loops by lignans could lead to the observed inhibition of RTK activity. The NADPH oxidase (Nox) localized at the plasma membrane generates extramitochondrial superoxide and hydrogen peroxide in mitogenic signaling, and the activity of this enzyme has been seen to increase in cancer, including prostate cancer (Kumar *et al.* 2009). Furthermore, physiological levels of androgen increase ROS production in LNCaP cells (Ripple *et al.* 1997), and the androgen-induced ROS levels in prostate epithelial cells play

a key role in prostate cancer occurrence, recurrence as well as progression (Basu *et al.* 2009). Inhibition of Nox activity or use of general antioxidants reduces Akt signaling, proliferation and survival of prostate cancer cells, including the LNCaP cells (Kumar *et al.* 2009). Interestingly, also TRAIL induces ROS production through activation of Nox in a caspase-dependent manner (Choi *et al.* 2010). The effect, however, is opposite to the mechanisms of RTK signaling, as ROS was found to counteract TRAIL-induced apoptosis by inhibiting caspase-3 activation. Sesamin and honokiol are lignans that have been reported to inhibit Nox activity in aortic cells and endothelial cells, respectively (Nakano *et al.* 2008, Sheu *et al.* 2008), and ROS is an important mediator of several signaling pathways that lignans have been shown to suppress, including the PI3K/Akt and MAPK pathways. Thus, it would be worth examining if DBL lignans are able to inhibit production of extramitochondrial ROS in LNCaP cells, and thereby reduce RTK activity and sensitize to TRAIL-induced apoptosis. The location of Nox enzymatic activity at the plasma membrane makes it also available for DBL lignan activity from the outside of the cell.

The observed effects of DBL lignans on growth factor signaling may account for some of the health benefits that mammalian lignans have been attributed with, including reduced risk of diabetes, cardiovascular diseases and cancer (reviewed by Adolphe *et al.* 2010). Whether dietary DBL lignans are a sufficient source for these effects to be detected in human subjects, warrants further studies. In our *in vitro* studies, the cellular effects were observed with DBL lignan doses in micromolar range, and doses that high can be challenging for sufficient drug delivery into the tumor site. The results from these *in vitro* studies are likely to be relevant for intravenous administration protocols in the preclinical uses, but not for nutritional intake of lignans. Our preliminary *in vivo* results in an experimental mouse model demonstrate that NTG is non-toxic at 15 mg/kg daily dose when administered intravenously. The future studies will address, how NTG influences *in vivo* growth of TRAIL resistant tumor xenografts in combination with TRAIL treatment. The stability as well as solubility of plant lignans, like MAT and NTG within the blood may have to be increased by carries systems such as liposomes or other nanoparticulate systems, previously applied for delivery of other polyphenols (Fang *et al.* 2006, Yuan *et al.* 2006). It must, however, be noted that these delivery systems should allow extracellular release of the lignan cargo as the effects observed in this study are likely to be mediated by lignans from outside of the cancer cell plasma membrane. Also the halflife of TRAIL in blood circulation can be increased via conjugation to albumin (Müller *et al.* 2010) or encapsulation to liposomes (Martinez-Lostao *et al.* 2010). Furthermore, TRAIL can be targeted to tumor site for increased local concentration by expression of biologically active soluble TRAIL in a bacterial strain that selectively grows inside tumors (Zhang *et al.* 2010).

3. TARGETING OF HYBRID SILICA NANOPARTICLES INTO CANCER CELLS

3.1 Mesoporous silica nanoparticles can be targeted to folate receptor expressing cancer cells by poly(ethylene imine) and folic acid surface functionalization (III)

Liposomes and polymer nanoparticles have been the leading materials for current nanomedical research, but novel approaches may help to meet the properties of the optimal nanocarrier, namely the carrier biocompatibility, size control, stability and versatile surface functionalization possibilities as well as high drug-loading capacity and controlled drug

release without premature leakage of drug molecules (reviewed by Vivero-Escoto *et al.* 2010). The beneficial features of mesoporous silica include tunable pore dimensions in the nanometer range, high pore volume and surface area, morphology control, and possibility for versatile surface function and controlled release strategies. As a biocompatible material, mesoporous silica has recently been used in development of potential nanocarriers for drug delivery and bioimaging approaches (Vivero-Escoto *et al.* 2010). The preceding work had established the synthesis of poly(ethylene imine), PEI –functionalized porous hybrid silica nanoparticles (Rosenholm *et al.* 2006, Rosenholm & Lindén 2008). In this novel hybrid nanoparticle system, the covalently conjugated hyperbranched PEI layer creates a positive charge at the particle surface preventing particle aggregation, but may also provide stealth properties, molecular gate properties, RES evasion and endosomal escape ability as well as a high surface density of reactive groups for further functionalization (Figure 17).

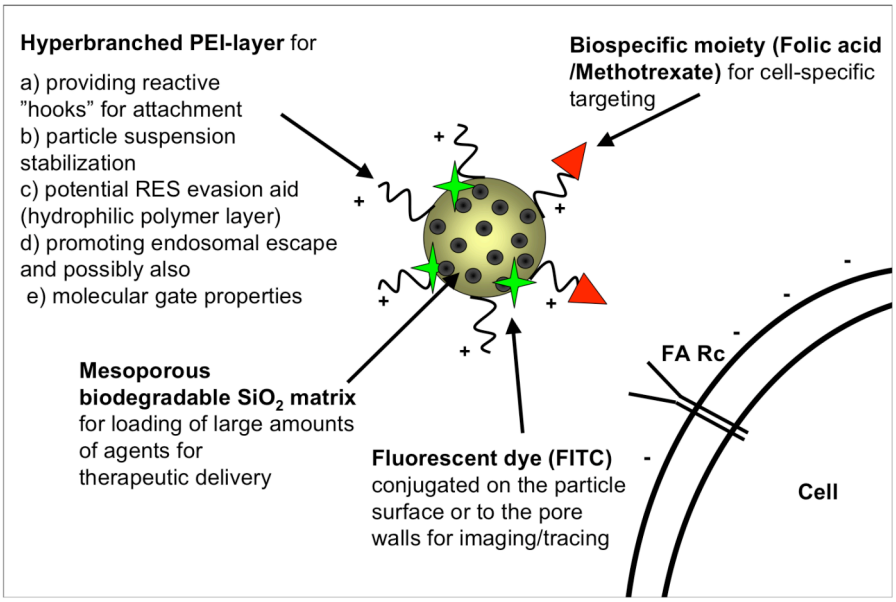


Figure 17. Mesoporous hybrid silica nanoparticles.

We sought to further test the developed system for its applicability to cancer cell targeting under *in vitro* conditions. To this end, the particles were functionalized with FITC molecules for imaging with fluorescence microscopy and analysis by flow cytometry (Figure 17). The mean diameter of the synthesized MSNs in our study was approximately 400 nm, as surface-functionalized particles with smaller size (100 nm) had previously been shown to readily enter cells in the absence of targeting ligands (Slowing *et al.* 2006). Although it has been demonstrated that active targeting provides only a relatively modest improvement in tumor tissue accumulation of drug delivery systems when compared to non-targeted systems, a clear difference is observed in cellular uptake of the nanoparticles and the cytotoxic drug action at the tumor site (reviewed by Pirollo & Chang 2008). Due to this advantage, we introduced a targeting ligand to the MSNs by covalent conjugation of FA to the PEI layer. FA has high affinity to folate receptors (FRs) that are normally expressed at the luminal surface of certain epithelial cells, inaccessible to the blood stream

(Weitman *et al.* 1992). However, approximately one third of human cancers, including ovarian, endometrial, colorectal, breast, lung, renal cell carcinoma, overexpress the FR (reviewed by Sudimack & Lee 2000, Paulos *et al.* 2004). Furthermore, FRs have been observed to undergo internalization and cell surface recycling at a regular rate in cancer cells as well as tumors *in vivo* (Paulos *et al.* 2004), which provides effective means for receptor-mediated endocytosis of an FA-conjugated nanocarrier. Interestingly, FA-targeted liposomes can also enter FR expressing tumor-associated macrophages, a feature that may be utilized in targeted cancer therapy (Turk *et al.* 2004). These things make FA a promising ligand for tumor-targeted drug delivery, which is why both FA-targeted therapeutic drugs and a FA-linked imaging agent are currently under evaluation in human clinical trials for cancer therapy (reviewed by Low & Kularatne 2009).

Our strategy to selectively transport the MSN carriers into cancer cells by FR-targeting would ideally allow delivery of therapeutic agents to the interior of the cancer cells and specific induction of apoptosis in targeted cell population. MSNs have previously been conjugated to FA with the aim of active cancer targeting *in vitro* (Slowing *et al.* 2006, Liong *et al.* 2008), but the particle design in these studies differs significantly from ours concerning the particle coating. The conventional particle functionalization with aminosilanes (Slowing *et al.* 2006) or phosphonate silanes (Liong et al. 2008) does not provide the advantageous properties of PEI described above. To evaluate the cellular internalization of the PEI-functionalized and FA-targeted MSNs, we employed the HeLa cervical carcinoma cells as a cancerous cell model that expresses high amount of FR on the cell surface and human embryonic kidney 293 cells as a FR negative non-cancerous cell type (III, Figure 7). Prior to analysis of the cell samples, extracellular fluorescence was quenched with the cell-impermeable label, Trypan blue, in order to detect solely the MSNs that have been internalized (Slowing *et al.* 2006). Our result show that after 4h incubation with the particles, approximately 20% of HeLa cells internalize the PEI-functionalized MSNs (FITC/PEI), while further conjugation of a FA targeting ligand (FITC/PEI/FA) increases the fraction of particle-containing cells to 40% (III, Figure 4). The cationic PEI layer is likely to enhance MSN interaction with the negatively charged plasma membrane, and thereby contribute to non-targeted particle endocytosis. Xia *et al.* (2009) have recently observed that non-covalent coating with PEI enhances cellular MSN uptake and cargo delivery, but compromises cell viability if the polymer size is increased. In the approach of Slowing *et al.* (2006) the FA-conjugation significantly increased positive charge of the aminosilane-coated MSNs and thereby attraction to plasma membrane, which complicates evaluation of the suggested targeting into HeLa cells.

The uptake efficiency of the FITC/PEI/FA particles by HeLa cells was observed to decrease in a concentration-dependent manner, when free FA was added to the medium (III, Figure 5), suggesting that competition with free FA inhibits specific particle endocytosis. When the effect of free FA on non-targeted uptake of MSNs was recently examined in our laboratories, the higher FA concentrations (>2mM) where found to also influence the unspecific endocytosis of FITC/PEI –particles in HeLa cells (Mamaeva *et al. submitted for publication*). However, the non-targeted uptake remained unaffected at 1mM free FA, while FA-targeted endocytosis is reduced 40–50% (Mamaeva *et al.*). It is important to note that ideally the non-targeted uptake should be examined using particles that are conjugated to a mock/scrambled ligand, as the physicochemical properties of the particle may change when no ligand is present. New means of diminishing the non-targeted MSN uptake, for example by optimization of the FA and PEI density or by including additional targeting-ligands on the MSNs, will be investigated in the future.

Importantly, our data demonstrate that the uptake of FITC/PEI/FA particles is substantially lower in non-cancerous 293 cells that also have very low FR surface expression (III, Figure 8 and Supplementary Figure 6). Taking into account both the fraction of cells that have internalized particles and the amount of particles internalized per cell, the observed total particle internalization in HeLa cells is ten times higher than in 293 cells. Moreover, a significant difference in targeted endocytosis of FA-conjugated MSNs is observed when HeLa cells and 293 cells are co-cultured. In these conditions the FITC/PEI/FA particles preferentially accumulate to HeLa cells (III, Figure 9). Ectopic expression of FR in 293 cells or in other FR-low cell types would elucidate the mechanism other than FR expression that can mediate the selective uptake of the FR-targeted MSNs by cancer cells. The recent results of Mamaeva *et al.* show that there are, indeed, cell type-specific differences that dictate the total level of FITC/PEI/FA uptake independent of the level of FR surface expression. However, when the uptake of FA-conjugated MSNs is compared to internalization of non-targeted MSNs in each cell type, FA-mediated uptake correlates well with the FR surface expression level (Mamaeva *et al.*). For example, while the FA-conjugation increases particle uptake in HeLa cells, 293 cells internalize even less particles when FA is added to the MSNs (Mamaeva *et al.*). These results validate the cancer cell targeting capacity of the developed MSN system and serve as an important basis for the *in vivo* application of the drug carrier system.

3.2 FA-functionalized MSNs can be employed for targeted delivery of hydrophobic cargo molecules into the cytoplasm of folate receptor expressing cancer cells (IV, V)

The mechanisms of nanoparticle internalization are varied and depend greatly on the particle size, shape and surface properties (reviewed by Hillaireau & Couvreur 2009). According to our results, internalization of the FA-functionalized MSNs is sensitive to actin cytoskeleton inhibition as well as cold temperature (IV, Figure 1C), indicating that particle uptake requires active endocytosis mechanisms. The pathway by which FR is internalized may involve several modes of endocytosis with many sorting events in multiple organelles (Paulos *et al.* 2004). Our data do not clarify the existing confusion of the FR recycling mechanisms (Paulos *et al.* 2004), as actin has been suggested to play a role in most, if not all endocytic pathways from caveolin-mediated and clathrin-mediated endocytosis to phagocytosis and macropinocytosis (reviewed by Engqvist-Goldstein & Drubin 2003). However, others have recently proposed that FA-conjugated MSNs may be preferentially endocytosed via the clathrin-dependent pathway (Fisichella *et al.* 2010). After successful entry into target cells, the drug cargo needs to be released from the nanoparticle carrier as well as from the endosomal compartment within the desired timeframe. The ability of the FITC/PEI/FA particles to release their cargo through endosomal escape was evaluated by loading 1wt% non-toxic poorly water-soluble molecules into the MSNs. The fluorescent dyes, DiI and DiO, were used as model cargo and their trafficking in cells was observed by live-cell confocal microscopy. We were able to image the release of DiI from the FITC-labeled MSNs and from the endocytic vesicles into the cytoplasm within 24h, as well as to demonstrate that equal cargo release occurs when DiI and DiO are simultaneously loaded into the particles (IV, Figure 2). Furthermore, quantitative analysis demonstrates that selective model drug delivery can be accomplished to FR-expressing HeLa cells, as the FR-negative 293 cells internalize only one third of the DiO amount delivered to HeLa cells (IV, Figure 3).

Our results indicate that the developed hybrid MSN system may be employed for FR-targeted co-delivery of multiple drug molecules. Lately, Chen *et al.* (2009b) simultaneously delivered Bcl-2 targeting siRNA molecules and the cancer drug doxorubicin to cells by hybrid MSNs, further highlighting the extensive drug-loading capacity of MSNs. The inherently fluorescent doxorubicin was shown to evade the polyamidoamine (PAMAM) –functionalized MSNs and also somewhat from the endosomal compartment, but the MSN-delivered doxorubicin resided at the perinuclear region and for an unknown reason did not accumulate to the nucleus like the free doxorubicin did. The endosomal escape of MSN cargo inside the cells is likely to stem from the proton 'sponge property' of particle coating when dendritic structures, like PEI or PAMAM that promote endosome rupture at lowering pH, are used for surface functionalization (Demeneix *et al.* 2004, Rosenholm *et al.* 2010). Indeed, we observed that within 4h the particles colocalize with an acidotropic probe, LysoTracker, suggesting that the intracellular compartment where the particles resign becomes acidified (V, Figure 7B). Interestingly, we can see that the MSNs remain compartmentalized inside the cell up to 72h (V, Figure 7A). The intracellular localization of non-functionalized MSNs was reported to depend on particle size, as only MSNs ≤420 nm localized in the lysosomal compartment (He et al. 2009). PEI may also function as a 'gate-keeper' and contribute to the fact that the loaded DiI is not released to a neutral pH-buffered solution during 24h incubation (data not shown). Further studies will address how other release mechanisms, such as pH, redox, light, heat or magnet-controlled release systems, could be introduced to the developed drug carrier system (reviewed by Slowing *et al.* 2008). For example, azobenzene derivative -functionalized MSNs have been designed to release the loaded molecules in response to specific light wavelength, while modification of light intensity and excitation time allows graded drug release (Lu *et al.* 2008).

3.3 FA-conjugated MSNs are non-toxic, but cellular uptake of methotrexate-conjugated MSNs induces apoptosis specifically in folate receptor expressing cancer cells (III,IV,V)

Nanoparticle toxicity to living organisms is a critical issue that researchers must carefully manage in order to develop truly biocompatible drug carrier systems (reviewed by Nel *et al.* 2009). Materials such as silica have the advantage of being biodegradable as well as being synthesized by methods that permit careful control over the particle size, morphology, porosity and surface properties, all of which are factors that determine how the particle will interact in a biological system and whether the particle is going to be cytotoxic. It has been demonstrated that mesoporous silica is less cytotoxic than non-porous (amorphous) silica (Lin & Haynes 2010), and thiol- or amino-functionalization, that creates a positive charge on the particle surface, further reduces cytotoxic effects of MSNs *in vitro* (Di Pasqua *et al.* 2008, Tao *et al.* 2009). Importantly, the surface functionalized MSNs have not induced acute toxicity in animal models indicating that the drug carrier system is likely to have clinically relevant implications (reviewed by Rosenholm *et al.* 2010).

According to our results, the PEI- and FA-functionalized hybrid MSN carrier is non-toxic *in vitro*, but only within certain concentrations and time limits. A microscopic and flow cytometric analysis of HeLa cells after 24h incubation with 10µg/ml FITC/PEI/FA particles suggested a normal nuclear morphology (III, Figure 6). However, quantification

of the nuclear integrity after long-term incubation (72h) with 10 µg/ml MSNs shows a detectable increase in apoptotic cell death of HeLa cells (V, Supplementary Figure 2A). The reason for toxicity was found to not be the particle *per se*, but DMSO that is used as a solvent, because particles suspended in HEPES were not toxic in same circumstances. However, DMSO provides better stability of the synthesized particles. To avoid these undesired effects of DMSO dissolved particles, we chose to use a ten times lower particle concentration (1 µg/ml), which was harmless to cells, when long term incubations were required (V, Figure 6). Our results support the previous reports showing that functionalized MSNs are non-toxic when used at low concentrations (Radu *et al.* 2004, Di Pasqua *et al.* 2008, Tao *et al.* 2009). The information concerning the potentially cytotoxic effects of mesoporous silica or PEI is limited, and the only reported data suggest that very high doses (>200µg/ml) of non-functionalized mesoporous silica may induce puncture of cell membranes, generation of reactive oxygen species, mitochondrial dysfunction and caspase activation (Di Pasqua *et al.* 2008, Heikkilä *et al.* 2010), whereas high molecular weight PEI promotes mitochondrial depolarization (Xia *et al.* 2009). However, the doses of silica and PEI used in our experiments, however, are considerably lower.

Our next aim was to investigate if the targeted MSN system could be used for selective induction of apoptosis in FR-expressing cancer cells. To this end, we took advantage of the folate analog, MTX, which is a commonly used cytostatic anticancer drug that inhibits the biosynthesis of nucleotides and proteins (reviewed by Schmiegelow 2009). Currently, the dose-related toxic side effects and occurrence of drug resistance limit the clinical application of MTX in cancer therapy. Linking MTX to macromolecular carrier systems, such as albumin or PAMAM dendrimers, has previously been shown to reduce the side effects, increase the plasma half-life as well as promote the antitumor efficacy of MTX *in vivo* (Wosikowski *et al.* 2003, Kukowska-Latallo *et al.* 2005). Because of the structural similarity between FA and MTX, both are internalized by common cellular uptake mechanisms, which is why we were able to use MTX as an FR-targeting ligand for MSNs in addition to its function as an active drug molecule. In folate free culture conditions, we observed that the cellular endocytosis of MSNs with covalently conjugated MTX is slightly more efficient than the uptake of FA-conjugated MSNs, but still a clear difference in particle endocytosis can be seen between FR-expressing HeLa cells and 293 cells that have low FR expression (V, Figure 4). The total amount of endocytosed MTX-conjugated MSNs was calculated to be ten times higher in HeLa cells than in 293 cells, a difference large enough to affect the delivered drug concentration. Therefore, the developed drug carrier with MTX functioning both as a targeting ligand and as a cytotoxic agent may be used to selectively induce apoptosis of FR-expressing cells.

Our results show that non-cancerous 293 cells and HeLa cancer cells have very similar sensitivity to free MTX, with concentrations of 200ng/ml and upwards clearly inducing apoptotic cell death within 72h in both cell types (V, Figure 2). To demonstrate that utilizing the MTX-conjugated MSNs instead of free MTX increases selectivity of MTX-induced cell death, we incubated 293 and HeLa cells with FA-conjugated control particles or MTX-conjugated particles in a concentration corresponding to 20ng/ml of free MTX. In the free drug form this concentration was found to induce approximately 10–15% of nuclear fragmentation in the treated samples (V, Figure 2), while the corresponding values for particle-attached MTX were 33% in HeLa cells and 7% in 293 cells, the latter being close to level of apoptosis in response to free MTX (V, Figure 6). These data suggest that MTX-conjugation is able to promote selective MSN uptake to FR-expressing cells and subsequently compromise cell survival in the targeted cell population. Administration of

MTX-conjugated MSNs to a co-culture of both cell types would further test the targeting capability of the developed carrier system. It is likely that the free MTX and the particle-conjugated MTX are transported via different endocytic pathways, which may lead to reduced drug-efflux and higher intracellular accumulation of MTX when it is in the particle-attached form. Such mechanisms could mediate the enhanced MTX cytotoxicity that was observed in HeLa cells upon treatment with MTX-functionalized hybrid MSNs. Liong *et al.* (2008) reported cell growth inhibition by FA-functionalized MSNs containing the cancer drug camptothecin adsorbed inside the particle pores. In their study, about 40% of FR-expressing human pancreatic carcinoma cells were viable after 24h incubation, while 70% of the human foreskin fibroblasts, which do not express FR, were viable at the same time point (Liong *et al.* 2008). Also Zhu *et al.* (2009) reported a fairly high cytotoxicity in the non-targeted cells as compared to aptamer-targeted cancer cells (60% vs. 40% viability, respectively) when the cells were treated with doxorubicin loaded MSNs. Both studies show that the cytotoxic action is selectively increased in the FR expressing cell type when the targeting ligand is attached to the MSN carrier system. However, as no comparison to efficacy of the free anticancer agent was included in the studies, it is difficult to judge the therapeutic benefit of the new administration approach.

Interestingly, the covalently attached MTX exerted cytotoxic activity in the cytoplasm, implying that the amide bond between MSN and MTX is broken in the cell interior and that MTX seems to preserve the original structure of the active drug molecule upon detachment. Recently, reduction of the disulfide bond released covalently linked cysteine from thiol-functionalized MSNs when the particles entered the intracellular environment where higher redox-potential prevails (Mortera *et al.* 2009). It is currently unknown, how MTX is released from the endosomal compartment where the fluorescent particles remain up to 72h (V, Figure 7A), but the enzymatic activity of lysosomal proteases may be responsible for the bond cleavage. We were surprised to find that MTX demonstrated potent cytotoxicity in HeLa cells even when it was covalently conjugated to pore walls prior to PEI-functionalization of the MSN surface, implying that efficient release of MTX must take place inside the cells (V, Supplementary Figure 3). In addition to providing a carrier for passive tumor targeting, conjugation of MTX to the hybrid MSNs would allow simultaneous delivery of other cancer drugs loaded inside the mesoporous matrix. This approach could reduce the dose of MTX-conjugated MSNs that is needed for effective apoptosis induction, and subsequently decrease the off-target toxicity in cells that do not overexpress FR.

The *in vivo* biodistribution and elimination of functionalized MSNs is only starting to be understood, and the relevance of active targeting in the *in vivo* application of MSNs is currently being evaluated. Lu *et al.* (2010) recently demonstrated that MSN-based drug delivery systems are biocompatible and eliminated from the body without causing any adverse effects. However, they did not observe any difference in antitumor efficacy of non-targeted and FA-conjugated MSNs loaded with camptothecin, suggesting that passive targeting mechanisms play a major role in the characterized MSN drug delivery system (Lu *et al.* 2010). The physicochemical properties of silica nanoparticles, such as size (Cho *et al.* 2009) and surface modifications (He *et al.* 2008, Park *et al.* 2009b, Souris *et al.* 2010) have a clear impact on the biodistribution and degradation kinetics of the particles *in vivo*. Mamaeva *et al.* have recently tested the *in vivo* tumor targeting capacity of the hybrid MSN system that was characterized *in vitro* during the course of this thesis work. Rapid clearance of the particles by renal excretion was observed and no toxic adverse effects were detected, but more importantly, prolonged tumor accumulation and significantly

enhanced inhibition of tumor growth were seen, when the FA targeting ligand was conjugated to the PEI-functionalized hybrid MSNs loaded with an anticancer agent (Mamaeva *et al.*).

Alternative ligands will be needed for targeted delivery of drugs into tumors that do not overexpress FR. For example, prostate cancer may require other approaches for MSN delivery and androgen receptors could serve as potential receptors for targeting. In addition to binding nuclear ARs, the lipofilic androgen has been shown to bind factors on the plasma membrane (Kampa *et al.* 2002). The membrane ARs are selectively overexpressed in aggressive prostate tumors in comparison to healthy prostate tissue, and engagement of these receptors by albumin-conjugated androgen in LNCaP prostate cancer cells induces endocytosis of the complex as well as cellular apoptosis (reviewed by Papadopoulou *et al.* 2009). Liposomes with androgen as a targeting ligand have previously been used for cancer drug delivery into AR expressing tissues *in vivo* (Mishra *et al.* 2009). As membrane AR activation has also been shown to enhance the anticancer effects of paclitaxel in prostate cancer cells (Papadopoulou *et al.* 2009), loading of membrane AR targeted hybrid MSNs with various cytotoxic agents, including paclitaxel, would allow efficient drug delivery for targeted elimination of prostate cancer cells.

CONCLUDING REMARKS

Cancer-specific cell surface receptors may serve as signposts for drug delivery vehicles, mediating selective uptake of nanometer scale particles that contain therapeutic agents. Other types of receptors, called death receptors, have the ability to induce intracellular signaling cascades leading to apoptotic cell death in a cancer cell-specific manner. In this thesis project, I set out to investigate methods of targeted cancer cell elimination with minimal toxicity to non-cancerous cells.

Active targeting of cancer drugs to tumor cells is currently explored as means to reduce the adverse effects of chemotherapy, to increase the therapeutic efficacy of drugs and to enable the use of cancer drugs that previously have been too toxic or too insoluble for systemic administration. This thesis work aimed at evaluating the cancer cell targeting capacity of a novel drug carrier system consisting of mesoporous silica nanoparticles with hyperbranched poly(ethylene imine) coating. The tested MSNs were functionalized with folic acid to enable their targeted internalization by folate receptor overexpressing cancer cells. The results presented in this thesis demonstrate that the developed carrier system can be employed *in vitro* for cancer cell-specific delivery of adsorbed or covalently conjugated molecules and furthermore, for selective induction of apoptotic cell death in folate receptor expressing cancer cells. Due to the high drug-loading capacity of the hybrid MSNs, several anticancer agents may be incorporated to this system in the future, as combinatorial chemotherapy is likely to be required for successful cancer treatment. The biocompatibility and *in vivo* tumor targeting ability of the folic acid -functionalized hybrid MSNs have recently been studied with promising results, and therefore, this drug carrier system has good potential for clinical application in cancer therapy. The challenges of nanotechnology remain especially in finding new and more specific cancer-targeting ligands and designing nanocarriers that optimally deliver their cargo and become then completely eliminated from the body. Also reaching the targeted cell populations that may resign in poorly inaccessible locations, such as the brain or weakly vascularized tumors, needs to be addressed in future studies.

During this thesis work, I have also investigated the anticancer effects that lignans have been reported to induce. Earlier studies have examined the influence of lignans on cell signaling, and shown that lignans can downregulate the activity of various signaling pathways. These studies, however, have been non-systematic in the sense that they only investigated a few lignan compounds at a time. The survival mechanisms of cancer cells, often involving deregulated receptor tyrosine kinase activity, are critical for the malignant cell behavior, whereas inhibition of these signaling pathways provides new opportunities for cancer cell elimination. The findings of this thesis present a novel mechanism of action for lignans, namely sensitization to death receptor-mediated apoptosis, and provide further support for lignans as potent inhibitors of cell survival signaling. Furthermore, increased cross talk between death receptor-mediated signaling and mitochondrial apoptosis pathway was observed upon lignan pretreatment, while lignans alone were non-toxic. Our structure-activity relationship analysis of lignans demonstrated that only dibenzylbutyrolactone lignans are capable of increasing susceptibility of prostate cancer-cells to induction of apoptosis by the tumor-targeting death ligand TRAIL.

Of the tested lignans, NTG was identified as the most efficient inhibitor of Akt survival signaling as well as sensitizer to TRAIL-induced apoptosis. This interesting molecule is not found in human diet, but can be extracted from knots of coniferous trees. Dietary lignans or lignans used as chemotherapeutic agents could be employed for priming cancer cells to therapeutic TRAIL receptor agonists, which provides interesting possibilities for cancer treatment. The observations of efficient and rapidly reversible TRAIL-sensitizing activity in androgen-dependent prostate cancer cells, but not in non-malignant prostate cells, encourage to employ NTG in *in vivo* studies together with TRAIL treatment in a mouse model for TRAIL-resistant prostate cancer. Furthermore, potential cancer preventive effects should be examined closer as dibenzylbutyrolactone lignans may increase efficacy of the endogenous TRAIL-mediated immunosurveillance against cancer types that rely on deregulated growth factor signaling for their survival. In fact, it is possible that the combined anticancer effects of plant-derived factors, including lignans and other polyphenols, in human diet can have a significant impact on the life-time risk of cancer.

Taken together, the results of this thesis describe two novel approaches for targeted cancer cell elimination, the first taking advantage of the receptor-mediated endocytosis of multifunctional nanocarriers and the second employing lignans for sensitization to death receptor-mediated apoptosis. Due to acquisition of therapeutic resistance towards single-agent cancer treatments, development of effective and safe treatment protocols for multi-drug cancer therapy is at the focus of cancer research. Recurrence of cancer is currently thought to originate from the multi-drug resistant tumor stem cell population that has capacity for self-renewal and tumor regeneration. Application of the approaches presented in this thesis to targeted elimination of cancer stem cells can provide new and interesting lines of research.

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