Univerzita Karlova v Praze

Přírodovědecká fakulta

Studijní program: Biochemie

Studijní obor: Biochemie

Sandra Lettlová

Úloha Mst1/FoxO dráhy při indukci apoptózy

Role of the Mst1/FoxO Pathway in Apoptosis Induction

Bakalářská práce

Vedoucí závěrečné práce: Prof. RNDr. Karel Bezouška, DSc.

Školitel: Prof. Ing. Jiří Neužil, CSc.

Praha, 2011

Prohlášení:

Prohlašuji, že jsem závěrečnou práci zpracovala samostatně a že jsem uvedla všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

V Praze,

.....

Podpis

Abstract

Vitamin E analogue α-tocopheryl succinate (α-TOS) from the group of mitocans, the drugs targeting mitochondria, is a selective inducer of apoptosis in various cancer cell types, which involves the accumulation of reactive oxygen species (ROS). It was found that ROS generation causes p53-independent upregulation of the pro-apoptotic protein Noxa that induces apoptosis by displacement of the BH3-only protein Bak from its inactive complex with the anti-apoptotic protein Mcl-1 to form a pore in outer mitochondrial membrane. Current research has demonstrated that generation of ROS causes activation of Mst1, a component of the Hippo pathway that presents a universal size-control mechanism in all metazoans, whose deregulation is linked to tumorigenesis. Treatment of Jurkat cells with α-TOS revealed that activated Mst1 kinase phosphorylates the Forkhead box O1 (FoxO1) transcription factor that then translocates to the nucleus and activates transcription of genes important for apoptosis induction, including *NOXA*. This explains the p53 independent apoptosis induction and presents Mst1-FoxO1-Noxa as a new pathway involved in the process. Current research has also documented that activated Mst1 kinase controls the expression of the *c-MYC* oncogene and its target genes, whose products are involved in glucose and glutamine metabolism. These findings suggest that Mst1 kinase, in response to α-TOS, is responsible for activation of several pathways leading to apoptosis induction in cancer cells and their restriction in the provision of nutrients, whereby impacting on inhibition of cancer development. (In English)

Key words:

Apoptosis, Mst1, FoxO, mitochondria, neoplastic diseases, vitamin E analogues

Abstrakt

Bylo prokázáno, že analog vitaminu E, α-tokoferyl sukcinát (α-TOS), jenž patří do skupiny mitokanů, která se vyznačuje tím, že účinkuje prostřednictvím mitochondrií, selektivně indukuje apoptózu v různých typech nádorových buněk pomocí generace reaktivních forem kyslíku (ROS). Akumulace ROS způsobuje nezávisle na přítomnosti proteinu p53 expresi pro-apoptotického proteinu Noxa. Zvýšená hladina Noxa proteinu vytěsní „BH3-only" protein Bak z inaktivního komplexu s anti-apoptotickým proteinem Mcl-1, iniciuje tak tvorbu kanálů ve vnější mitochondriální membráně a tím indukci apoptózy. Současné výsledky demonstrují, že generace ROS způsobuje aktivaci Mst1 kinázy, která je součástí signalizační dráhy Hippo. Tato dráha představuje mechanismus zodpovědný za správný vývoj a velikost orgánů u mnohobuněčných organismů a její deregulace je spojena s vývojem nádorového onemocnění. Působením látky α-TOS na buňky linie Jurkat bylo prokázáno, že aktivovaná Mst1 kináza fosforyluje transkripční faktor Forkhead box O1 (FoxO1), který se poté přemístí do jádra, kde aktivuje transkripci pro-apoptotických genů mezi nimiž je i *NOXA*. To vysvětluje indukci apoptózy nezávisle na p53 a představuje Mst1-FoxO1-Noxa dráhu jako nový mechanismus indukující apoptózu. Současný výzkum rovněž ukazuje, že Mst1 kináza ovlivňuje expresi onkogenu *c-MYC* a genů podílejících se metabolismu glukózy a glutaminu, jejichž expresi c-Myc jako transkripční faktor moduluje. Tyto nálezy naznačují, že Mst1 kináza v reakci na léčbu látkou α-TOS aktivuje několik drah vedoucích k indukci apoptózy a restrikci příjmu energie u rakovinných buněk a tím k inhibici nádorového onemocnění. (In English)

Klíčová slova:

Apoptóza, Mst1, FoxO, mitochondrie, nádorová onemocnění, analogy vitaminu E

Contents

List of abbreviations

AIF	apoptosis-inducing factor
ANT	adenine nucleotide transporter
Apaf -1	apoptosis-protease-activating factor-1
Bcl	B cell lymphoma
BH	Bcl-2 homology
BTG-1	B-cell translocation gene
CAD	caspase-activated DNase
CARD	caspase recruitment domain
CI	complex I
cIAP	cellular IAP
Crb	Crumbs
cyt c	cytochrome c
DBE	Daf-16 family member-binding element
DDB1	damage-specific DNA-binding protein 1
DED	death effector domain
DISC	death inducing signalling complex
DR	death receptor
Ds	Dachsous
Endo G	endonuclease G
Ex	Expanded
FADD	Fas-associated death domain
FasL	Fas ligand
FasR	Fas receptor
Fj	Four-jointed
FKH	forkhead
FoxO	forkhead box O
Ft	Fat
G6Pase	glucose-6-phosphatase
GADD45	growth arrest and DNA damage-inducible protein-45
HK	hexokinase
Hpo	Hippo

HSP	heat-shock protein
Hth	Homothorax
IAP	inhibitor of apoptosis protein
ICAD	inhibitor of caspase-activated DNase
IGF-1	insulin-like growth factor-1
IMM	inner mitochondrial membrane
Mer	Merlin
MitoQ	mitochondrially targeted coenzyme Q
MitoVES	mitochondrially targeted vitamin E succinate
MnSOD	manganese superoxide dismutase
MOMP	mitochondrial outer membrane permeabilisation
Mst	mammalian sterile 20-like
mtDNA	mitochondrial DNA
NF	nuclear factor
OMM	outer mitochondrial membrane
PDK1	3-phosphoinositol-dependent protein kinase-1
PEPCK	phosphoenolpyruvate carboxykinase
PI3K	phosphatidylinositol 3-kinase
PIP_3	phosphatidilinositol 3,4,5-triphosphate
PP2A	phosphoprotein phosphatase-2A
PPARγ	peroxisome proliferator-activated receptor γ
Q_D	distal UbQ-binding site
Q_P	proximal UbQ-binding site
RB	retinoblastoma
RIP1	receptor-interacting protein 1
ROS	reactive oxygen species
Sav	Salvador
Sd	Scaloped
SDH	succinate dehydrogenase
SMase	sphingomyelinase
SOD	superoxide dismutase
SODD	silencer of death domain
tBid	truncated Bid

TM	trans-membrane
TNF	tumour necrosis factor
TNFR1	TNF receptor-1
TRADD	TNFR-associated death domain
TRAF2	TNF receptor-associated factor-2
TRAIL	TNF-related apoptosis-inducing ligand
TTF-1	thyroid transcription factor-1
UbQ	ubiquinone
VDAC	voltage-dependent anion channel
VE	vitamin E
Wts	Warts
XIAP	X-chromosome-linked IAP
YAP	Yes-associated protein
Yki	Yorkie
$\Delta\Psi_m$	mitochondrial inner trans-membrane potential
α-TOH	α-tocopherol
α-TOS	α-tocopheryl succinate
ρ_0	mitochondrial DNA-deficient cells

1. Introduction

Cancer is the term for a group of diseases whose common feature is the lapse of normal cell behaviour, resulting in uncontrolled cell growth and spread out of abnormal cells within the organism that ultimately causes death. The number of people diagnosed with cancer is on the rise due to a variety of reasons, including the lifestyle and the environment. The lack of exercise, smoking, unhealthy diet, obesity, stress, environmental pollutants, toxins, radiation and many other factors are involved in cancer induction and progression. According to the American Cancer Society (2010) one of two men and one of three women are likely to be diagnosed with cancer during their lifetime (American Cancer Society, 2010). Although cancer used to be diagnosed primarily in the aging society, today it occurs increasingly also in young people. Cancer is exceedingly hard to treat and is highly "unpredictable" because of its ability to undergo a multitude of various mutations that can result in the increase of its aggressiveness and resistance to current anti-cancer treatments. Naturally, neoplastic diseases present a huge socio-economic burden. All these features make cancer research a focus of many laboratories around the world.

1.1. Basic features of cancer cells

The transformation of normal cells into cancer cells is a multistep process, which includes many genetic alterations. These changes are presented by irreversible mutations in the DNA. The cells have several protective mechanisms to detect such changes and repair them. In case they cannot be repaired, the cells usually undergo apoptosis. However, when the cells do not die, the mutations can lead to cancer development. For cancer genesis, the changes in genetic information that are related to alterations in cell cycle regulation, cell proliferation and cell differentiation are of ultimate importance. The key genes, whose alterations can result in carcinogenesis are divided into two groups: oncogenes and tumour suppressor genes. Oncogenes are genes that promote cell growth and reproduction. Tumour suppressor genes are genes that inhibit cell growth and promote the induction of apoptosis (Weinberg, 1994). These genetic alterations contribute to the acquisition of properties that are important for cancer development. The main features of cancer cells are:

1. Sustaining proliferative signalling – cancer cells deregulate their growth and cell cycle to ensure ongoing ability to divide. They do so, for example, by enhancing the level of

growth factor receptors or by mutations of the downstream components of these receptor-dependent signalling pathways, which leads to the activation of transcription factors that regulate the expression of genes needed for cell division. Among the most frequently mutated genes are those encoding growth factors, hormone and growth factor receptors, tyrosine kinases, serin/threonin kinases, G-proteins and transcription factors.

2. Evading growth suppressors - in addition to the ability to sustain proliferation, cancer cells need to circumvent the mechanisms that negatively regulate cell growth. These are usually under the control of tumour suppressor genes such as *p53* and retinoblastoma (*RB*). Unlimited capacity to divide also requires overcoming the mechanism of contact inhibition.
3. Resisting cell death - upon irreversible damage that could lead to cancer development, normal cells are able to avoid this by committing apoptosis. Cancer cells possess various mechanisms how to circumvent apoptosis. These include the loss of the function of *p53* that induces apoptosis by activation of expression of pro-apoptotic genes or upregulation of anti-apoptotic genes.
4. Enabling replicative immortality - normal cells are able to undergo only a limited number of cell divisions, which is connected with senescence and irreversible entry of the cells into the non-proliferative state. Cancer cells exert the ability to overcome this barrier and undergo unlimited number of divisions. This is connected to increased expression of telomerase, found in almost 90 % of malignant, immortalized cells.
5. Inducing angiogenesis - cancer cells, similar to normal cells, need to be supplied with nutrients and oxygen and they also need to remove the ‘waste material’ and carbon dioxide. They are able to induce the growth of new blood vessels to promote tumour growth.
6. Activating invasion and metastasis - this is a multistep process, in which cancer cells acquire the ability to penetrate and infiltrate surrounding tissues or the walls of lymphatic or blood vessels and form a new (secondary) tumour in other site in the patient. The process of the formation of secondary tumours is called metastasis.
7. Reprogramming of energy metabolism - in order to fuel the energy requirements of proliferation, cancer cells switch their energy formation (generation of ATP) from oxidative phosphorylation to generation of ATP via glycolysis starting from glucose, even in the presence of oxygen. This process is called the Warburg effect.

8. Evading immune destruction - cancer cells also exhibit the ability to circumvent the immune system detection or, at least to limit the extent of their killing by cells of the immune system (Hanahan and Weinberg, 2011).

Cancer cells undergo numerous alterations that contribute to their specific phenotype. Each key feature of cancer cells seems to be regulated in a robust manner, which means that the specifically targeted anti-cancer treatment that can inhibit one of the core features of cancer cells not necessarily inhibits the various signalling pathways. It can result in activation of different mechanisms that function in a similar way. Variability of genomic mutations gives rise to resistance of cancer cells to current specifically targeted anti-cancer treatments. According to the comprehensive genomic analysis performed in 24 samples of human pancreatic cancers and 22 samples of human glioblastoma multiformae biopsies, many mutations differ in the same type of cancer (Jones et al., 2008; Parsons et al., 2008). These results point out to the importance to develop drugs that specifically target particular, invariant components of various altered signalling pathways. Using this notion, agents may be developed that would effectively inhibit cancer development and destroy cancer cells across the landscape of different cancer types.

1.2. Motivation of this bachelor thesis

The vitamin E (VE) analogue α-tocopheryl succinate (α-TOS) has been documented as an efficient anti-cancer drug that selectively induces apoptosis in various types of cancer cells (Neuzil et al., 2001). It was shown that this agent, acting by targeting mitochondria, is responsible for production of reactive oxygen species (ROS) that result in p53-independent expression of the pro-apoptotic protein Noxa and in apoptosis induction (Prochazka et al., 2010). The current studies have suggested that these upstream events involve activation of the Hippo signalling pathway, playing a role in control mechanism in organ size, which presents a new signalling pathway that controls apoptosis in cancer cells exposed to anti-cancer drugs (Valis et al., 2011). In this thesis, I would like to summarize the current knowledge of mechanisms by which α-TOS activates signalling pathways that induce apoptosis in cancer cells with the main focus on Hippo signalling.

2. Apoptosis

Programmed cell death or apoptosis is an evolutionary conserved mechanism of self-destruction of cells and has an indispensable role in the physiological processes of multicellular organisms. It is involved in normal animal development as well as in certain pathophysiological processes that may result in disease development. Therefore, the balance between cell survival and apoptosis has to be tightly regulated. Both the lack in apoptosis and too much apoptosis may have dangerous consequences.

The development of all metazoans is characterized by over-production of cells. The excess cells must be removed to ensure the right size, structure, and function of the parts of the body. For example, it was found that in the most frequently studied nematode *Caenorhabditis elegans* precisely 131 of its 1090 somatic cells generated during its development undergo apoptotic cell death. Apoptosis is also responsible for proper separation of fingers on the hands and feet of vertebrates. These are initially connected by webbing which is eliminated by this process. The tail of tadpoles is disposed of by apoptosis as well as the larval tissues of insects (Voet, 2004; Alberts et al., 2008). During the mammalian nervous system development, only neurons with proper tissue connections receive adequate survival factors, while more than 50% of them die by apoptosis (Mazarakis et al., 1997).

Apoptosis is an essential control mechanism whereby organisms eliminate abnormal, misplaced, malfunctional or any potentially dangerous cells. Apoptosis also plays a crucial role in the immune system development and function. It eliminates lymphocytes lacking appropriate receptors. Death of lymphocytes also regulates the extension and duration of the immune responses to infection (Marsden and Strasser, 2003). Upon infection with viral particles, cells undergo apoptosis to avoid viral replication. Cells with irreparably damaged DNA undergo apoptosis to protect the organism from pathological states, such as cancer.

The human body produces “zillions” of new cells by mitosis and similar amount of cells die every day by apoptosis to maintain homeostasis. Apoptosis is thus a part of normal physiology and is required that no harm is presented to neighbouring cells, which would occur upon necrosis. Necrosis is disorganized cell death, which is often caused by acute injury, such as abrupt anoxia, sudden depletion of energy (ATP), extremes of temperatures, presence of detergents, mechanical injuries etc. Necrotic cells are

characterized by cellular swelling resulting in plasma membrane rupture. Individual organelles also swell and burst, and the cytoplasmic content is then released into the surrounding tissue, causing extensive inflammatory responses that are deleterious to the surrounding cells (Denecker et al., 2001). In contrast to necrosis, apoptosis is a way of cell death, by which organisms remove cells without harming the surrounding tissues. The most characteristic features of apoptosis are chromatin condensation and nuclear fragmentation. The double-stranded DNA is cleaved by caspase-activated enzymes into fragments with the length of multiplies of ~180-200 bp. Apoptotic cell loses its contact with neighbouring cells, and plasma membrane shrinks and starts to bleb. The blebs are finally separated from the original cell to form apoptotic bodies. These contain the intracellular content enclosed by plasma membrane and are subsequently phagocytosed by surrounding cells, in particular macrophages. Macrophages recognize apoptotic cells from living cells by means of phosphatidylserine, which is presented on the apoptotic cells providing the so-called "eat-me" signals. This process in not accompanied by any inflammatory response, because the intracellular content is not released into the surrounding tissue and because apoptotic cells are rapidly phagocytosed and macrophages lower the production of pro-inflammatory cytokines (Ziegler and Groscurth 2004; Kurosaka et al., 2003).

2.1. Mechanism of apoptosis

The mechanism of apoptosis is a tightly regulated and sophisticated sequence of reactions that leads to the activation of the caspase cascade. Caspases (cysteine aspartate-specific proteases) constitute a family of proteases that cleave target proteins after aspartate using cysteine residues as the catalytic nucleophile. Ten major caspases have been identified in mammals. These have been divided into groups according to their functions as initiator caspases (caspase-2, -8, -9 and -10), effector caspases (caspase-3, -6 and -7), and inflammatory caspases (caspase-1, -4, and -5) (Lamkanfi et al., 2002; Ashe and Berry 2003). Caspases are expressed in organisms as inactive enzyme precursors referred to as pro-caspases. Pro-caspases are single-chain proteins consisting of two domains. The N-terminal domain is called pro-domain and is proteolytically removed during activation. Pro-domains have different lengths and contain different domains depending on the caspase function. The initiator and inflammatory caspases carry long pro-domains

(~100 residues), while effector caspases feature short pro-domains (~30 residues). Initiator caspases (caspase-8 and -10) contain in their pro-domains the death effector domains (DEDs). The other initiator caspases (caspase-2 and -9) and inflammatory caspases (caspase-1, -4 and -5) contain caspase recruitment domains (CARDs). The second catalytic domain consists of the large (α) and small (β) subunit. Caspase activation requires cleavage of the catalytic domain into small and large subunits. These immediately interact with each other and form a dimer with two other large and small subunits and the active caspase is a dimer with αββ'α' symmetry containing two active sites.

Initiator caspases are activated by dimerisation following auto-catalytic cleavage facilitated by their enhanced concentration and proximity at the adaptor proteins such as the Fas-associated death domain (FADD) or the apoptosis-protease activating factor-1 (Apaf-1). Pro-caspases bind to these adaptor proteins by their DEDs or CARDs. Effector caspases dimerise shortly after their synthesis and are activated by cleavage of their domain linkers mediated by initiator caspases (Ashe and Berry, 2003; Pop and Salvesen 2009). Once effector caspases are activated the cells crosses the so-called "point of no return" because the cells can no longer be rescued and are committed to die. The reason is that effector caspases cleave many proteins that constitute the so-called death substrate, which results in cell death. Among them are many major proteins of the cytoskeleton (actin, myosin, tubulins, dynein, keratins etc.), proteins forming the nuclear membrane (lamin), proteins participating in cell-cell and cell-extracellular matrix interactions, as well as proteins with a function in transcription and translation and ribosomal RNAs. Another protein that becomes activated by effector caspases is caspase-activated DNase (CAD) which is in healthy cells complexed with the inhibitor of CAD (ICAD). During apoptosis ICAD is cleaved by caspases to liberate CAD that causes DNA fragmentation (Taylor et al., 2008).

There are two main pathways leading to activation of caspases: the extrinsic pathway and the intrinsic pathway. Both pathways are connected and can affect each other.

2.2. Extrinsic pathway of apoptosis

The extrinsic apoptotic pathway participates mainly in eliminating excess cells during development and during immune system evolvement. These pathways (Fig. 1, page 16) involve trans-membrane receptors, which belong to the tumour necrosis factor (TNF)

receptor super-family whose members share similar cysteine-rich extracellular domains responsible for ligand binding and intracellular death domains of ~80 amino acids transducing the initial apoptotic signal down-stream. Members of the TNF receptor family trigger apoptosis upon ligand binding. Among the best characterized death receptors and their ligands are the Fas receptor (FasR) also called Apo-1 or CD95 with its ligand (FasL) also called CD95L, TNF receptor-1 (TNFR1) with its ligand TNF-α, as well as death receptor-4 (DR4) and DR5 with their ligand TNF-related apoptosis-inducing ligand (TRAIL) also called Apo2L (Ashkenazi and Dixit, 1998).

FasL is a homotrimeric protein which binds to three molecules of FasR, whose cytosolic death domains then trimerise. This recruits the adaptor protein FADD to the trimeric FasR via its N-terminal DED, which is followed by association with three molecules of pro-caspase-8 via their own DEDs. The complex including FasR, FasL, FADD and pro-caspase-8 is referred to as the death-inducing signalling complex (DISC). Formation of DISC induces autocatalytic cleavage and activation of caspase-8 and -10. In the type I cells, the DISC formation is robust enough to promote cleavage of effector caspases directly by caspase-8. On the other hand, type II cells form low level of DISC and active caspase-8, which is followed by activation of the intrinsic pathway to amplify the incoming weak signal. This involves cleavage of B cell lymphoma 2 (Bcl-2) family Bcl-2 homology (BH) 3-only protein Bid by caspase-8. This truncated Bid (tBid) then causes mitochondria-mediated activation of caspase-9 (see below) (Curtin and Cotter, 2003).

TRAIL induces apoptosis by mechanism similar to that of FasL. The ligand can bind two receptors, DR4 and DR5, which both contain cytoplasmatic death domains. Upon TRAIL binding these receptors trimerise and bind FADD and pro-caspases-8 to form DISC and to activate caspase-8 (Almasan and Ashkenazi, 2003).

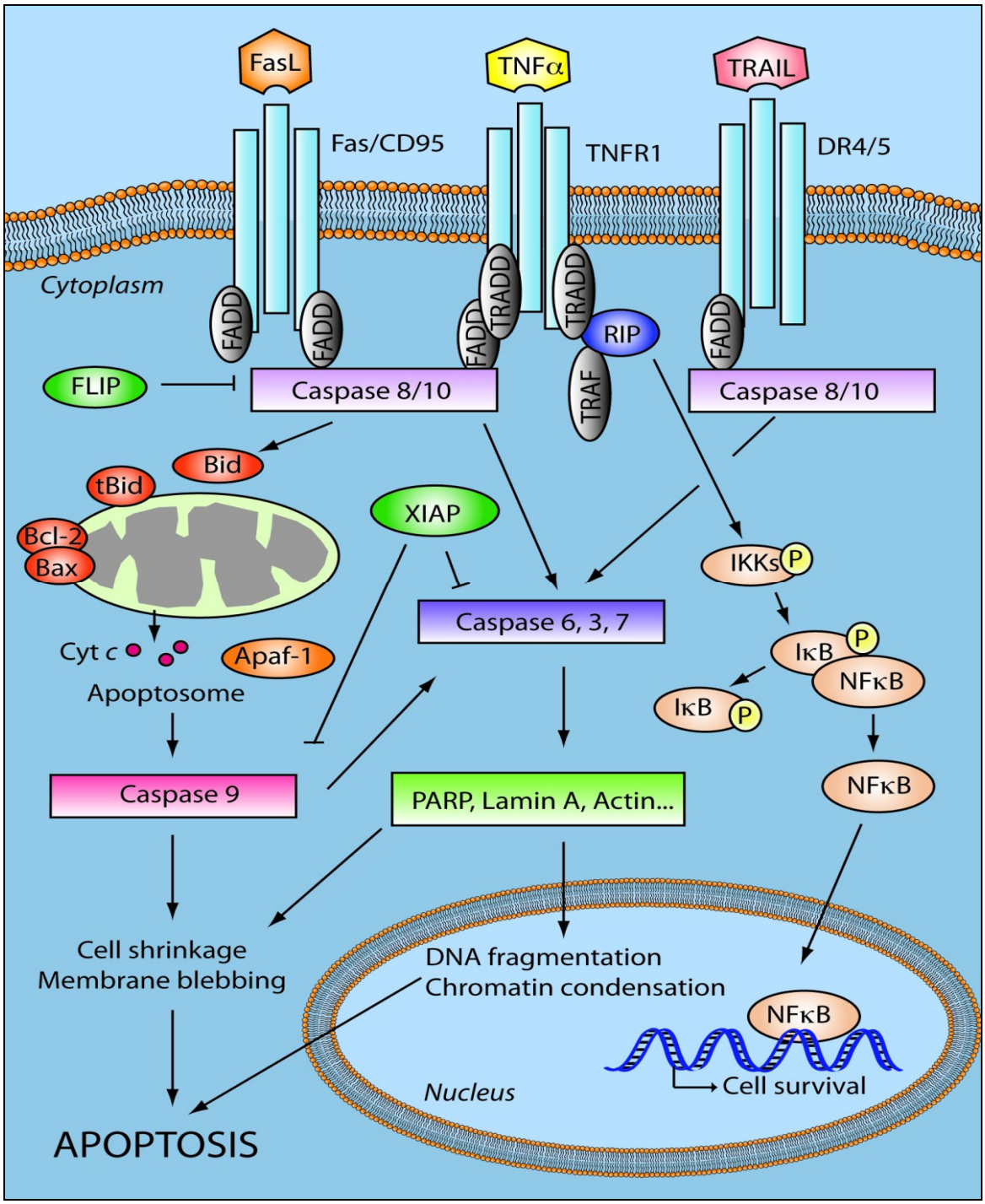

Figure 1. Induction of extrinsic apoptotic pathways through FasL, TNF-α and TRAIL. Schematic view of apoptotic signal transduction involving also the intrinsic apoptotic pathway and the inhibitors of apoptosis FLIP and XIAP, and activation of NF-κB (adapted from Krakstad and Chekenya, 2010).

TNF-α induces apoptosis by binding to TNFR1. TNFR1 initially binds the silencer of death domain (SODD), which inhibits TNFR1-mediated apoptotic signalling. Persistent

interaction between TNFR1 and TNF causes dissociation of SODD, trimerisation of TNFR1 and recruitment of the TNFR-associated death domain (TRADD). TRADD binds to the trimerised intercellular death domain of TNFR1 by its own death domain, and presents a platform for recruitment of other proteins participating in the TNF-α apoptotic signalling, including the receptor-interacting protein 1 (RIP1), FADD and the TNF receptor-associated factor-2 (TRAF2). Interaction of TRADD with FADD induces activation of caspase-8 and apoptotic death similar to that mediated by FasL. RIP1 association can activate caspase-2, which in type II cells can induce tBid-mediated apoptosis. However, RIP1 is also involved in nuclear factor-κB (NF-κB) activation including interaction with TRAF2 (Bixby et al., 2005). The activation of NF-κB results in induction of expression of a number of genes producing anti-apoptotic proteins like the inhibitor of apoptosis prteins (IAPs), cFLIP, TRAF1 and TRAF2, as well as the Bcl-2 family proteins (Bfl1/A-1, Bcl-x_L and Bcl-2). Cellular IAPs (cIAPs) inhibit caspases by direct binding to caspase-3 and -7 as well as by preventing activation of pro-caspase-8 and -9. The best characterised IAP member is the X chromosome-linked IAP (XIAP), which inhibits caspase-3, -7 and -9. The inhibitory protein cFLIP suppresses activation of caspase-8 by competition for binding sites in DISC (Lin and Karin, 2003).

2.3. Intrinsic pathway of apoptosis

The intrinsic apoptotic pathway is activated in response to a plethora of stress stimuli such as DNA damage, chemotherapeutic drugs, ionising radiation, oxidative stress, viral or bacterial infections, oncogene activation, intrinsic organelle destabilisation, etc. (Boatright and Salvesen, 2003). The pivotal event leading to caspase activation is the release of cytochrome c (cyt c) from the mitochondrial inter-membrane space to the cytosol. Cyt c has an important role in the respiratory chain as an electron shuttle between complex III (CIII) and CIV in the inner mitochondrial membrane (IMM), but it is also a fundamental molecule in mitochondria-mediated cell death (Voet, 2004). In the cytosol, cyt c forms a complex called apoptosome by association with the adaptor protein Apaf-1 and pro-caspase-9. Apaf-1 is composed of three functional domains: N-terminal CARD, central nucleotide-binding and oligomerisation domain, and the C-terminal domain containing multiple WD-40 repeats. Upon cyt c binding to the C-terminal domain, Apaf-1 undergoes a conformational change that allows its activation and, in the presence of ATP/dATP,

Apaf-1/cyt c oligomerise into a heptameric complex (Fig. 2). This structure exposes seven N-terminal CARDs that bind pro-caspases-9 via their own CARDs. The increased concentration of pro-caspase-9 induces its auto-catalytic cleavage, dimerisation and their activation (Schafer and Kombluth, 2006).

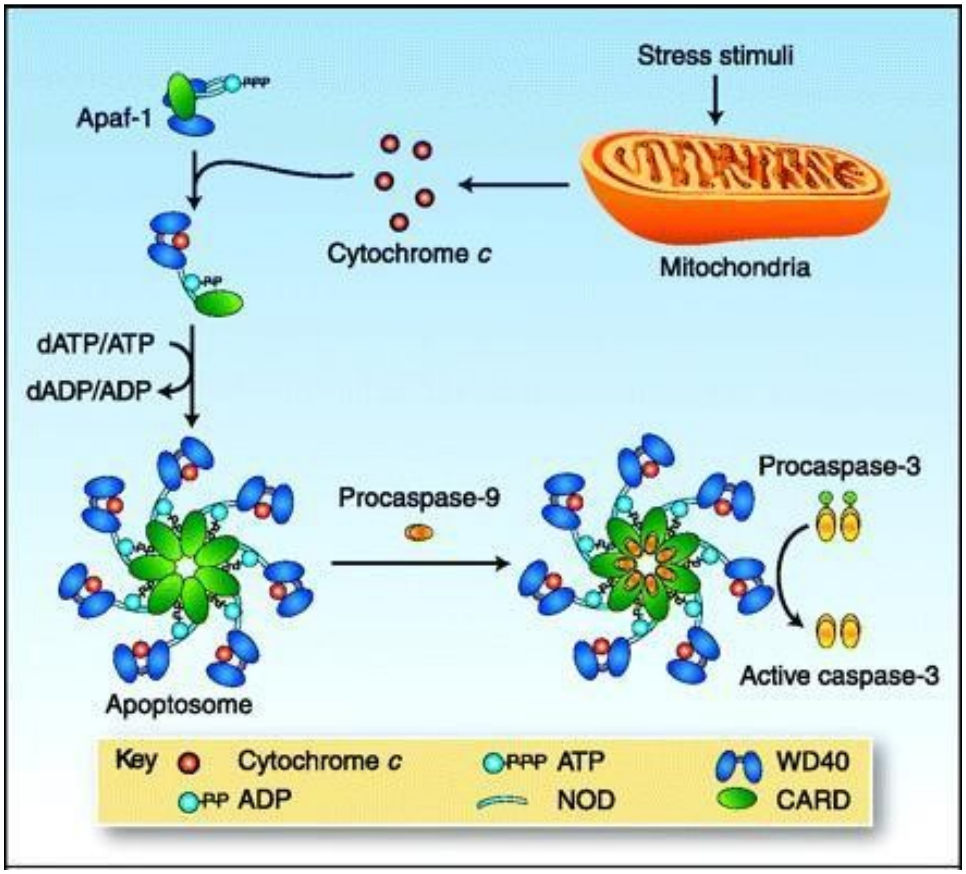

Figure 2. Cyt c released from mitochondria induces apoptosome formation and caspase-9 activation (adapted from Ledgerwood and Morison, 2009)

Together with cyt c, other proteins such as Smac/DIABLO, the flavoprotein apoptosis-inducing factor (AIF) and endonuclease G (Endo G) are released from the mitochondrial inter-membrane space to the cytosol. Smac/DIABLO interacts with IAPs and thus inactivates their function as caspase inhibitors. AIF and Endo G can induce apoptosis in caspase independent manner by direct promotion of DNA fragmentation (Garrido et al., 2006).

The release of cyt c from the inter-membrane space into the cytosol requires the mitochondrial outer membrane permeabilisation (MOMP), which is regulated by the Bcl-2 family proteins. Members of this family contain at least one BH domain, and are divided into three sub-groups according to their function and structure as anti-apoptotic and

pro-apoptotic proteins, and BH3-only proteins (Fig. 3). The anti-apoptotic proteins include Bcl-2, Bcl-x_L, Bcl-w, Mcl-1, and A-1, and they function as inhibitors of apoptosis. All of them share four BH domains and most of them have also trans-membrane domain (TM) through they are integrated within the OMM. The pro-apoptotic proteins Bak, Bax and Bok are characterised by the presence of three BH domains and also TM. Bak and Bax can homo-oligomerise and form pores within OMM. The BH3-only members are Bim, Bad, Bid, Puma and Noxa. They contain only the BH3 motif and some of them also TM, and are responsible for signal transduction to other Bcl-2 proteins (Chipuk et al., 2010).

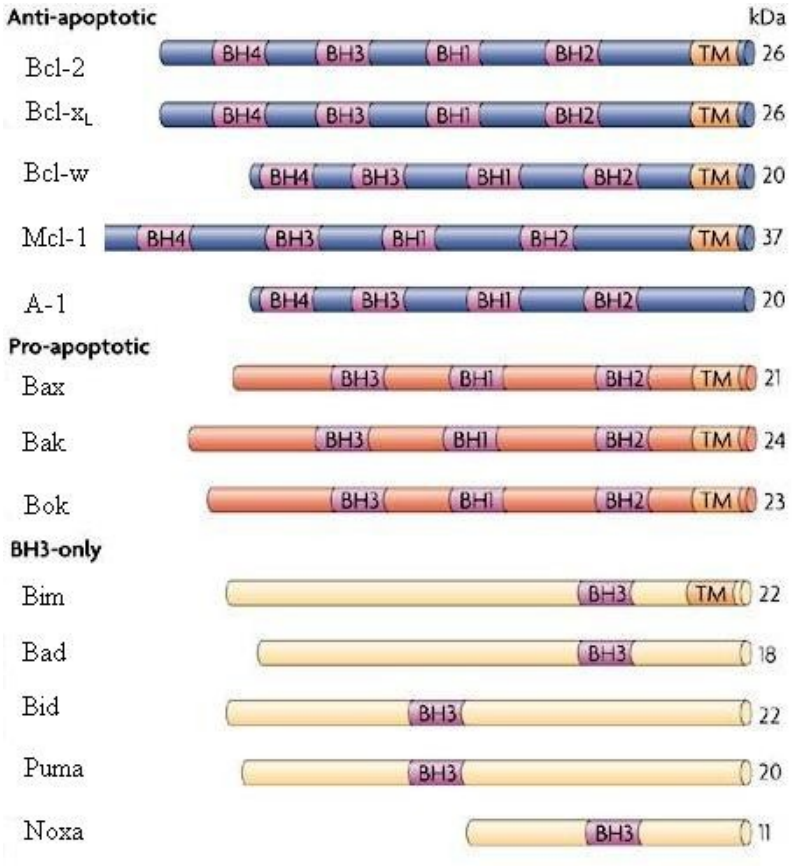

Figure 3. The Bcl-2 family of proteins and their classification according to function and domains they comprise (adapted and modified from Taylor et. al., 2008)

Upon activation, Bax translocates from the cytosol to insert into the OMM via its TM (Bak is already inserted in the OMM). Bak and Bax undergo a conformational change, dimerise and subsequently homo-oligomerise to form large complexes creating pores in the OMM. The mechanism of Bak and Bax activation and pore formation is not exactly known. Two models, direct and indirect, propose that the BH3-only proteins induce

apoptosis by direct binding to Bak or Bax or by inactivating the Bcl-2 anti-apoptotic proteins, respectively (Fig. 4) (Dewson and Kluck, 2009). The mechanism of MOMP induction by BH3-only protein is based on the fact that each protein of the BH3-only subfamily can bind some or all Bcl-2 family members with different affinity. Bim, tBid, and Puma can interact with all Bcl-2 anti-apoptotic members. Bad binds Bcl-2, Bcl-x_L, and Bcl-w, and Noxa binds A-1 and Mcl-1. The indirect model of Bak and Bax activation presumes that all Bax and Bak are inactivated by interacting with the Bcl-2 anti-apoptotic proteins Bcl-2, Bcl-x_L, and Mcl-1. The interaction of these proteins with BH3-only members releases Bak and Bax from their sequestration and enables them to form pores in the OMM. The direct model is based on direct activation of Bax and Bak by the BH3-only proteins tBid and Bim (and possibly Puma and p53). In this case the other BH3-only family members Bad, Noxa and Puma function as "sensitisers" that cannot activate Bax and Bak directly, but can bind anti-apoptotic proteins and cause the release of BH3-only proteins. Bad binds Bcl-2 and Bcl-x_L to liberate tBid and Bim, and Noxa binds Mcl-1 to liberate Bim (Fig. 4) (Brenner and Mak, 2009; Brunelle and Letai, 2009).

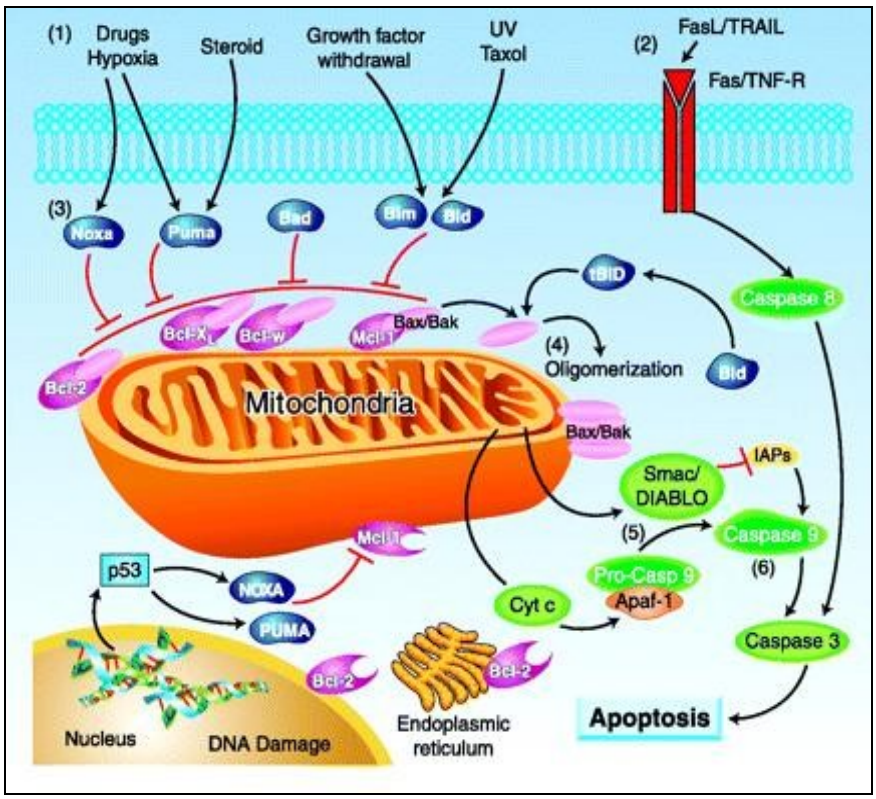

Figure 4. The intrinsic apoptotic pathway and the role of Bcl-2 family proteins (adapted from Kang and Reynolds, 2009)

The induction of the OMM pore is regulated by post-translation modifications of BH3-only proteins. The death receptor signalling activates caspase-8 that cleaves Bid. Cleavage and subsequent N-myristylation produce active tBid. This mechanism presents the main link between the extrinsic and intrinsic pathways. In the presence of growth factors, Bad is phosphorylated by several kinases such as Akt and is inactivated by sequestration with the 14-3-3 protein. Upon growth factor withdrawal, this pathway is disrupted and Bad is activated. Bim is regulated by transcriptional and post-transcriptional mechanisms. The forkhead box O (FoxO) transcription factors induce transcription of the Bim-coding gene, and several post-transcriptional modifications regulate the Bim function (Chipuk et al., 2010). Transcriptional regulation of Bcl-2 family proteins plays an important role. The FoxO transcriptional factors can cause expression of the death ligands FasL/TRAIL and suppress expression of Bcl-x_L (Fu and Tindall, 2008). The most common tumour suppressor p53 is responsible for apoptosis induction via activation of expression of genes encoding the death receptor proteins Fas, DR4 and DR5, Bax and BH3-only proteins (Puma and Noxa), Apaf-1 and caspase-6. The protein p53 can also act by suppressing the expression of genes coding for Bcl-2 anti-apoptotic proteins (Bcl-2, Bcl-x_L) (Yu and Zhang, 2005).

3. Targeting apoptotic pathways in cancer therapy

As we can see above, damaged cells possess several mechanisms to activate their suicide programme, but these are usually altered in cancer cells, and the deregulation of apoptosis is a main aspect of cancer pathogenesis. The current treatment of cancer largely relies on the induction of apoptosis, and alterations in apoptotic signalling pathways are often associated with cancer cell resistance to anti-cancer therapies (chemotherapy or radiation). The most common alteration is mutation in the *p53* gene that occurs in more than half cancer patients. Normally, upon DNA damage, p53 alerts the cell to mend its DNA. When the damage is irreversible, p53 induces apoptosis. Thus, mutations in the *p53* gene lead to inhibition of apoptosis induction because expression of pro-apoptotic proteins like Puma and Noxa is compromised. NF-κB is also deregulated in many cancers, which lead to over-expression of pro-survival genes encoding proteins like IAPs, cFLIP, Bcl-2, Bcl-x_L, etc. Among other common alterations in apoptotic pathways are mutations in the *Fas* gene or upregulation of the PI3K kinase (Ghobrial et al., 2005).

Circumventing the activation of apoptotic pathways in cancer cells is the main task of anti-cancer treatment. For example, a considerable potential in cancer therapy can be ascribed to the TRAIL-induced extrinsic pathway because it is relatively intact in many cancers and is largely p53-independent. Pro-apoptotic agonists targeting DR4 and DR5 have been developed including the recombinant human TRAIL (activates both DR4 and DR5) as well as several monoclonal antibodies including the human DR5-activating Apomab. Both approaches seem to cause significant growth regression of tumours without secondary toxicity (Ashkenazi, 2008). The intrinsic pathways and their components present many therapeutic targets. An intriguing group of potential anti-cancer agents is presented by agents referred as mitocans, a group comprising small molecules acting via mitochondria.

3.1. Mitocans - drugs targeting mitochondria

Mitochondria are the main source of energy but, as mentioned above, they also contain many important mediators of apoptosis, thereby they are essential both for the cell survival and for the induction of apoptotic cell death. Mitochondria mediate apoptosis in response to a wide range of death stimuli. The upstream pathways leading to apoptosis are often inactivated in cancer cells by various mutations, which are of diverse nature. The genome variability and thus large range of various mutations in cancer cells cause that cancer is a disease characterised by highly variable characteristics in each patient. This is one of the major obstacles in the development of efficient anti-cancer therapies.

Mutations in mitochondria are relatively rare and most tumours retain the ability of oxidative phosphorylation (Cairns et al., 2011). Thus, mitochondria present a potential target for anti-cancer treatment of various cancer types. The group of compounds termed mitocans include structurally distinct molecules that have one common feature. They target mitochondria and cause destabilisation of these organelles, which leads to induction of mitochondria-mediated apoptosis, often selectively in cancer cells. Mitocans are a large group of anti-cancer agents that act by different mechanisms, according to which they can be divided into eight sub-classes. The hexokinase inhibitors suppress the function of hexokinase (HK) I and II that stabilise the channel formed by the interaction of the voltage-dependent anion channel (VDAC) in the OMM and the adenine nucleotide transporter (ANT) in the IMM. In this way, HK prevents Bax from binding to VDAC as

well as from induction MOMP. The group of BH3 mimetic acts by targeting the BH3 domains of anti-apoptotic proteins Bcl-x_L and Bcl-2, whereby disrupts their interaction with Bax and Bak. The thiol redox inhibitors and VDAC/ANT-targeting drugs act by modification of VDAC or ANT that result in MOMP. The class of electron transport chain-targeting drugs includes a range of drugs targeting components of mitochondrial complexes, causing accumulation of ROS and induction of apoptosis. Lipophilic cations targeting the IMM act by dissipating the mitochondrial membrane potential ($\Delta\Psi_m$) with ensuing mitochondrial destabilisation and apoptosis induction. Several compounds have been shown to target mitochondrial mtDNA. Classification of mitocans according to their targeting sites in mitochondria and examples are summarised in Table 1 (Neuzil et al., 2007a).

Table 1 Classification of mitocans

Class number	Type	Examples
I	Hexokinase inhibitors	3-bromopyruvate, 2-deoxyglucose
II	BH3 mimetics	gossypol, antimycin, α-TOS, HA14-1
III	Thiol redox inhibitors	isothiocyanates, PITC, phenylarseneoxide, arsenite, Cu-phenanthroline
IV	VDAC/ANT targeting drugs	lonidamine, bisphosphonate, MT21, steroid analogues like CD437, ATRA
V	Electron transport chain targeting drugs	4-OH retinamide, α-TOS, tamoxifen, resveratrol, dicumarol
VI	Lipophilic cations targeting inner membrane	rhodamine-123, F16, $(KLAKKLAK)_2$ peptide, MKT-077, dequalinium
VII	Drugs targeting mtDNA	Menadione, MitoVES
VIII	Drugs targeting other (unknown) sites	betulinic acid, phenoxodiol, sesquiterpene lactones (parthenolide)

(adapted from Neuzil et al., 2007a, Ralph and Neuzil, 2009)

3.2. Vitamin E analogue α-tocopheryl succinate as a mitocan

The term VE refers to group of lipid-soluble compounds including tocopherols and tocotrienols. These agents have indispensible functions in a variety of organisms. Important is their antioxidant role that prevents damage to tissues inflicted by free radicals, primarily preventing oxidation of lipids in (sub)cellular membranes. They have a function in the reproduction as well as in the proper function of the nervous system. The most

abundat VE form, α-tocopherol (α-TOH), has also a function in modulation of signalling. It decreases protein kinase C activity and increases the activity of phosphoprotein phosphates 2A (PP2A). γ-TOH is a nucleophile that reacts with electrophilic mutagens. Members of the VE group also modulate important processes with a role in the prevention of tumorigenesis (Brigelius-Flohé and Traber, 1999; Wang and Quinn, 1999).

The VE ester analogue α-TOS is a mitocan belonging to the group of BH3 mimetics as well as electron transport-targeting drugs. It has been documented as an efficient inducer of apoptosis in a wide range of cancer cells including breast, colon and lung cancer, mesotheliomas and melanomas, while being non-toxic to normal cells and tissues (Neuzil et al., 2001, 2004; Wang et al., 2006).

3.3. Selectivity of α-tocopheryl succinate

The apoptogenic activity and selectivity of α-TOS result from its structure as well as from differences between normal and tumour cells. Fig. 5 shows structural features of α-TOS and α-TOH, both containing three major domains. The hydrophobic and signalling domains are identical for both compounds, while they differ in the functional domain. The hydrophobic domain is responsible for docking the agents in biological membranes and circulating lipoproteins. The signalling domain regulates certain signalling pathways such as the protein kinase C and PP2A pathway, and the functional domain determines whether the compounds possess apoptogenic activity. While α-TOH does not exert apoptogenic activity, its ester α-TOS induces apoptosis in a wide range of cancer cell lines. Experiments with VE analogues with different functional domains revealed that for the analogues with apoptogenic activity, presence of free carboxylate in the functional domain

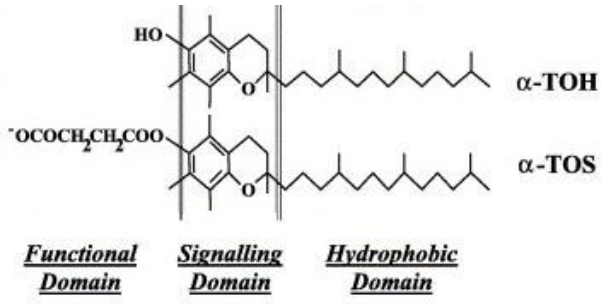

Figure 5. Three major domains in α-TOH and α-TOS (adapted from Birringer et al, 2003).

(e.g. succinate, maleate or fumarate) is essential (Birringer et al, 2003).

In contrast to normal cells, their malignant counterparts produce ATP not only via oxidative phosphorylation but also (and in some cases primarily) by glycolysis even in the presence of sufficient amounts of oxygen. This phenomenon is called the Wargurg effect and results in conversion of glucose into lactate in cancer cells (Vander Heiden et al., 2009). The excess production of lactate leads to transient acidification of the cytosol. Cytosolic pH is maintained neutral by the proton pump that moves protons to the extracellular space and causes its acidification from neutral pH to 6.3-6.6. α-TOS is a weak acid with pK_a of 5.64 (Birringer et al., 2003) that can cross the plasma membrane by free diffusion in its protonated form. At neutral pH almost 99 % of the α-TOS pool is in the deprotonated state while at pH of ~6.2, the amount of protonated form of α-TOS increases ~25-fold. Thus, the acidic interstitium of solid tumours favours the selective uptake of α-TOS by cancer cells (Neuzil et al., 2002). The other reason for α-TOS selectivity is that normal cells feature high esterase activity that converts α-TOS to the non-apoptogenic α-TOH. Tumour cells lack this activity, whereby resulting in α-TOS accumulation with ensuing ROS production (Neuzil et al., 2004). The third reason for α-TOS selectivity is the production of ROS in response to this agent. Normal cells have mechanisms by which they can more efficiently dispose of ROS. These include increased activity of anti-oxidant enzymes like superoxide dismutase (SOD) or catalase, whose expression is usually lower in cancer cells (Neuzil et al., 2007b).

3.4. Mechanism of ROS production in response to α-tocopheryl succinate

It has been shown that ROS production in response to α-TOS treatment precedes induction of apoptosis. α-TOS-treated cells show increased levels of ROS, altered mitochondrial structure and hallmarks of mitochondria-mediated apoptosis. Experiments with mitochondrial DNA deficient (ρ_0) cells revealed that these cells are resistant to α-TOS-induced apoptosis (Weber et al., 2003). Recent studies documented that α-TOS induces ROS generation by interfering with the mitochondrial electron redox chain. Mitochondrially targeted antioxidant MitoQ that is known to interact with the mitochondrial CII (succinate dehydrogenase, SDH), suppressed ROS production induced by α-TOS. CII has been proposed to be a direct target for α-TOS (Neuzil et al., 2007b).

It is a multi-enzyme complex bound to the IMM that passes electrons from succinate oxidation to ubiquinone (UbQ) and reduces it to ubiquinol (Voet, 2004). Molecular modelling on crystal structure of porcine CII revealed that α-TOS binds to the proximal (Q_p) and distal (Q_d) UbQ sites in CII. Because the energy of binding of α-TOS at the Q_p and Q_d site is similar to that of UbQ, α-TOS is assumed to compete for these binding sites. Displacement of UbQ from CII by α-TOS means that electrons from succinate oxidation cannot pass to UbQ, thereby recombining with oxygen to produce superoxide anion radicals (Dong et al., 2008). SOD then converts superoxide to the highly diffusible hydrogen peroxide that can activate intrinsic apoptotic pathways by mechanisms discussed below.

3.5. α-Tocopheryl succinate-mediated apoptotic signalling along the Noxa-Bak axis

Several mechanisms have been proposed to explain α-TOS-induced apoptosis. It has been shown that α-TOS treatment causes activation of sphingomyelinase (SMase), lysosomal destabilisation and ROS generation (Neuzil et al., 2007a). SMase is an enzyme that converts sphingomyelin in the plasma membrane to the apoptogenic ceramide. Phosphorylation of the Bcl-2 protein on serine residue Ser70 is required for its anti-apoptotic function. Ceramide was shown to activate PP2A, which result in Bcl-2 dephosphorylation and apoptosis induction (Ruvolo et al., 1999). Another event leading to apoptosis induction is α-TOS-mediated lysosomal destabilisation. This stems from experiments with cells deficient in cathepsin D (Neuzil et al., 2002) that were relatively resistant to α-TOS-induced apoptosis. However, the experiment with ρ_0 cells showed that these events may not be crucial and rather amplify the main mitochondria-mediated pathway. α-TOS was shown to interact with the BH3 domain of anti-apoptotic proteins Bcl-2 and Bcl-x_L. This interaction liberates Bak from the Bcl-x_L and Bcl-2 heterodimer formation and enables Bak to form pores in the OMM (Shiau et al., 2006). However, ROS generation is the main reason for apoptosis induction in response to α-TOS. Hydrogen peroxide formed by dismutation of superoxide can cause dimerisation of two cytosolic monomers of Bax. Computer simulation revealed that disulfide bridge formation changes conformation of the Bax monomer and enables formation of Bax dimers and their incorporation into the OMM (Neuzil et al., 2006). Oxidative stress can also activate the

transcription factor p53 and thus expression of BH3-only proteins Puma and Noxa that are able to displace Bak from Mcl-1 binding to form pores in the OMM (Neuzil et al., 2007b).

However, recent studies shed new light on the role of ROS in apoptosis induction in response to α-TOS, such that it causes p53-independent upregulation of the protein Noxa (Prochazka et al., 2010). This was confirmed by experiments with cell lines expressing wild type p53 and those either without p53 or with transcriptionally silent p53. In response to α-TOS, all cell lines underwent apoptosis and show increased levels of the Noxa protein, while the levels of other Bcl-2 family members weres not altered. To document that Noxa upregulation is a result of ROS generation, the level of ROS was suppressed by MitoQ, which caused suppression of Noxa and apoptosis induction. For generation of pores in the OMM, the proteins Bax and Bak have to first undergo conformational changes. All cell lines tested showed high level of conformational changes of Bak in response to α-TOS, while Bax underwent a conformational change only in one cell line. Thus, increased levels of the Noxa protein cause release of Bak from Mcl-1 and enable it to form pores in the OMM (Prochazka et al., 2010).

The next question is by which mechanism the *NOXA* gene becomes upregulated in response to α-TOS treatment of cancer cells. Recent data strongly indicate that this occurs via activating the Hippo signalling pathway, as discussed in the next paragraphs.

4. The role of the Hippo signalling pathway in apoptosis induction

The Hippo signalling pathway presents the main control mechanism in the regulation of organ growth, whose size has to be strictly regulated. The Hippo pathway maintains the balance between cell proliferation and cell death, which is fundamental for the precise function of multicellular organisms, such that their organs reach the required size, after which their growth is terminated. This control mechanism is obvious for example during liver regeneration. After partial hepatectomy, hepatocytes undergo rapid cell division to restore the liver mass. Upon reaching the original size, hepatocytes stop dividing to prevent over-growth of the regenerating liver (Fausto et al. 2006). Further, deregulation of this pathway contributes to the loss of contact inhibition and the ability of anchorage-independent growth, whereby promoting tumorigenesis.

4.1. Key components of the Hippo pathway

The Hippo pathway was first defined in *Drosophila melanogaster* and its components (Fig. 6, page 29) were discovered by the development of the technology of genetic screening, which allowed to observe the results of genetic mutations in genes, whose alterations often lead to lethality (Zhao et al., 2008). The core components of the Hippo pathway in *Drosophila* are protein kinases Warts (Wts), Salvador (Sav), Hippo (Hpo) and Mats (Pan, 2010). These tumour suppressors form a kinase cascade in which Hpo phosphorylates and interacts with Sav (Wu et al., 2003). The complex Hpo-Sav then phosphorylates Mats and activates its function as a co-activator of the Wts kinase, which is phosphorylated by Hpo (Wei et al. 2007). The final component of this pathway is the transcriptional co-activator Yorkie (Yki).

The Hippo pathway is evolutionary conserved in mammals and includes two homologues of the Hpo kinase, the mammalian sterile 20-like kinase-1 and -2 (Mst1 and Mst2), one Sav homologue (Sav1 or WW-45), two Wts homologues (Lats1 and Lats2) and two Mats homologues (MOBKL1A and MOBKL1B), often referred to jointly as Mob1. This cascade phosphorylates and inactivates the mammalian Yki homologue, the transcriptional co-activator Yes-associated protein (YAP) and the transcriptional co-activator with PDZ domain (TAZ) (Pan, 2010). A homologue of Hpo kinase, the Ste20 protein, was also identified in yeast as a main inducer of apoptosis in response to hydrogen peroxide (Ahn et al., 2005).

4.2. Upstream regulation of the Hippo pathway

The mechanism of upstream regulation of the Hippo signalling pathway has not been elucidated. Recent research found several tumour suppressors, whose signalling pathways impact on the Hippo cascade and whose mutations result in deregulation of this pathway and consequent growth defects (Fig. 6, page 29). These include Merlin (Mer), Expanded (Ex), Fat (Ft), Dachsous (Ds), Kibra and Crumbs (Crb) in *Drosophila* (Pan, 2010). The membrane proteins Ft and Ds are atypical cadherins. Ft is a cell surface molecule with a role not only in regulation of cell growth but also in planar cell polarity, for which it interacts with Ds of neighbouring cells as well as with the Four-jointed (Fj) kinase, which phosphorylates the extracellular domains of Ft and Ds. Fj and Ds regulate the Hippo

pathway in relation to their cellular expression (Willecke et al., 2008). The proteins Mer and Ex contain the FERM domain, which contributes to their localisation in the plasma membrane. These are likely to interact with the Kibra protein and together interact with the Hpo-Sav complex, and transfer the signal from the plasma membrane to the Yki protein (Yu et al., 2009). Others reported that Ex regulates growth by direct interaction with Yki, whereby causing its inactivation (Badouel et al., 2009). It was shown that Ft regulates the cellular level and membrane localisation of Ex, which transfers the signal to Hpo. It was also proven that Ft regulates Wts by an unconventional myosin Dachs. The exact mechanism of signal transmission from Ft to the Hpo kinase is not fully clarified (Feng and Irvine, 2007). An important role in regulation of the Hippo pathway has also been reported for the RASSF protein, which belongs to the Ras family of proteins. The Drosophila orthologue dRASSF negatively regulates the Hippo pathway by competitive inhibition (Polesello et al., 2006).

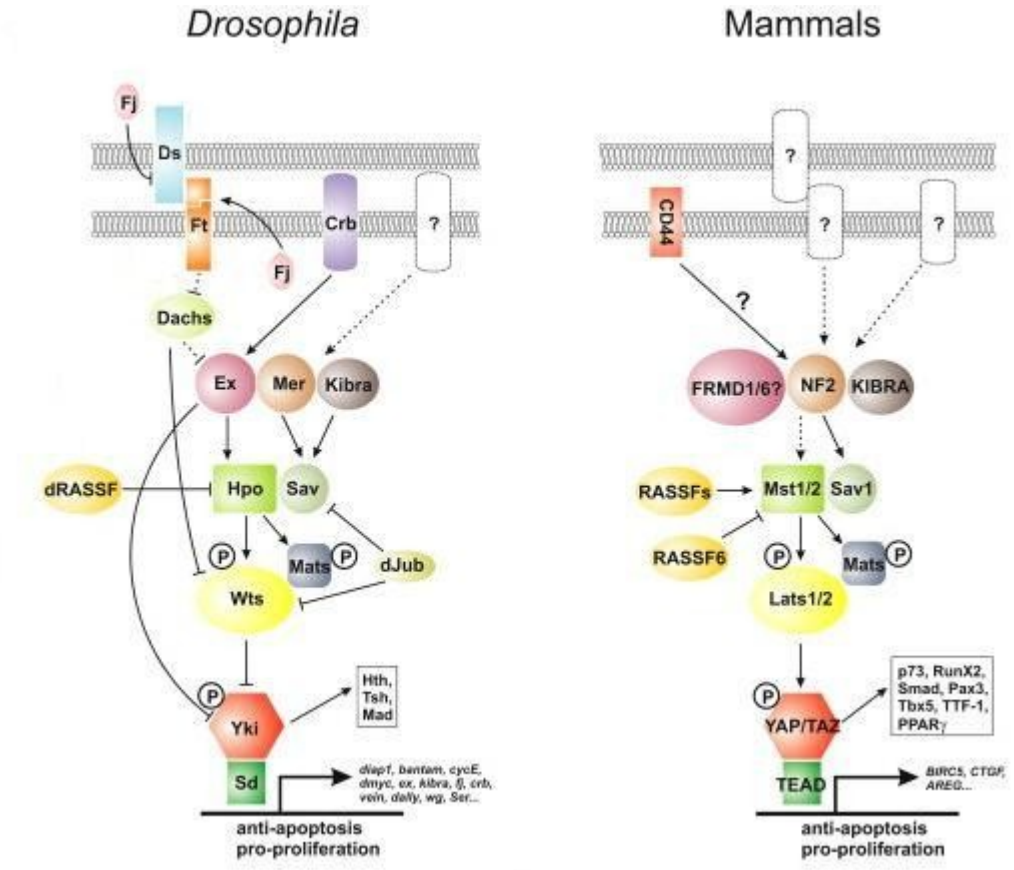

Figure 6. The Hippo signalling pathway in *Drosophila melanogaster* and mammals (adapted and modified from Pan, 2010).

The mammalian genome contains homologues of all mentioned components of the *Drosophila* Hippo pathway except for Dachs. In mammals, there are two Kibra homologues KIBRA or WWC1 and WWC2, two Ex homologues FRMD6/EX1 and FRMD1/EX2, one Mer homologue NF2, one Ft homologue Fat4/Fat-j, two Ds homologues Dchs1 and Dchs2 and one Fj homologue Fjx1. Mutations in some of these proteins cause deregulation of the Hippo pathway. Among proteins whose mutations influence the Hippo pathway is NF2, which is linked to the wide spectrum of cellular functions and effector pathways, with an important role in contact inhibition mediated by cell surface receptors such as the receptor for hyaluronic acid CD44 (Pan, 2010). Another potential tumour suppressor is the Kibra protein, which was shown to interact with NF2 but not with FRMD6, MST2 or RASSF6 (Genevet et al., 2009). The Sav1 protein also interacts with NF2. It has been suggested that stimulation of the Hippo pathway by NF2 is probably conserved in mammals, but the exact regulation is unclear (Zhang et al., 2010).

4.3. Yki/YAP/TAZ as targets of Hippo signalling and co-activators of targeting genes

As mentioned above, the transcription co-activator Yki is the target of the Hippo signalling pathway in *Drosophila*. Yki is phosphorylated by the serin/threonin kinase Wts on Ser168. This phosphorylation facilitates interaction of Yki with the 14-3-3 proteins and its consequent nuclear export, cytoplasmic retention and inactivation. Yki can be inactivated in both phosphorylation-dependent and -independent manner, the latter involving the WW domain of Yki. This domain interacts with the PPXY motif of Ex, Wts and Hpo, whereby inactivating Yki (Oh et al., 2009), and appears to be important also for its transcriptional activity. Yki binds the transcription factor Scaloped (Sd) from the family of TEAD/TEF transcription factors, which directly binds to the regulatory sequences of the Yki target gene *Diap1*, and Homothorax (Hth), whose direct target is the miRNA gene *bantam*. The known Yki target genes play an important role in cellular growth (*dMyc, bantam*), in inhibition of apoptosis (*diap1*) and in regulation of cell cycle (*cyclin E*). Yki also influences genes encoding upstream regulators of the Hippo signalling cascade (Kibra. Ex, Fj), and genes encoding proteins that play a role in intercellular signalling (E-cadherin, Serrate, Wingles, Vein, etc.) (Oh and Irvine, 2010; Pan, 2010).

In mammals the target molecule of the Hippo signalling pathway is the Yki homologue, the transcription co-activator YAP and its paralogue TAZ. The Lats kinases

phosphorylate serine residues (Ser127 in YAP and Ser89 in TAZ) in the conserved HXRXXS sequence, and whereby facilitating the interaction of YAP and TAZ with 14-3-3 proteins and their transport from the nucleus to the cytoplasm (Zhao et al., 2008). The WW domains of YAP and TAZ can interact with transcription factors containing the PPXY motif, such as RUNX and SMAD. Several reports point at the pro-apoptotic function of YAP in the complex with the transcription factor p73, which also contains the PPXY motif. The N-terminal part of YAP and TAZ is homologous to Yki and contains the TEF/TEAD-binding domain. TAZ interacts with other transcriptional factors such as peroxisome proliferator-activated receptor-γ (PPARγ), the thyroid transcription factor-1 (TTF-1), PAX3, TBX5, etc. (Wang et al., 2009). Predicted target genes of the Hippo pathway in mammals are the anti-apoptotic genes and genes supporting cellular growth such as *c-MYC, SOX4, H19, AFP, survivin* or *cIAP1* (Zeng and Hong, 2008; Pan, 2010).

The precise regulation of this pathway is essential for proper organism development. Its deregulation leads to the loss of contact inhibition and uncontrolled cellular growth, which are the main features of cancer cells. The re-activation of this pathway could contribute to the inhibition of cellular grow and induction of apoptosis in malignant cells with implications for treatment of cancer patients.

4.4. FoxO transcription factors as target for Mst1 kinase

FoxO transcription factors are a subgroup from the super-family of proteins that share a highly conserved DNA-binding domain called forkhead (FKH). This domain contains three main α-helices and two wing-like loops, therefore these transcription factors are often called “wingled helix”. They are conserved from *C. elegans* to mammals, where four members of this group FoxO1 (FKHR), FoxO3 (FKHRL1), FoxO4 (AFX) and FoxO6 have been identified (Huang and Tindall, 2007). FoxO factors interact as monomers with the motif called the DAF-16 family member-binding element (DBE) with the conserved consensus core sequence TTGTTTAC that was find in promoters of many genes (Xuan and Zhang, 2005). FoxOs regulate transcription of genes participating in cell cycle regulation and metabolism, some of which are listed in Fig. 7 (page 32). However, the most important genes for us are those causing apoptosis induction, i.e. *BIM*, *FASL*, *TRAIL*, *PUMA* (Zhang et al., 2011a) and *NOXA* (Valis et al., 2011).

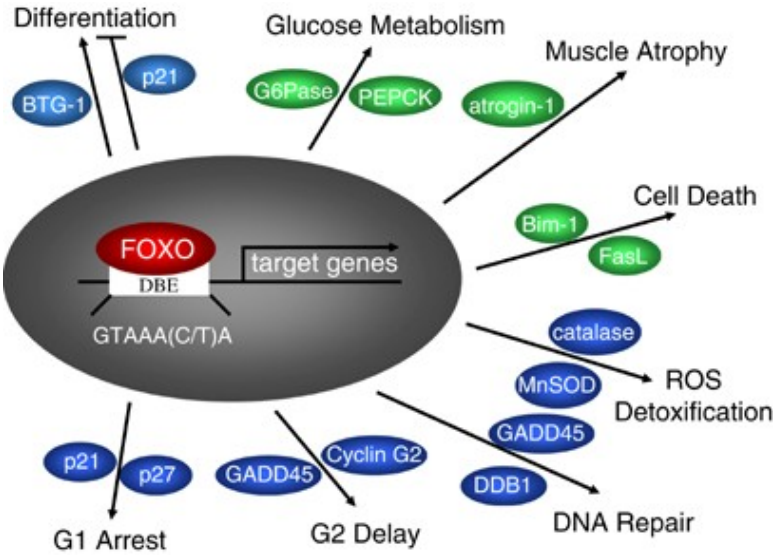

Figure 7. FoxO target genes and their cellular roles (adapted from Greer and Brunnet, 2005).

Many external stimuli such as insulin, insulin-like growth factor (IGF-1), other growth factors, nutrients, cytokines and oxidative stress are involved in regulation of activity and localisation of FoxO transcription factors. These external stimuli regulate FoxO by post-translational modifications, which include phosphorylation, ubiquitylation, acetylation and protein-protein interactions. FoxO proteins are phosphorylated and localised to the cytoplasm in the presence of growth and survival factors, but in response to various stress stimuli they are phosphorylated by different kinases and de-localise to the nucleus even when the growth factors are still present (Calnan and Brunet, 2008; Huang and Tindall, 2007). Phosphorylation of FoxO factors and their sequestration in the cytoplasm is performed mainly by the Akt kinase via the phosphatidylinositol 3-kinase (PI3K) - Akt pathway. This pathway is involved in regulation of many biological processes including cell growth, proliferation or survival, and its dysregulation is a hallmark of a wide spectrum of cancers. Insulin and growth factors activate PI3K kinase via binding to their receptors. Activation of PI3K results in formation of phosphatidilinositol 3,4,5-triphosphate (PIP_3). PIP_3 functions as a second messenger that creates membrane binding sites for the serine/threonine kinase Akt. Translocation of Akt to the plasma membrane and its recruitment to PIP_3 results in its phosphorylation and activation by 3-phosphoinositide-dependent protein kinase 1 (PDK1) (Vivanco and Sawyers, 2002). Activated Akt translocates to the nucleus, where it phosphorylates FoxO proteins at three

sites (Thr32, Ser253 and Ser315 of FoxO3, Thr24, Ser256 and Ser319 of FoxO1), whereby facilitating interaction of FoxO proteins with 14-3-3 proteins and their cytoplasmic sequestration (Brunet et al., 1999; Rena et al., 1999).

It has been found that in response to oxidative stress, active Mst1 mediates apoptosis by direct phosphorylation of FoxO factors. Active Mst1 phosphorylates FoxO3 at Ser207. This phosphorylation inhibits the Akt-mediated phosphorylation of Ser253 of FoxO3, disrupting the interaction between FoxO3 and the 14-3-3 proteins. FoxO3 then translocates to the nucleus where it inactivates the transcription of target genes (Lehtinen et al., 2006). Upon survival factor deprivation, Mst1 phosphorylates Ser212 of FoxO1, corresponding to Ser207 of FoxO3, thus triggering the dissociation of FoxO1 from 14-3-3 and its translocation to the nucleus where it activates the translation of target genes (Yuan et al., 2009). Recent studies also suggest that the Mst1-FoxO1 pathway mediates apoptosis in cancer cells exposed to α-TOS (Valis et al., 2011).

4.5. Mst1-FoxO1-Noxa pathway

As mentioned above, the Noxa-Bak axis has been shown to be required for α-TOS-induced apoptosis in cancer cells. Although the *NOXA* gen is known to be transcriptionally regulated by p53, it has been reported to be also upregulated in p53-deficient cells, presenting a new mechanism of Noxa upregulation in cancer cells. The *ab initio* analysis revealed conserved DBE FoxO motif in the *NOXA* gene promoter. Previous studies indicated that transcription of *NOXA* and thus apoptosis induction is regulated by FoxO3 (Czymai et al., 2010; Obexer et al., 2007). In this model, *NOXA* is not regulated by FoxO3, but by FoxO1. Both proteins were assessed for their nuclear localisation in Jurkat cells in response to α-TOS and both proteins were detected in the nucleus. While FoxO3 was detected in the nucleus of control cells and during treatment its nuclear level increased, FoxO1 translocated to the nucleus within 2 h and its level remained unchanged. Knocking down FoxO1 and FoxO3 by siRNA in p53-deficient cells and in Jurkat cells showed that only FoxO1 plays a role in the increase of *NOXA* mRNA. Additionally, upregulation of the pro-apoptotic *FASL* and *TRAIL* genes was also observed to be induced by Mst1-FoxO1 pathway (Valis et al., 2011).

These results present the Mst1-FoxO1 signalling as a new pathway involved in apoptosis induction in cancer cells. To date, this pathway has been verified in two types of

cancer cells, and it is important to find out whether it is operational in different types of cancer cells and whether it promotes apoptosis in response to apoptogens other than α-TOS. It also remains to be shown that this pathway is responsible for the anti-apoptotic effect of α-TOS and other anti-cancer agents *in vivo*.

4.6. Activation of Mst1 kinase

Current studies suggest that Mst1 kinase activation plays an important role in tumour suppression, but the mechanisms of its activation are still obscure. One possibility is that Mst1 is activated by caspase-dependent cleavage. Caspases cleave Mst1 in the regulatory part of the C-terminus, which generates a fragment that includes the N-terminal kinase domain, which is almost 10-fold more active than the parental enzyme. The cleavage removes the C-terminal sequences that excludes Mst1 from the nucleus. The N-terminal part of the cleaved Mst1 translocates from the cytosol to the nucleus, where it phosphorylates histone H2B at Ser14 and histone H2AX at Ser139, leading to chromatin condensation and apoptosis induction (Lee et al., 2001; Cheung et al., 2003; Wen et al., 2010). The N-terminal fragments of Mst1 were observed in the nucleus of α-TOS-treated Jurkat cells within 2 h of exposure, while caspases were activated only after 6 h (Valis et al., 2011). This indicates that the kinase is activated, possibly by hydrogen peroxide, before caspase activation. Mst1 has been reported to be phosphorylated at Thr183 via intermolecular auto-phosphorylation enhanced by its homodimerisation (Glantschnig et al., 2002). Increased levels of Mst1 phosphorylated at Thr183 were observed within 2 h of α-TOS exposure of Jurkat cells (Valis et al., 2011). The crystal structure revealed that the Mst1 protein is present in cells as a homodimer (http://www.pdb.org/pdb/explore/explore.do?structureId=3COM).

Molecular modelling revealed two surface cysteine residues, Cys80 and Cys203, in the Mst1 protein, which are accessible to oxidation by hydrogen peroxide, most likely provoking a conformational change, auto-phosphorylation and cleavage of the protein. Cys203 was found to lie in the proximity to His141. Histidine deprotonates cysteine sulfhydryls and creates a nucleophilic environment that attacks the peptide carbonyl carbon, whereby cleaving the peptide bond, suggesting a mechanism for the autocatalytic cleavage of Mst1 (Valis et al., unpublished data). These preliminary data suggest the role

of surface cysteine in Mst1 activation by hydrogen peroxide and subsequent cleavage, but the mechanism is still not clear and requires further research.

4.7. *c-MYC* oncogene as a potential target of Mst1 kinase

As mentioned above, cancer cells are dependent on glycolysis even in the presence of sufficient amount of oxygen. To meet the increased energy demands, cancer cells upregulate the glucose uptake system to produce enough ATP. In addition to glucose, cancer cells also depend on high level of exogenous glutamine and exhibit the so-called 'glutamine addiction'. Glutamine is metabolised in the process called 'glutaminolysis', being converted to ammonia and glutamate, further catabolised to α-ketoglutarate that enters the Krebs cycle to generate NAPDH and fatty acids. Thus, glutamine serves as a nitrogen and carbon donor for the synthesis of nucleotides, amino acids, and lipids and also generates the reduced equivalents NADPH for lipid and nucleotide synthesis (Wise and Thompson, 2010).

Altered cancer cell metabolism is linked to the transcription factor c-Myc that is known to regulate many important cellular processes including cell cycle, growth, metabolism, differentiation, and apoptosis, and its increased levels are involved in carcinogenesis of ~40 % of human cancers. c-Myc regulates the expression of genes encoding proteins for glucose and glutamine metabolism (such as lactate dehydrogenase, hexokinase 2, phosphofructokinase, enolase, the glucose transporter GLUT1, the glutamine transporters SLC38A5, and SLC1A5), and also for nucleotide metabolism and DNA replication (Wise and Thompson, 2010; Dang et al., 2009). This highlights c-Myc as a potential target for anti-cancer drugs. The *Drosophila* Myc expression was found to be directly regulated by the Hippo-Yki pathway (Neto-Silva et al., 2010). Recent pilot data suggest that this pathway is conserved in mammals as well (Valis et al, unpublished data). As mentioned, the Mst1 kinase has been reported to be activated by hydrogen peroxide generated during α-TOS treatment. Exposure of Jurkat cells to α-TOS caused significant decrease of c-Myc mRNA and protein levels within 6 h of treatment. The connection between c-Myc and Mst1 was also confirmed by Mst1 silencing by siRNA, resulting in lack of c-Myc mRNA downregulation during the treatment. Assessment of the expression of the c-Myc-regulated gene *GLUT1* shows a decrease in its expression as well (Valis et al., unpublished data). This means that activation of the Mst1 kinase by hydrogen peroxide in response to α-TOS

treatment of Jurkat cells would result not only in FoxO1/Noxa-dependent activation of apoptosis but also in starvation of cancer cells for energy. Validation of this propose is the current goal of the studies of our laboratory (Valis et al., unpublished data).

4.8. The Hippo pathway as a pivotal tumour suppressor and target for cancer therapy

It was found that deregulation of the Hippo pathway constituents contributes to a wide range of human cancer diseases, and it was also observed that Hippo signalling causes resistance of cancer cells to certain anti-cancer drugs. Elevated expression and nucleus localisation of YAP was found in many human cancers (Steinhardt et al., 2008; Zhao et al., 2007). The testing of YAP level expression and nuclear localisation in 284 samples of human ovarian cancer revealed high expression level and nuclear localisation of YAP in 14% cases. These results correlate with the poor patient prognosis, because YAP causes independent growth and contributes to overcoming contact inhibition. YAP also stimulates invasive character of ovarian cancer cells and makes them resistant to established anti-cancer drugs cisplatin and Taxol (Zhang et al., 2011b). The survival rate of patients with YAP over-expression was significantly lower than in case of patients deficient in the YAP protein (Wang et al., 2010).

Mst1 and Mst2 are the main regulators of cellular growth in cells and the loss of their function leads to inhibition of YAP1 phosphorylation on Ser127 and its localisation in the nucleus. This results in massive over-growth of organs such as liver and to the development of hepatocellular carcinoma. In liver cells, Mst1 and Mst2 regulate the transcriptional co-activator YAP1 through a kinase distinct from Lats. The loss of activated Mst1 and Mst2 and the low YAP1 phosphorylation of Ser127 have been shown in ~30% of human hepatocellular carcinomas (Zhou et al., 2009). Elevated level of the TAZ transcriptional co-activator was found in ~70% of breast cancer cell lines and in ~20% of invasive breast cancer tissues. TAZ over-expression causes higher proliferation, cellular migration and transformation. Elevated TAZ has been recently detected in breast cancer cells resistant to the anti-cancer drug Taxol (Lai et al., 2011).

Heat shock protein 90 (HSP90) controls many signalling pathways leading to tumorigenesis and has been presented as a new target for anti-cancer treatment. Many inhibitors of HSP90 are under clinical trials, during which agents like the 17-allylamino-17-demethoxygeldana-mycin (17-AAG) radical and PU-H71 caused depletion of the Lats1

and Lats2 proteins in the cells, lowering phosphorylation of YAP. Thus HSP90 inhibitors disrupt the Lats tumour suppressor signalling and contribute to tumorigenesis (Huntoon et al., 2010). During prostate cancer treatment with the anti-cancer drug cisplatin, expression of HSP70 is induced. However, HSP70 mediates degradation of the Mst1 kinase, whereby decreasing its pro-apoptotic activity and increasing resistance of tumour cells to cisplatin (Ren et al., 2008). The other potential therapeutic target is CD44. Increased expression of CD44 was observed in many malignant tumours, most recently in glioblastoma multiformae, which is the most aggressive brain tumour that is characterised by resistance to chemotherapy and radiotherapy. CD44 inhibits the activity of the Hippo signalling pathway. In glioblastoma multiformae, the loss of CD44 function results in activation of the signalling pathways leading to apoptosis, suggesting CD44 as a potential target of anti-cancer therapy (Xu et al., 2010).

These few examples show that the Hippo pathway has an important role in regulating the malignancy of cancer cells and also presents a potential target for anti-cancer therapy, including its upstream and downstream members and regulators.

5. Conclusions and further perspectives

This thesis focuses on the recently found molecular mechanisms of action of the anti-cancer agent from the group of VE analogues, α-TOS, the drug that was shown to selectively induce apoptosis in various types of cancer cells. It was found that the direct target of this agent resides in mitochondria, epitomising an intriguing, invariant target for anti-cancer treatment due to their infrequent mutations. α-TOS targets the mitochondrial CII and by displacing UbQ from its binding sites causes generation of ROS. This event was shown to induce the expression of the Noxa protein, resulting in mitochondrial permeabilisation by way of formation of Bak pores in the OMM. Noxa was previously known as a direct target of p53, but in this model Noxa is upregulated in a p53-independent manner. This is of importance, since many tumours carry mutations in the *p53* gene that contributes to their resistance towards anti-cancer agents.

The current research demonstrates that ROS production causes activation of Mst1 kinase, the component of Hippo signalling. Deregulation of particular components of this pathway contributes to the loss of contact inhibition and unlimited growth, hallmarks of cancer cells. Although it is known that Mst1 is activated by ROS, the mechanism of this

process remains obscure. Activated Mst1 has been shown to regulate several downstream components. Mst1 inhibits the transcription co-activator YAP via the Lats kinase. Collectively, this will result in the inhibition of transcription of anti-apoptotic genes and genes supporting cellular growth such as *c-MYC, SOX4, H19, AFP, survivin,* or *cIAP1*. Of these, the inhibition of *c-MYC* and its target genes encoding proteins of glucose and glutamine metabolism by Mst1 in response to α-TOS treatment is a current focus of our research. The validation of inhibition of expression of c-Myc target genes would mean that besides inducing apoptosis, α-TOS also causes starvation of cancer cells for energy sources. The *NOXA* gene expression was demonstrated to be regulated by the FoxO1 transcription factor, which is directly phosphorylated and thus activated by the Mst1 kinase. The other pro-apoptotic genes *FASL* and *TRAIL* were also found to be upregulated via the Mst1-FoxO1 pathway. The importance of all these findings is further accentuated by the fact that Noxa is upregulated in p53-independent manner, involving the Mst1 kinase. It will be important to show whether these proof-of-principle results are of universal importance for a variety of cancer types and whether they still hold in tumour suppression in animal cancer models.

The finding that α-TOS acts through the Mst1 kinase by several mechanisms leading to inhibition of tumour development and apoptosis induction in cancer cells highlights the potential of the kinase as an important target of anti-cancer drugs and also presents α-TOS as an effective anti-cancer agent. This points out to the fact that it is of importance to search for new therapeutic agents that can destroy cancer cells by inducing signalling routes such as the Hippo pathway. There is some hope in this regard. Recently an analogue of α-TOS tagged with the positively charged triphenylphosphonium, the mitochondrially targeted vitamin E succinate (MitoVES), has been shown to accumulate in mitochondria and promote apoptosis much more efficiently than the parental α-TOS (Dong et al, 2011a). Importantly, MitoVES also utilises the Mst1-FoxO1 pathway (Dong et al., 2011b), corroborating its importance as an intriguing target for cancer therapy.

6. References

Ahn, S.H., Cheung, W.L., Hsu, J.Y., Diaz, R.L., Smith, M.M., Allis, C.D.: Cell 120, 25-36 (2005)

Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K., Walter, P.: Molecular Biology of the Cell, 5th Edition, Garland science, New York, Abingdon (2008)

Almasan, A., Ashkenazi, A.: Cytokine Growth Factor Rev. 14, 337-348 (2003)

American Cancer Society. Cancer Facts & Figures 2010. Atlanta: American Cancer Society (2010)

Ashe, P.C., Berry, M.D.: Prog. Neuropsychopharmacol Biol. Psychiatry 27, 199-214 (2003)

Ashkenazi, A., Dixit, V.M.: Science 281, 1305-1308 (1998)

Ashkenazi, A.: Cytokine Growth Factor Rev. 19, 325-331 (2008)

Badouel, C., Gardano, L., Amin, N., Garg, A., Rosenfeld, R., Le Bihan, T., McNeill, H.: Dev. Cell 16, 411-420 (2009)

Birringer, M., EyTina, J.H., Salvatore, B.A., Neuzil, J.: Br. J. Cancer 88, 1948-1955 (2003)

Bixby, J., Ray, T.D., Chan, F.K.M.: Curr. Med. Chem. 4, 557-567 (2005)

Boatright, K.M., Salvesen, G.S.: Curr. Opin. Cell Biol. 15, 725-731 (2003)

Brenner, D., Mak, T.W.: Curr. Opin. Cell Biol. 21, 871-877 (2009)

Brigelius-Flohé, R., Traber, M.G.: FASEB J. 13, 1145-1155 (1999)

Brunelle, J.K., Letai, A.: J. Cell Sci. 122, 437-441 (2009)

Brunet, A., Bonni, A., Zigmond, M.J., Lin, M.Z., Juo, P., Hu, L.S., Anderson, M.J., Arden, K.C., Blenis, J., Greenberg, M.E.: Cell 96, 857-868 (1999)

Cairns, R.A., Harris, I.S., Mak, T.W.: Nat. Rev. Cancer 11, 85-95 (2011)

Calnan, D.R., Brunet, A.: Oncogene 27, 2276-2288 (2008)

Cheung, W.L., Ajiro, K., Samejima, K., Kloc, M., Cheung, P., Mizzen, C.A., Beeser, A., Etkin, L.D., Chernoff, J., Earnshaw, W.C., Allis, C.D.: Cell 113, 507-517 (2003)

Chipuk, J.E., Moldoveanu, T., Llambi, F., Parsons, M.J., Green, D.R.: Mol. Cell 12, 299-310 (2010)

Curtin, J.F., Cotter, T.G.: Cell. Signal. 15, 983-992 (2003)

Czymai, T., Viemann, D., Sticht, C., Molema, G., Goebeler, M., Schmidt, M.: J. Biol. Chem. 285, 10163-10178 (2010)

Dang, C.V., Le, A., Gao, P.: Clin. Cancer Res. 15, 6479-6483 (2009)

Denecker, G., Vercammen, D., Declercq, W., Vandenabeele, P.: Cell. Mol. Life Sci. 58, 356-370 (2001)

Dewson, G., Kluck, R.M.: J. Cell Sci. 122, 2801-2808 (2009)

Dong, L.F., Jameson, V.J.A., Tilly, D., Cerny, J., Mahdavian, E., Marín-Hernández, A., Hernández-Esquivel, L., Rodríguez-Enríquez, S., Strusa, J., Witting, P.K., Stantic, B., Rohlena, J., Truksa, J., Kluckova, K., Dyason, J.C., Ledvina, M., Salvatore, B.A., Moreno-Sánchez, R., Coster, M.J., Ralph, S.J., Smith, R.A.J., Neuzil, J.: J. Biol. Chem. 286, 3717-3728 (2011b)

Dong, L.F., Jameson, V.J.A., Tilly, D., Prochazka, L., Rohlena, J., Valis, K., Truksa, J., Zobalova, R., Mahdavian, E., Kluckova, K., Stantic, M., Strusa, J., Wang, X.F., Freeman, R., Witting, P.K., Norbert, E., Goodwin, J., Salvatore, B.A., Novotna, J., Turanek, J., Ledvina, M., Hozak, P., Zhivotovsky, B., Coster, M.J., Ralph, S.J., Smith, R.A.J., Neuzil, J.: Free Radic. Biol. Med. 50, 1546-1555 (2011a)

Dong, L.F., Low, P., Dyason, J.C., Wang, X.F., Prochazka, L., Witting, P.K., Freeman, R., Swettenham, E., Valis, K., Liu, J., Zobalova, R., Turanek, J., Spitz, D.R., Domann, F.E., Scheffler, I.E., Ralph, S.J., Neuzil, J.: Oncogene 27, 4324-4335 (2008)

Fausto, N., Campbell, J.S., Riehle, K.J.: Hepatology 43, 45-53 (2006)

Feng, Y., Irvine, K.D.: Proc. Natl. Acad. Sci. USA 104, 20362-20367 (2007)

Fu, Z., Tindall, D.J.: Oncogene 27, 2312-2319 (2008)

Garrido, C., Galluzzi, L., Brunet, M., Puig, P.E., Didelot, C., Kroemer, G.: Cell Death Differ. 13, 1423-1433 (2006)

Genevet, A., Wehr, M.C., Brain, R., Thompson, B.J., Tapon, N.: Dev. Cell. 18, 300-308 (2010)

Ghobrial, I.M., Witzig, T.E., Adjei, A.A.: CA Cancer J. Clin. 55, 178-194 (2005)

Glantschnig, H., Rodan, G.A., Reszka, A.A.: J. Biol. Chem. 277, 42987-42996 (2002)

Greer, E.L., Brunet, A.: Oncogene 24, 7410-7425 (2005)

Hanahan, D., Weinberg, R., A.: Cell 144, 646-674 (2011)

http://www.pdb.org/pdb/explore/explore.do?structureId=3COM (31.5.2011)

Huang, H., Tindall, D.J.: J. Cell Sci. 120, 2479-2487 (2007)

Huntoon, C.J., Nye, M.D., Geng, L., Peterson, K.L., Flatten, K.S., Haluska, P., Kaufmann, S.H., Karnitz, L.M.: Cancer Res. 70, 8642-8650 (2010)

Jones, S., Zhang, X., Parsons, D.W., Lin, J.C., Leary, R.J., Angenendt, P., Mankoo, P., Carter, H., Kamiyama, H., Jimeno, A., Hong, S.M., Fu, B., Lin, M.T., Calhoun, E.S.,

Kamiyama, M., Walter, K., Nikolskaya, T., Nikolsky, Y., Hartigan, J., Smith, D.R., Hidalgo, M., Leach, S.D., Klein, A.P., Jaffee, E.M., Goggins, M., Maitra, A., Iacobuzio-Donahue, C., Eshleman, J.R., Kern, S.E., Hruban, R.H., Karchin, R., Papadopoulos, N., Parmigiani, G., Vogelstein, B., Velculescu, V.E., Kinzler, K.W.: Science 321 1801-1806 (2008)

Kang, M.H., Reynolds, C.P.: Clin. Cancer Res. 15, 1126-1132 (2009)

Krakstad, C., Chekenya, M.: Mol. Cancer 9, 135 (2010)

Kurosaka, K., Takahashi, M., Watanabe, N., Kobayashi, Y.: J. Immunol. 171, 4672-4679 (2003)

Lai, D., Ho, K.C., Hao, Y., Yang, X.: Cancer Res. 71, 2728-2738 (2011)

Lamkanfi, M., Declercq, W., Kalai, M., Saelens, X., Vandenabeele, P.: Cell Death Differ. 9, 358-61 (2002)

Ledgerwood, E.C., Morison, I.M.: Clin. Cancer Res. 15, 420-424 (2009)

Lee, K.K., Ohyama, T., Yajima, N., Tsubuki, S., Yonehara, S.: J. Biol. Chem. 276, 19276-19285 (2001)

Lehtinen, M.K., Yuan, Z., Boag, P.R., Yang, Y., Villén, J., Becker, E.B., DiBacco, S., de la Iglesia, N., Gygi, S., Blackwell, T.K., Bonni, A.: Cell 125, 987-1001 (2006)

Lin, A., Karin, M.: Semin. Cancer Biol. 13, 107-114 (2003)

Marsden, V.S., Strasser, A.: Annu. Rev. Immunol. 21, 71-105 (2003)

Mazarakis, N., Edwards, A., Mehmet, H.: Arch. Dis. Child Fetal Neonatal Ed. 77, 165-170 (1997)

Neto-Silva, R.M., de Beco, S., Johnston, L.A.: Dev. Cell 19, 507-520 (2010)

Neuzil, J., Dong, L.F., Ramanathapuram, L., Hahn, T., Chladova, M., Wang, X.F., Zobalova, R., Prochazka, L., Gold, M., Freeman, R., Turanek, J., Akporiaye, E.T., Dyason, J.C., Ralph, S.J.: Mol. Aspects Med. 28, 607-645 (2007a)

Neuzil, J., Dyason, J.C., Freeman, R., Dong, L.F., Prochazka, L., Wang, X.F., Scheffler, I., Ralph, S.J.: J. Bioenerg. Biomembr. 39, 65-72 (2007b)

Neuzil, J., Tomasetti, M., Mellick, A.S., Alleva, R., Salvatore, B.A., Birringer, M., Fariss, M.W.: Curr. Cancer Drug Targets 4, 355-372 (2004)

Neuzil, J., Wang, X.F., Dong, L.F., Low, P., Ralph, S.J.: FEBS Lett. 580, 5125-5129 (2006)

Neuzil, J., Weber, T., Gellert, N., Weber, C.: Br. J. Cancer. 84, 87-89 (2001)

Neuzil, J., Zhao, M., Ostermann, G., Sticha, M., Gellert, N., Weber, C., Eaton, J.W., Brunk, U.T.: Biochem. J. 362, 709-715 (2002)

Obexer, P., Geiger, K., Ambros, P.F., Meister, B., Ausserlechner, M.J.: Cell Death Differ. 14, 534-547 (2007)

Oh, H., Irvine, K.D.: Trends Cell Biol. 20, 410-417 (2010)

Oh, H., Reddy, B.V., Irvine, K.D.: Dev. Biol. 335, 188-197 (2009)

Pan, D.: Dev. Cell 19, 491-505 (2010)

Parsons, D.W., Jones, S., Zhang, X., Lin, J.C., Leary, R.J., Angenendt, P., Mankoo, P., Carter, H., Siu, I.M., Gallia, G.L., Olivi, A., McLendon, R., Rasheed, B.A., Keir, S., Nikolskaya, T., Nikolsky, Y., Busam, D.A., Tekleab, H., Diaz, L.A. Jr., Hartigan, J., Smith, D.R., Strausberg, R.L., Marie, S.K., Shinjo, S.M., Yan, H., Riggins, G.J., Bigner, D.D., Karchin, R., Papadopoulos, N., Parmigiani, G., Vogelstein, B., Velculescu, V.E., Kinzler, K.W.: Science 321, 1807-1812 (2008)

Polesello, C., Huelsmann, S., Brown, N.H., Tapon, N.: Curr. Biol. 16, 2459-2465 (2006)

Pop, C., Salvesen, G.S.: J. Biol. Chem. 284, 21777-21781 (2009)

Prochazka, L., Dong, L.F., Valis, K., Freeman, R., Ralph, S.J., Turanek, J., Neuzil, J.: Apoptosis 15, 782-794 (2010)

Ralph, S.J., Neuzil, J.: Mol. Nutr. Food Res. 53, 9-28 (2009)

Ren, A., Yan, G., You, B., Sun, J.: Cancer Res. 68, 2266-2274 (2008)

Rena, G., Guo, S., Cichy, S.C., Unterman, T.G., Cohen, P.: J. Biol. Chem. 274, 17179-17183 (1999)

Ruvolo, P.P., Deng, X., Ito, T., Carr, B.K., May, W.S.: J. Biol. Chem. 274, 20296-20300 (1999)

Schafer, Z.T., Kornbluth, S.: Dev. Cell 10, 549-561 (2006)

Shiau, C.W., Huang, J.W., Wang, D.S., Weng, J.R., Yang, C.C., Lin, C.H., Li, C., Chen, C.S.: J. Biol. Chem. 281, 11819-11825 (2006)

Steinhardt, A.A., Gayyed, M.F., Klein, A.P., Dong, J., Maitra, A., Pan, D., Montgomery, E.A., Anders, R.A.: Hum. Pathol. 39, 1582-1589 (2008)

Taylor, R.C., Cullen, S.P., Martin, S.J.: Nat. Rev. Mol. Cell Biol. 9, 231-241 (2008)

Valis, K., Prochazka, L., Boura, E., Chladova, J., Obsil, T., Rohlena, J., Truksa, J., Dong, L.F., Ralph, S.J., Neuzil, J.: Cancer Res. 71, 946-954 (2011)

Vander Heiden, M.G., Cantley, L.C., Thompson, C.B.: Science 324, 1029-1033 (2009)

Vivanco, I., Sawyers, C.L.: Nat. Rev. Cancer 2, 489-501 (2002)

Voet, D., Voet, J.G.: Biochemistry, 3rd Edition, Wiley, New York (2004)

Wang, K., Degerny, C., Xu, M., Yang, X.J.: Biochem. Cell Biol. 87, 77-91 (2009)

Wang, X., Quinn, P.J.: Prog Lipid Res. 38, 309-336 (1999)

Wang, X.F., Dong, L., Zhao, Y., Tomasetti, M., Wu, K., Neuzil, J.: Mol. Nutr. Food Res. 50, 675-685 (2006)

Wang, Y., Dong, Q., Zhang, Q., Li, Z., Wang, E., Qiu, X.: Cancer Sci. 101, 1279-1285 (2010)

Weber, T., Dalen, H., Andera, L., Nègre-Salvayre, A., Augé, N., Sticha, M., Lloret, A., Terman, A., Witting, P.K., Higuchi, M., Plasilova, M., Zivny, J., Gellert, N., Weber, C., Neuzil, J.: Biochemistry 42, 4277-4291 (2003)

Wei, X., Shimizu, T., Lai, Z.C.: EMBO J. 26, 1772-1781 (2007)

Weinberg, R.A.: CA Cancer J. Clin. 44:160-170 (1994)

Wen, W., Zhu, F., Zhang, J., Keum, Y.S., Zykova, T., Yao, K., Peng, C., Zheng, D., Cho, Y.Y., Ma, W.Y., Bode, A.M., Dong, Z.: J. Biol. Chem. 285, 39108-39116 (2010)

Willecke, M., Hamaratoglu, F., Sansores-Garcia, L., Tao, C., Halder, G.: Proc. Natl. Acad. Sci. USA 105, 14897-14902 (2008)

Wise, D.R., Thompson, C.B.: Trends Biochem. Sci. 35, 427-433 (2010)

Wu, S., Huang, J., Dong, J., Pan, D.: Cell 114, 445-456 (2003)

Xu, Y., Stamenkovic, I., Yu, Q.: Cancer Res. 70, 2455-2464 (2010)

Xuan, Z., Zhang, M.Q.: Mech. Ageing Dev. 126, 209-215 (2005)

Yu, J., Zhang, L.: Biochem. Biophys. Res. Commun. 331, 851-858 (2005)

Yu, J., Zheng, Y., Dong, J., Klusza, S., Deng, W.M., Pan, D.: Dev. Cell 18, 288-299 (2010)

Yuan, Z., Lehtinen, M.K., Merlo, P., Villén, J., Gygi, S., Bonni, A.: J. Biol. Chem. 284, 11285-11292 (2009)

Zeng, Q., Hong, W.: Cancer Cell 13, 188-192 (2008)

Zhang, N., Bai, H., David, K.K., Dong, J., Zheng, Y., Cai, J., Giovannini, M., Liu, P., Anders, R.A., Pan, D.: Dev. Cell. 19, 27-38 (2010)

Zhang, X., George, J., Deb, S., Degoutin, J.L., Takano, E.A., Fox, S.B., AOCS Study group, Bowtell, D.D., Harvey, K.F.: Oncogene Epub ahead of print (2011b)

Zhang, X., Tang, N., Hadden, T.J., Rishi, A.K.: Biochim. Biophys. Acta. Epub ahead of print (2011a)

Zhao, B., Lei, Q.Y., Guan, K.L.: Curr. Opin. Cell Biol. 20, 638-646 (2008)

Zhao, B., Lei, Q.Y., Guan, K.L.: Curr. Opin. Cell Biol. 20, 638-646 (2008)

Zhao, B., Wei, X., Li, W., Udan, R.S., Yang, Q., Kim, J., Xie, J., Ikenoue, T., Yu, J., Li, L., Zheng, P., Ye, K., Chinnaiyan, A., Halder, G., Lai, Z.C., Guan, K.L.: Genes Dev. 21, 2747-2761 (2007)

Zhou, D., Conrad, C., Xia, F., Park, J.S., Payer, B., Yin, Y., Lauwers, G.Y., Thasler, W., Lee, J.T., Avruch, J., Bardeesy, N.: Cancer Cell 16, 425-438 (2009)

Ziegler, U., Groscurth, P.: News Physiol. Sci. 19, 124-128 (2004)

I agree with the lending of this work for study purposes and I ask for the proper record of the borrowers.

Name and surname with address	Number of OP	Date of rental	Note