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**The study of the influence of vitamin E analogues
on malignant mesothelioma**

**Studium vlivu analogů vitamínu E
na maligní mezoteliom**

PhD Thesis

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Statement of originality

I declare that I have accomplished this final thesis independently and that I have included all cited literature and used sources of information. Neither the work as the whole nor any its substantial part has been previously submitted to achieve a different or similar academic degree.

Prague

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Abstract

Cancer is a leading cause of death in the western world and is increasing in frequency world-wide. Although diagnosis, treatment and therapeutic approaches to cancer have improved, many types of cancer are still lethal due to the lack of radical treatment. One of the fatal neoplastic disease types with poor prognosis is represented by malignant mesothelioma (MM). MM is characterised by very high mortality rate and limited therapeutic options. The etiology of the disease is mainly associated with exposure to asbestos fibres. The incidence of MM is increasing in many countries. The search for novel molecular targets, anti-cancer strategies and drugs, which would considerably improve the treatment is of great importance. Certain new drugs, especially those with specific molecular targets, show high selectivity in their action to cancer cells, and have considerably increased the cure rate in some types of cancer. Mitochondria have recently emerged as a very promising target for anti-cancer agents. A group of compounds with anti-cancer activity that induce apoptosis by way of mitochondrial destabilisation, termed 'mitocans', have been a recent focus of research. Several mitocans have been shown to selectively induce apoptosis in cancer cells and suppress the growth of many types of carcinomas in pre-clinical models.

The aim of this PhD thesis was to study the pro-apoptotic effect of mitocans from the group of vitamin E analogues epitomised by the redox-silent α -tocopheryl succinate (α -TOS) and its modified form, mitochondrially targeted analogue of vitamin E succinate (MitoVES) on MM cells. The experiments were performed on MM cells due to the very aggressive nature of this treatment-resistant cancer. Both vitamin E (VE) analogues were known from previous studies to selectively induce apoptosis in cancer cells and suppress the growth of many types of carcinomas in pre-clinical models. At the molecular level they act as Bcl-2 homology domain 3 (BH3) mimetics and sensitise cancer cells to other drugs. More importantly, they induce apoptosis by affecting the mitochondrial complex II (CII) of the electron redox chain. Thereby they interfere with ubiquinone, the natural acceptor for electrons generated by the succinate dehydrogenase activity of CII during conversion of succinate to fumarate. The electron flow is disrupted and reactive oxygen species (ROS) are formed. These ROS diffuse into cytoplasm in the form of hydrogen peroxide and are converted into more reactive radical species that catalyse activation of the pro-apoptotic proteins, viz. oligomerisation of Bax or Bak, resulting in the formation of a megachannel in the mitochondrial outer membrane. This leads to the translocation of cytochrome c into cytosol via the channel, and the downstream mitochondrial pathway of apoptosis is initiated. In contrast to the untargeted prototypic VE analogue α -TOS, MitoVES has been modified with a triphenylphosphonium (TPP⁺) group, which is important for the directed docking and accumulation of the compound in the mitochondrial inner membrane of a cancer cell, providing a much stronger apoptogenic efficacy.

In this work, it has been demonstrated that MitoVES is significantly more efficient in killing MM cells than α -TOS with IC₅₀ lower by up to two orders of magnitude. Further, the mitochondrial accumulation of MitoVES in MM cells has been proved by confocal microscopy using its fluorescently tagged analogue (MitoVES-F). Shortening the aliphatic chain of MitoVES spanning the chromanol and TPP⁺ groups has reduced the efficacy of the agent, suggesting that its possible target might be the mitochondrial CII. The function of CII in the activity of MitoVES has been confirmed, as MM cells with suppressed

succinate quinone reductase were resistant to the agent. Further, MitoVES has induced apoptosis in MM cells via mitochondrial destabilisation, resulting in the decrease of mitochondrial membrane potential and generation of ROS. A mouse model has been used to show the superior anti-MM activity of MitoVES to α -TOS. All data obtained within this work have proven that mitochondrial targeting of vitamin E succinate endows the agent with considerably higher efficacy to kill MM cells, bringing it to the vicinity of its molecular target.

Another part of this PhD project involves a proteomic and genomic study of the influence of α -TOS on MM cells. Using 2-dimensional gel electrophoresis, a variety of up- or down-regulated proteins have been identified after the treatment of MM cells with the VE analogue. Some of these proteins have been confirmed by other techniques, including RT-PCR, real-time quantitative RT-PCR and western blot. We have focused on the cytoskeletal protein septin 11, which has been found to be down-regulated after the treatment with α -TOS by all used techniques. Unfortunately, the subsequent gene silencing experiments have failed. Due to the oncoming clinical trials (planned by our laboratory), there was a need to focus more on the project described above, i.e. the mechanisms of killing of MM cells by MitoVES. Notwithstanding this focus, it is worth to complete the second part of this project, as the obtained results seem to be very interesting since septin 11 and the several other identified proteins have been described by previous studies to play important roles in a variety of functions of (cancer) cells, including regulation of apoptosis, cell proliferation, carcinogenesis and metastasis. It is planned that this part of the project will be completed in a follow up study in our laboratory.

The context of this work is within the potential cure of MM, a thus far fatal disease. Scientifically, it is believed that the acquired outcomes from this work will contribute to the emerging notion that mitocans hold a substantial promise to be developed into efficient anti-cancer drugs that will be utilised to help curb the increasing incidence of neoplastic diseases, as epitomised here by the extremely hard-to-treat mesothelioma.

Souhrn

Rakovina je nejčastější příčinou smrti v západním světě a frekvence jejího výskytu stále stoupá. Přestože k dnešní době došlo k výraznému zlepšení v diagnostice a přístupu k léčbě, mnoho typů rakoviny je stále letální díky nedostatečné radikální terapii. Jedním z fatálních neoplastických druhů této choroby je maligní mezoteliom (MM). MM je charakterizován vysokou úmrtností a limitovanými terapeutickými možnostmi. Etiologie tohoto onemocnění je především spojována s inhalací azbestových vláken. Výskyt MM se zvyšuje v mnoha zemích; proto je velmi důležité hledat nová molekulární zásahová místa, protirakovinné strategie a léčiva, která by výrazně pomohla zkvalitnit terapii. Určitá nová léčiva, především ta, která mají specifické molekulární cíle a vykazují se vysokou selektivitou pro rakovinné buňky, významně zvýšila úspěšnost léčby některých druhů rakoviny. Velmi slibný molekulární cíl pro protirakovinné látky tvoří mitochondrie. V posledních letech se výzkum zaměřil na skupinu protirakovinných sloučenin, zvaných 'mitokany', které indukují apoptózu v rakovinných buňkách mitochondriální destabilizací. Bylo publikováno, že některé mitokany vyvolávají apoptózu selektivně v rakovinných buňkách a inhibují růst mnoha druhů karcinomu v preklinických modelech.

Cílem této doktorské práce bylo studium proapoptotického vlivu mitokanů ze skupiny derivátů vitamínu E, reprezentovaných α -tokoferylsukcinátem (α -TOS) a jeho modifikovanou formou - mitochondriálně cíleným analogem vitamín E sukcinátu (MitoVES) na MM buňky. MM buňky byly vybrány pro testování zmíněných látek kvůli agresivnímu charakteru této rezistentní formy rakoviny. Oba analogy vitamínu E byly známy již z předchozích studií pro jejich schopnost indukovat apoptózu v rakovinných buňkách a potlačovat růst mnoha druhů rakoviny v preklinických modelech. Na molekulární úrovni působí tyto látky jako BH3 (Bcl-2 homology domain-3) mimetika, která zvyšují citlivost rakovinných buněk vůči jiným protirakovinným léčivům. Oba mitokany indukují apoptózu zasažením mitochondriálního komplexu II (CII) elektronového redoxního řetězce. Přitom interferují s ubichinonem, přirozeným akceptorem elektronů, vzniklých aktivitou sukcinátdehydrogenázy CII elektronového transportního řetězce během přeměny sukcinátu na fumarát. Tok elektronů je tímto způsobem přerušen a dochází k tvorbě kyslíkových radikálů, které difundují do cytoplazmy ve formě peroxidu vodíku a které jsou následně přeměněny na více reaktivní typy radikálů, katalyzující aktivaci proapoptotických proteinů, konkrétně oligomerizaci proteinů Bax nebo Bak. Následkem této oligomerizace je vytvoření multimerního kanálu v mitochondriální vnější membráně. Tímto kanálem dochází k uvolnění cytochromu c do cytosolu a následné aktivaci mitochondriální apoptotické dráhy. Na rozdíl od prototypického analogu vitamínu E, α -TOSu, je MitoVES modifikován trifenylofosfóniovou skupinou (TPP^+), která je zásadní pro cílené ukotvení a akumulaci této látky v mitochondriální vnitřní membráně rakovinné buňky a zajišťuje tak mnohem silnější apoptotickou účinnost látky.

Tato práce ukazuje, že MitoVES je výrazně účinnější v zabíjení MM buněk ve srovnání s α -TOSem s hodnotami IC_{50} nižšími až o dva řády. Dále je v práci dokumentována akumulace MitoVESu v mitochondriích MM buněčných liniích pomocí konfokální mikroskopie a fluorescenčně označeného analogu MitoVESu. Zkracování alifatického řetězce MitoVESu, které spojuje skupinu TPP^+ a chromanolovou část molekuly, vede ke snížení účinnosti látky. Toto pozorování indikuje, že mitochondriální CII by mohl být cílem působení MitoVESu. Úloha CII v aktivitě MitoVESu byla dále potvrzena tím,

že MM buňky s inhibovanou sukcinát-ubichinonreduktázou byly rezistentní vůči této látce. V dalších experimentech indukoval MitoVES apoptózu v MM buněčných liniích mitochondriální destabilizací, což se projevilo poklesem mitochondriálního membránového potenciálu a tvorbou volných kyslíkových radikálů. Výrazně vyšší anti-mezoteliomální aktivita MitoVESu nad α -TOSem byla také prokázána na myším modelu. Všechna data, získaná v tomto projektu dokazují, že cílení vitamín E sukcinátu do mitochondrií a dopravení k molekulárnímu zásahovému místu působení obdařuje tuto látku výrazně vyšší schopností zabíjet MM buňky.

Další část této doktorské práce zahrnuje proteomickou a genomickou studii vlivu α -TOSu na MM buňky. Pomocí 2-rozměrné gelové elektroforézy a hmotnostní spektrometrie bylo identifikováno několik proteinů se zvýšenou či sníženou expresí po působení zmíněného derivátu vitamínu E na MM buňky. Rozdílnou expresi některých z těchto proteinů či jejich kódujících genů se podařilo potvrdit jinými technikami, zahrnujícími RT-PCR, real-time kvantitativní RT-PCR a western blot. Zaměřili jsme se na cytoskeletální protein septin 11, který byl identifikován všemi zmíněnými metodami jako snížený po působení α -TOSu. V následujících experimentech se však nepodařilo utlumit gen, kódující protein septin 11. Z důvodu blížících se klinických testů plánovaných naší laboratoří bylo nezbytné zaměřit se na část projektu popsanou výše, tj. mechanismus zabíjení MM buněk MitoVESem. Přesto však má význam, sledovat a dokončit tento druhý projekt, jelikož získané výsledky mají velice zajímavou podstatu. Jak je známo z dřívější literatury, septin 11 a některé další identifikované proteiny hrají důležitou roli při různých funkcích, zahrnujících regulaci apoptózy, buněčné proliferace, karcinogeneze a tvorby metastáz. Proto je plánováno, že tato část projektu bude dokončena v následující studii v naší laboratoři

Tato práce je v kontextu s potenciální léčbou MM, který je doposud smrtelným onemocněním. Vědecky vzato mohou výsledky získané v této práci přispět ke vznikající nadějně předstávě, že mitokany mohou být vyvinuty v efektivní protirakovinná léčiva, která budou použita za účelem omezení rostoucího výskytu neoplastických onemocnění, jako je zde představený, velmi složitě léčitelný mezoteliom.

List of publications

1. **Kovarova J**, Bajzikova M, Vondrusova M, Stursa J, Goodwin J, Nguyen M, Zabalova R, Pesdar EA, Truksa J, Tomasetti M, Dong LF, Neuzil J (2013) Efficiently suppression of malignant mesothelioma by mitochondrially targeting of α -tocopheryl succinate. Under review in BMC Cancer.
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List of abbreviations

A	Adenine
ABCA1	ATP-binding cassette transporter 1
AcCoA	Acetyl-coenzyme A
ADP	Adenosine diphosphate
AIF	Apoptosis-inducing factor
AJCC	American Joint Committee on Cancer
ANT	Adenine nucleotide translocase
AldDeh (ALDH)	Aldehyde dehydrogenase
APAF1	Apoptotic protease activating factor-1
APC	Adenomatous polyposis coli
Apo	Apoptosis antigen
ARP3	ARP3 actin-related protein 3 homolog variant
α -, β -, γ -, δ -TOH	α -, β -, γ -, δ -Tocopherol
α -TOS	α -Tocopherylsuccinate
α -TOS-F	Fluorescently labelled α -Tocopherylsuccinate
ARF	ADP ribosylation factor
ATP	Adenosine triphosphate
ATP5B	β subunit of H^+ transporting F1 complex of mitochondrial ATP synthase
Bak	BCL-2 antagonist/killer-1
Bad	Bcl-2 antagonist of cell death
Bax	Bcl-2-associated X protein
Bcl	B-cell lymphoma
Bcl-2	B-cell lymphoma 2
Bcl-xl	B-cell lymphoma-extra large
BH domain	Bcl-2 homology domain
BH3	Bcl-2 homology domain 3
Bid	BH3-interacting-domain death agonist
tBid	Truncated Bid
Bik	Bcl-2-interacting killer
Bim (= Bod)	Bcl-2-like-11
BIR	Baculoviral IAP repeat
Bmf	Bcl-2 modifying factor

Bok	Bcl-2-related ovarian killer
3BP	3-bromopyruvate
BRCA	Breast cancer antigen
C	Cytosine
CI	Mitochondrial complex I
CII	Mitochondrial complex II
CIII	Mitochondrial complex III
CIV	Mitochondrial complex IV
CV	Mitochondrial complex V
CARD	Caspase recruitment domain
CCCP	Carbonyl cyanide m-chlorophenyl hydrazone
CEBP α	CCAAT-enhancer binding protein- α
cFLIP	Cellular FLICE inhibitory protein
CHOP	CEBP-homologous protein
cIAP	Cellular inhibitor of apoptosis protein
CL	Cardiolipin
CRD	Cysteine-rich domain
CT	computed tomography
Cyt c	Cytochrome c
DCA	Dichloroacetate
DCF-DA	Dichlorodihydro-fluorescein diacetate
DCPIP	2',6'-dichloroindophenol
DcR (1; 2)	Decoy receptor (1; 2)
DED	Death effector domain
DD	Death domain
DHE	Dihydroethidium
2DG	2-deoxyglucose
2-D GE	2-dimensional gel electrophoresis
DIABLO	Direct inhibitor of apoptosis protein-binding protein with low pI
DISC	Death-inducing signalling complex
DNA	Deoxyribonucleic acid
DR (3; 4; 5)	Death receptor (3; 4; 5)
$\Delta\psi_{m,i}$	Mitochondrial inner trans-membrane potential
$\Delta\psi_p$	Plasmatic membrane potential

EGFR	Epidermal growth factor receptor
Endo G	Endonuclease G
EPP	Extrapleural pneumonectomy
ER	Endoplasmic reticulum
ErbB-2	Erythroblastic leukaemia viral oncogene homolog 2
ERC	Electron respiratory chain
ETC	Electron transfer chain
EtOH	Ethanol
FAD	Flavine adenine dinucleotide
FADD	Fas-associated protein with a death domain
FADH ₂	Flavine adenine dinucleotide, reduced
Fas	also known as CD95
FasL	Fas ligand
FLICE	FADD-like interleukin-1 beta-converting enzyme (= caspase 8)
FLIP	Flice inhibitory protein
FMN	Flavin mononucleotide
FoxO1	Forkhead box protein O1
G	Guanine
Glut synth	Glutathione synthetase
GTP	Guanosine triphosphate
HER2	Human Epidermal Growth Factor Receptor 2
HPLC	High-performance liquid chromatography
HPNCC	Hereditary non-polyposis colon cancer
HRK	Harakiri
Hr-CNE	High resolution clear native electrophoresis
Hsp8	Heat shock70 kDa protein 8 isoform 1
HtrA2/Omi	High-temperaturerequirement protein A2
IAP	Inhibitor of apoptosis protein
IC ₅₀	Half maximal inhibitory concentration
IMIG	International Mesothelioma Interest Group
IMRT	Intensity-modulated radiotherapy
IMS	Mitochondrial intermembrane space
JNK	c-Jun N-terminal kinase
Mcl-1	Induced myeloid leukemia cell differentiation protein

MDM2	Mouse double minute 2 homolog
MEN	Multiple Endocrine Neoplasia
MIM (= IM)	Mitochondrial inner membrane
MitoVES	Mitochondrially targeted vitamin E analogue
MitoVES-F	Fluorescently labelled MitoVES
MM	Malignant mesothelioma
MOM (= OM)	Mitochondrial outer membrane
MOMP	Mitochondrial outer membrane permeabilisation
MS	Mass spectrometry
Mst1	Mammalian sterile 20-like kinase 1
mtDNA	Mitochondrial DNA
NAC	N-acetyl-L-cysteine
NADH	Nicotinamide adenine dinucleotide, reduced
NBE	Native blue electrophoresis
NFκB	Nuclear factor-κB
2'5'Oas	2'5'Oligoadenylatesynthetase
8OHdG	8-hydroxy-2'-deoxyguanosine
OXPPOS	Oxidative phosphorylation
p53	Protein 53
PCD	Programmed cell death
P/D	Pleurectomy/decortication
PDGF	Platelet-derived growth factor
PDI	Protein disulfide isomerase
PDIR5	Protein disulfide isomerase-related protein 5
P4H	Prolyl-4-hydroxylase
P _i	Inorganic phosphate
PI	Propidium iodide
PKC	Protein kinase C
PP2A	Protein phosphatase 2A
PUMA	p53 upregulated modulator of apoptosis
Q _d	Uniquinone distal binding site in CII
Q _i	Oxidation site
Q _o	Reduction site
Q _p	Uniquinone proximal binding site in CII

qRT-PCR	Quantitative real time polymerase chain reaction
ras	Retrovirus-associated DNA sequence
Rb	Retinoblastoma
ROS	Reactive oxygen species
RT	Radiation therapy; or Room temperature
RT-PCR	Reverse transcription polymerase chain reaction
SDH	Succinate dehydrogenase
SDHA;B;C or D	SDH subunit A;B;C or D
Smac	Second mitochondria-derived activator of caspases (= DIABLO)
SMase	Sphingomyelinase
SQR	Succinate quinone reductase
src	Rous sarcoma oncogene
SV40	Simian virus-40
T	Thymine
TBP7	Tat binding protein 7
TCA	Tricarboxylic acid
TM	Transmembrane domain
TMRM	Tetramethylrhodamine methyl ester
TNF	Tumour necrosis factor
TNFR	TNF receptor
TNM	Tumour-Node-Metastasis
TPP ⁺	Triphenylphosphonium
TRADD	TNFR type 1-associated DEATH domain protein
TRAIL	TNF-related apoptosis-inducing ligand (also known as Apo2L)
TRAIL-R (1;2;3;4)	TRAIL receptor (1; 2; 3; 4)
UbQ	Ubiquinone Q
UICC	International Union Against Cancer
VE	Vitamin E
VEGF	Vascular endothelial growth factor
VEGFR	VEGF receptor
VES	Vitamin E succinate
VDAC	Voltage-dependent anionic channel
WB	Western blot
XIAP	X-linked inhibitor of apoptosis protein

1 Introduction

1.1 Aims and objectives

Cancer presents one of the greatest challenges in clinical practice. Malignant cells exhibit high genetic instability, resulting in mutations with ensuing development of resistance against established drugs. This commonly involves tumour suppressor genes that are important for efficient anti-cancer activity of conventional anti-cancer agents as well as activation of proto-oncogenes, aggravating the treatment. Some types of cancer are completely resistant to treatment, so that patients die shortly after diagnosis. Further problem is the secondary harmful effect of many drugs to normal cells and tissues. Investigation of novel efficient anti-cancer agents overcoming these complications is of paramount importance.

In this work we aimed to study the pro-apoptotic effect of vitamin E (VE) analogues on malignant mesothelioma (MM) cells, consistent with the notion that VE analogues hold a considerable promise to be developed into selective and efficient anti-cancer agents. Our focus was on the novel form of α -tocopheryl succinate (α -TOS), the mitochondrially targeted vitamin E succinate (MitoVES), which is an epitome of a novel, efficient approach to cancer therapy.

Malignant mesothelioma (MM) is a very aggressive neoplastic pathology that arises primarily from the surface serosal cells mostly of the pleural and peritoneal cavities, as well as, less frequently, the pericardium or the tunica vaginalis. It is a relatively rare form of cancer, however, its incidence is stagnating or increasing world-wide. MM affects both males and females. Its mortality rate is very high with patient survival from diagnosis of less than 12 month. MM is resistant to conventional therapy and the treatment options for this aggressive malignancy are limited, hardly going beyond palliative care. One of the most promising mechanisms for the treatment of MM represents selective induction of apoptosis in MM cells (Tomasetti *et al.*, 2009).

Apoptosis or programmed cell death is a genetically controlled process, essential to maintain the homeostasis between proliferating and dying cells of an organism. Apoptotic

cell death occurs by either of two distinct pathways, the intrinsic or mitochondria-mediated pathway and the extrinsic or receptor-mediated pathway. These apoptotic pathways are significantly interconnected to amplify the total apoptotic response (Armstrong, 2006). Damaged or ‘abnormal’ cells undergo apoptosis and are, consequently, removed by surrounding cells and macrophages. Cancer cells can be induced to commit ‘suicide’ using a variety of agents, including VE analogues epitomised by α -TOS. The agent triggers mitochondria-mediated apoptotic pathway by increasing the intracellular reactive oxygen species (ROS) production and accumulation, which then triggers the apoptotic cascade. α -TOS has recently been modified to endow it with much more efficient targeting to cancer cell mitochondria. This modified form has been termed MitoVES, standing for mitochondrially targeted analogue of vitamin E succinate. Both VE analogues belong to the recently defined group of ‘mitocans’, which includes a number of selective anti-cancer agents (Neuzil *et al.*, 2007a,b).

1.2 Significance

Cancer is a major cause of death in industrialised countries and is becoming an increasing problem world-wide, even in countries where the incidence of cancer was low just a decade or so ago. For many types of cancer, such as MM, no effective treatment is available at present. Developing an anti-cancer drug that would cope with this human race-menacing disease would represent a significant breakthrough in cancer pathobiology. Since a number of mitocans are very efficient in killing cancer cells with high selectivity and since they are able to overcome various types of resistance towards other anti-cancer agents, they hold a substantial promise to be developed into efficient anti-cancer therapeutics.

2 Literature Review

2.1 Introduction: Cancer

A multicellular organism consists of a large number of different types of cells. Within a healthy organism, these cells grow, divide, die, and are eventually replaced with new cells in a highly regulated manner. When this orderly process goes wrong, cells proliferate uncontrollably, invading surrounding normal tissues, which leads to cancer development. The cancer cells may metastasise to distant parts of the body by the lymphatic system or the bloodstream. Cancer or malignant neoplastic pathologies include a wide category of diseases, which are all characterised by uncontrolled cell division. In humans, about 200 different types of cancer have been described (Cancer Research UK, 2012).

Cancer is always named for the location where it arose from, independently of where the neoplastic cells may spread. The majority of human malignancies arises in the epithelium. These cancer types are called carcinomas. Neoplastic diseases of the connective tissue of the body are referred to as sarcomas. Leukemias and lymphomas are cancers that develop from hematopoietic cells and cells of the immune system. Melanomas arise from darkly pigmented cells in the skin and gliomas originate in the nerve tissue (American Cancer Society, 2008).

Carcinogenesis is a multi-step process. Tumour formation often begins when a series of deleterious genetic mutations accumulates in an individual cell. These mutations allow for the cell to bypass the natural cellular anti-cancer defences, whereby the cell acquires a proliferation advantage and thus can be transformed to acquire a malignant phenotype (Doucas and Berry, 2006) (Figure 2.1). The sequence of events in which the damage occurs and how it progresses is rather unique to each cell, no two cancers are the same (Weinberg, 2007). Even after a tumour has developed, the genetic instability of cancer cells leads to further changes in the malignant phenotype. This variable nature of cancer allows to develop resistance to current anti-cancer treatments within the cancer progression. The incidence of most cancers grows with age, since genetic alterations that form the malignant phenotype of cancer generally appear over a long period of time (Doucas and Berry, 2006). Aging and growth of the world population together with an increasing adoption of cancer-

causing behaviour in economically developed countries makes cancer an increasing world-wide problem.

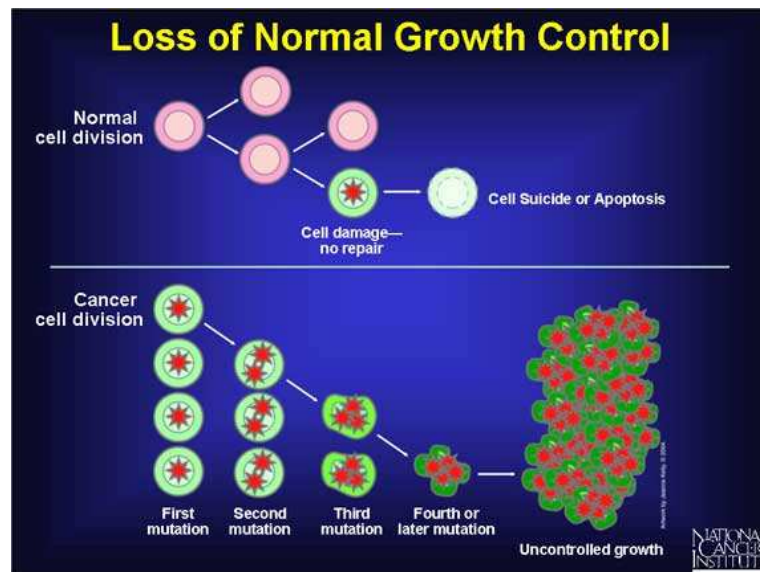


Figure 2.1: Loss of normal growth control.

(<http://www.cancer.gov/cancertopics/cancerlibrary/what-is-cancer>)

Cancer is now the leading cause of mortality in economically developed countries and the second most common cause of death in developed countries after cardiovascular disease. Based on the GLOBOCAN 2008 estimate, 12.7 million cancer cases and 7.6 million cancer deaths have been estimated to have occurred in 2008 world-wide, with more than half of those cases and deaths occurring in economically developed countries. The most frequently diagnosed cancers are breast cancer in females and lung cancer in males. They are the leading causes of cancer-related deaths in developed countries (Jemal *et al.*, 2011).

The incidence of various cancer types differs world-wide, depending on the influence of specific environmental factors. Certain life-style factors such as tobacco use, radiation, stress, obesity, diet, lack of physical activity, infections, and environmental pollutants can increase the risk of cancer development (Anand *et al.*, 2008). These risk factors develop their effects through the cellular genetic machinery. The environment is responsible for 90 to 95% cancer cases, whereas only 5 to 10% of all cancers are linked to genetics.

Tumourigenesis in humans is a multi-step process, reflecting genetic alterations that drive the progressive transformation of normal human cells into highly malignant derivatives

(Hanahan and Weinberg, 2000). Genes that play a crucial role in the development of cancer are oncogenes and tumour suppressor genes. In healthy cells, the activity of these genes is strictly controlled, and cell division proceeds in a correct manner and at the appropriate times, while in cancer, this balanced control of tissue growth is disordered (Doucas and Berry, 2006).

In normal cells, proto-oncogenes are regulatory genes participating in various intracellular signalling pathways controlling cell growth, proliferation and differentiation. A proto-oncogene can become an oncogene due to an increased expression or after acquired genetic alteration, which may lead to its abnormally increased or altered function. Such genetic alterations may include point mutations, insertion mutations, chromosomal translocations, retroviral integration and retroviral transduction. Oncogenes behave in a dominant manner, which means that only one of the two alleles of a proto-oncogene has to be mutated for the appearance of a biological effect (Doucas and Berry, 2006). There are many different categories used for the classification of oncogenes based on their normal role in cells and based on sequence homology to other proteins including growth factors or mitogens, receptor tyrosine kinases, cytoplasmic tyrosine kinases, cytoplasmic serine/threonine kinases, regulatory GTPases and transcription factors. Several examples for oncogenes are listed in Table 2.1 (Doucas and Berry, 2006).

Table 2.1: Examples of oncogenes
(adapted from Doucas and Berry, 2006)

Gene	Chromosome	Location of gene product within cell	Function
erbB2	7	membrane	growth factor receptor
myc	8	nucleus	transcription factor
ras	11	membrane	mediates signal transduction
src	20	cytoplasm	tyrosine kinase regulates activity of other proteins

ras: Retrovirus-associated DNA sequence; src: Rous sarcoma oncogene

Tumour suppressor genes, unlike oncogenes, behave usually in a recessive way. That means that both alleles in a gene have to be mutated before a biological effect is manifested. An exception represents the tumour suppressor p53 where the production of an altered form deactivates the normal allele (Baker *et al.*, 1990; Doucas and Berry, 2006).

Tumour suppressor genes code for inhibitory proteins, which in normal cells moderate or suppress the regulation of cell cycle and/or induce apoptosis and thus prevent cell growth and proliferation (Doucas and Berry, 2006). Tumour suppressor genes not only repress genes crucial for the continuation of the cell cycle, they also couple the cell cycle to DNA damage (Sherr, 2004). Thus, when the DNA damage is repaired, the cell cycle can proceed, otherwise the cell undergoes apoptosis. DNA repair proteins are commonly regarded as tumour suppressors. Alterations in their genes (e.g. BRCA, HNPCC and MEN) often lead to cancer development. Certain tumour suppressors, known as metastasis suppressors, exert their functions in cell adhesion and metastasis prevention (Yoshida *et al.*, 2000; Hirohashi and Kanai, 2003). Examples of tumour suppressor genes are mentioned in Table 2.2.

Table 2.2: Examples of tumour suppressor genes
(adapted from Doucas and Berry, 2006)

Gene	Chromosome	Location of gene product within cell	Function
APC	5	cytoplasm	regulation of co-transcriptional activators
Bcl-2	18	mitochondria	apoptosis
p53	17	nucleus	DNA repair and apoptosis
Rb	13	nucleus	regulation of cell cycle

Rb: Retinoblastoma; Bcl: B-cell lymphoma; APC: Adenomatous polyposis coli.

There are currently six known crucial modifications in cell physiology that altogether might influence/promote malignant transformation. These include: self-reliance on growth signals, insensitivity to growth-inhibitory signals, unlimited replicative potential, continuous angiogenesis, tissue invasion and metastasis, and avoiding of apoptosis (Figure 2.2) (Hanahan and Weinberg, 2000; Gogvadze *et al.*, 2008).

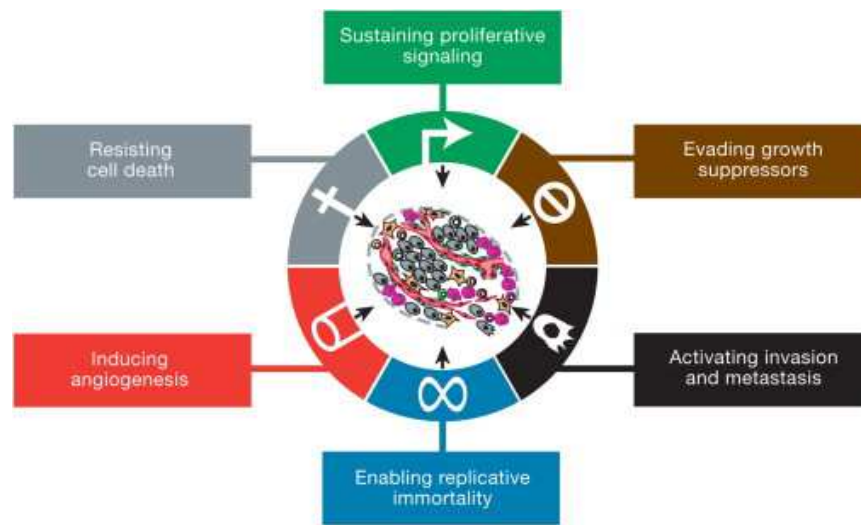


Figure 2.2: The hallmarks of cancer.
(Hanahan and Weinberg, 2011)

Several factors may influence the survival rates in different types of cancer, which may vary substantially. These include the type and location of the neoplasm as well as the stage of the disease at the onset of the treatment. In most cancer cases, it is almost impossible to ascribe a particular cause to a particular cancer type. However, several types of cancer exist that can be primarily linked to a single source, such as MM, which has been proven to be principally caused by exposure to asbestos fibers (American Cancer Society, 2002; Jemal *et al.*, 2011). These changes and alterations that arise gradually in the end give rise to tumours (Figure 2.3).

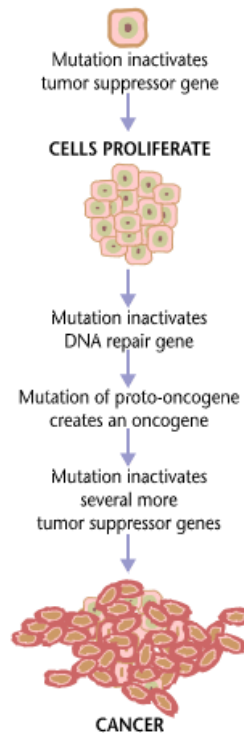


Figure 2.3: Simplified scheme of tumour generation.

(http://en.wikipedia.org/wiki/File:Cancer_requires_multiple_mutations_from_NIHen.png)

2.2 Malignant mesothelioma

Mesothelioma, usually referred to as malignant mesothelioma (MM), is a fatal neoplastic disease, whose patients are more-or-less restricted to palliative care (Robinson *et al.*, 2005). MM is a relatively rare form of cancer and the incidence varies considerably from one country to another (Table 2.3). The highest annual crude incidence rates (about 30 cases per million) are reported or estimated for Australia, Belgium and Great Britain, i.e. countries with history of high level of asbestos mining, processing and use (Bianchi and Bianchi, 2007).

Table 2.3: Estimated incidence of mesothelioma in various countries
(adapted from Bianchi C and Bianchi T, 2007)

Country	IR	Main source of the data	References
Australia	30	Mesothelioma Registry	National Occupational Health and Safety Commission (2004); Leigh <i>et al.</i> , 2002
Great Britain	30	Mesothelioma Mortality Registry	McElvenny <i>et al.</i> , 2005
Belgium	29	Researchers estimates	Bianchi <i>et al.</i> , 2000
The Netherlands	23*	Mortality data	Burdorf <i>et al.</i> , 2005
Italy	17*	Mortality data	Marinaccio <i>et al.</i> , 2005
Norway	16*	Cancer registry	Ulvestad <i>et al.</i> , 2003
New Zealand	15	Cancer registry	Kjellstrom, 2004
Denmark	13	Cancer registry	Kjaergaard and Anderson, 2000
Germany	13	Various	Hagemeyer <i>et al.</i> , 2006
Sweden	12*	Cancer registry	Burdorf <i>et al.</i> , 2005
France	10-13*	Mesothelioma Surveillance Program	Goldberg <i>et al.</i> , 2006
Finland	>10*	Cancer registry	Huuskonen and Rantanen 2006
Canada	9	Cancer registry	Comité aviseur sur l'amiante au Québec (2003)
United States	9*	SEER Program	Price and Ware, 2004; Weill <i>et al.</i> , 2004; Pinheiro <i>et al.</i> , 2004
Hungary	8	Mesothelioma Registry	Mándi <i>et al.</i> , 2000
Turkey	7.8	Researchers estimates	‡)
Croatia	7.4*	Cancer registry	Curin <i>et al.</i> , 2002; Saric, 2004
Japan	7	Mortality data	Furuya <i>et al.</i> , 2003
Romania	6	Researchers estimates	Bianchi <i>et al.</i> , 2000
Austria	5.6*	Cancer registry	Neuberger and Vutuc, 2003
Poland	4*	Mortality data	Szeszenia-Dabrowska and Wilczynska, 2004
Slovakia	4	Researchers estimates	Bianchi C <i>et al.</i> , 2000
Slovenia	4	Cancer registry	Kovac <i>et al.</i> , 2001
Spain	4*	Mortality data	Lopez Abente <i>et al.</i> , 2005
Estonia	3	Researchers estimates	Bianchi C <i>et al.</i> , 2000
Israel	3	Cancer registry	Ariad <i>et al.</i> , 2000
Latvia	3	Researchers estimates	Bianchi <i>et al.</i> , 2000
Macedonia	3	Researchers estimates	Bianchi <i>et al.</i> , 2000
Portugal	2-3	Researchers estimates	Bianchi <i>et al.</i> , 2000
Argentina	2.2*	Health Ministry Statistics	Rodriguez, 2004
Singapore	2	Cancer registry	Lee <i>et al.</i> , 2002
South Korea	1-2	Cancer registry	Paek, 2003

IR = annual crude incidencerates per million; * = pleural mesotheliomas ‡) Baris Y, personal communication.

Mesothelioma arises in the mesothelium, the protective lining that covers many of the internal organs and visceral cavities of the body. The primary cause of mesothelioma is exposure to asbestos fibers. Asbestos is the commercial name for a hydrated magnesium silicate fiber. There are two main families of asbestos fiber, the more carcinogenic amphibole (e.g., crocidolite, amosite and tremolite) and the less carcinogenic serpentine (chrysotile) forms. Serpentine fibers are curly, whereas amphibole fibers are needle-like (Rees *et al.*, 1999; Hodgson and Darnton, 2000; Barreiro and Katzman, 2006). The fibers can enter the body in two possible ways: either through the mouth and nose by inhalation or through the mouth by eating and drinking (Figure 2.4) (The Mesothelioma Center, 2013). In rare cases, mesothelioma has also been associated with irradiation, intrapleural thorium dioxide (Thorotrast) and inhalation of other fibrous silicates, such as erionite. Several studies have also suggested that Simian virus-40 (SV40) could have a carcinogenic or co-carcinogenic role in the initiation of mesothelioma (Cicala *et al.*, 1993, MacLachlan, 2002).

Mesothelioma is characterised by long latency periods between first exposure to asbestos and clinical diagnosis, ranging from 14 to 72 years, depending on the extent and type of exposure (Bianchi *et al.*, 1997). Duration and intensity of exposure to asbestos influence the development of mesothelioma. The tumour is mostly the consequence of long-time exposures, although even very short or very mild exposures to asbestos may be sufficient and are known to have lead to tumour formation (Bianchi and Bianchi, 2007). Long latency periods in MM indicate that numerous somatic genotoxic alterations are needed for the malignant transformation of mesothelial cells. A comprehensive heterogeneity of the structural chromosomal aberrations in MM points to an intrinsic susceptibility of the cells to amass genomic damage. Some of the accumulating genomic alterations can directly result from exposure to asbestos fibers or indirectly from cytokines and hormones released from neighbouring cells in response to the damage (Tomasetti *et al.*, 2009).

The carcinogenicity of asbestos is associated with its physical attributes (Hardy and Aust, 1995; Tomasetti *et al.*, 2009). Most of the carcinogenic asbestos types, such as amosite and crocidolite contain high levels of iron (27%), which indirectly causes DNA damage through the generation of ROS forms including the hydroxyl radical and superoxide anion radical, as well as by causing oxidative and mutagenic DNA damage (Takeuchi and

Morimoto, 1994; Kamp *et al.*, 1995; Tomasetti *et al.*, 2009). A major product of such DNA lesions is 8-hydroxy-2'-deoxyguanosine (8OHdG) (Takeuchi and Morimoto, 1994; Tomasetti *et al.*, 2009). 8OHdG induces A→C and G→T transitions, which are involved in spontaneously increased expression of oncogenes and may lead to cancer development (Moriya, 1993; Unfried *et al.*, 2002; Tomasetti *et al.*, 2009).

Asbestos fibers can also mechanically disturb the mitotic process by incorporation into the mitotic spindle, which may lead to such chromosomal aberrations as aneuploidy (Ault *et al.*, 1995; Deguen *et al.*, 1998; Tomasetti *et al.*, 2009). There are several chromosomal anomalies, which often occur in MM, including the polysomy for chromosome 5, 7 and 20, monosomy for chromosome 4 and 22, as well as the loss at 1p21-p22, 3p21, 6q15-q21, 9p21-p22 and 22q12 (Flejter *et al.*, 1989; Fletcher *et al.*, 1991; Taguchi *et al.*, 1993; Murphy and Testa, 1999; Tomasetti *et al.*, 2009). Furthermore, the chromosome 9p13-p22 deletion occurs in 50% of mesotheliomas (Murphy and Testa, 1999; Tomasetti *et al.*, 2009). Interestingly, tumour suppressor genes p14, p15 and p16 are located in this area.

Mesotheliomas can be classified into four main types based on the tumour location or the histological type. These types of mesothelioma are known as pleural, peritoneal, pericardial and testicular mesothelioma. All four types of the cancer are very aggressive. Cancer cells usually spread rapidly, and the median life expectancy of person with the disease is typically <12 months from diagnosis (Robinson *et al.*, 2005). The percentage of cases diagnosed for each type is represented in Figure 2.5 (The Mesothelioma Center, 2013).

Pleural mesothelioma is the most common type of mesothelioma with approximately 75% of all cases. It develops in the pleura, the thin serous lining of the lungs and the chest wall. Although symptoms may vary, the most common pleural mesothelioma symptoms include: persistent chest wall pain, pleural effusion or fluid surrounding the lung, shortness of breath (dyspnea), fatigue or anemia, wheezing, hoarseness, or coughing, blood in the sputum (fluid) coughed up (hemoptysis), difficulty swallowing (dysphagia), and night sweats/fever (Barreiro and Katzman, 2006).

Peritoneal mesothelioma arises in the peritoneum, the thin serous membrane that covers the abdominal organs and lines the walls of the abdominal and pelvic cavities. About 10 to 20% of all mesothelioma diagnoses are of the peritoneal type. Tumours that affect the abdominal cavity often do not cause symptoms until they are at a late stage. Patients with peritoneal mesothelioma may note symptoms resulting from the thickening of the peritoneal membrane and the build-up of fluid. Peritoneal mesothelioma symptoms typically include: abdominal pain, abdominal swelling, ascites or an abnormal buildup of fluid in the abdomen, thickening of the peritoneal membrane weight loss, nausea; constipation or diarrhea; and fatigue (<http://www.asbestos.com/mesothelioma/types.php>).

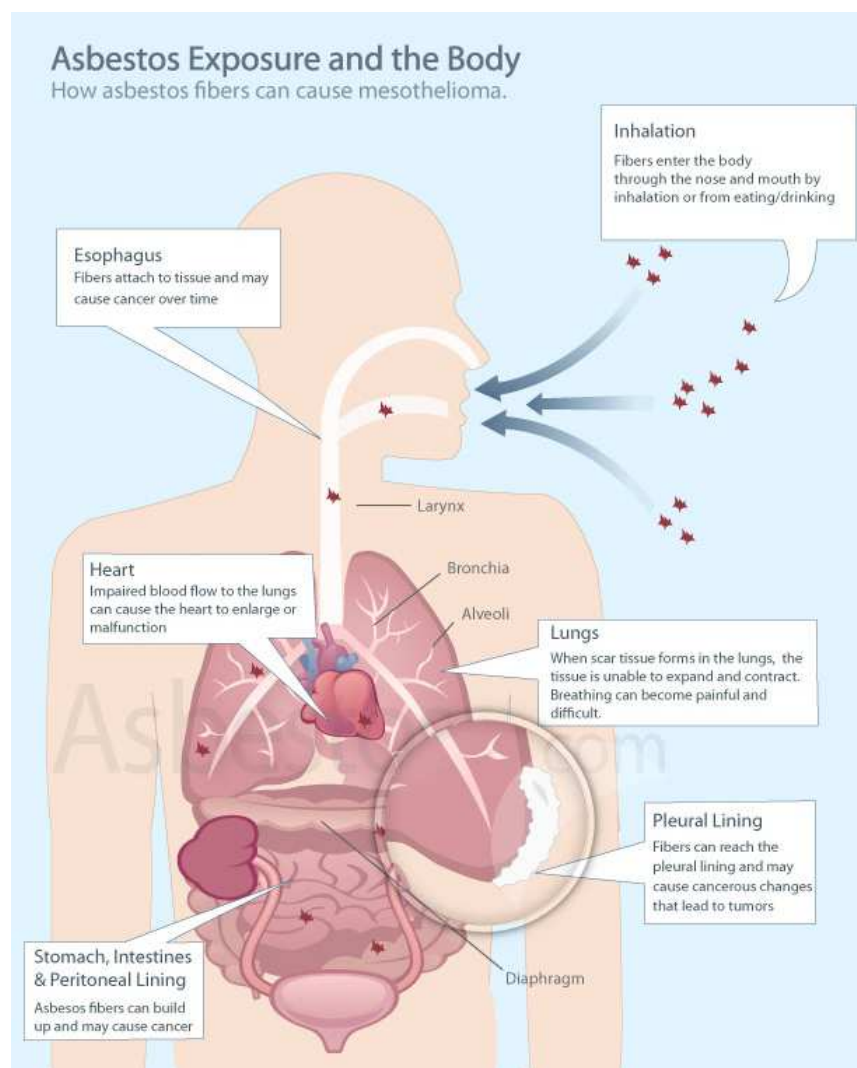


Figure 2.4: Two ways how asbestos fibers enter the body and the consequences relative to the intake.

(The Mesothelioma Center, 2013)

(<http://www.asbestos.com/mesothelioma/what-is-mesothelioma.php>)

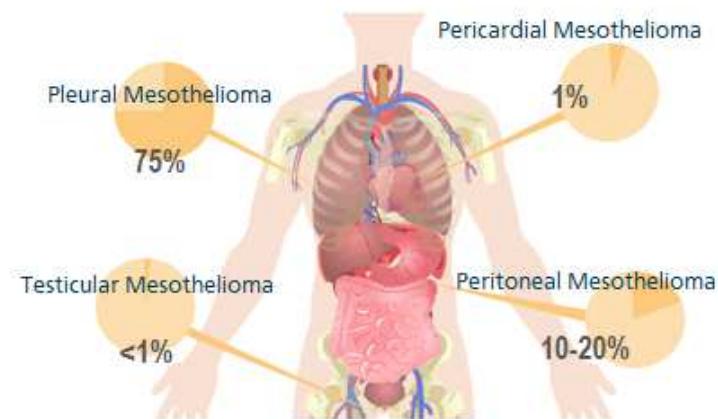


Figure 2.5: Main types of malignant mesothelioma and their occurrence.
(The Mesothelioma Center, 2013; <http://www.asbestos.com/mesothelioma/types.php>)

Pericardial mesothelioma forms in the pericardium, the fibroserous sac enclosing the heart and the roots of the great vessels, consisting of two layers - the visceral layer or epicardium and the outer parietal layer, which is lined with serous membrane. This type of mesothelioma is rare and accounts for about 1% of all mesothelioma cases. Symptoms develop when fluid builds up in the spaces between the layers of the pericardial membrane and may include: irregular heartbeat, chest pain, difficulty breathing, coughing, night sweats/fever and fatigue (<http://www.asbestos.com/mesothelioma/types.php>).

Mesothelioma can also be classified into four histological types: the epitheloid (40%), sarcomatoid or fibrous (around 20%), mixed type (epitheloid and sarcomatoid also called the biphasic type) (35%) and undifferentiated (5%) (Tomasetti *et al.*, 2009). These types of mesothelioma cells can be further subdivided into subtypes referred to as clear cell, small cell, acinar cell and tubopapillary cell mesotheliomas (cancerresearchuk.org).

Mesothelioma can be rather difficult to diagnose. For diagnosis and characterisation of the different types and subtypes of mesothelioma, histological samples are required to evaluate tumour morphology and, consequently, pathology (Carter and Otis, 1988; Cagle *et al.*, 1989; Ordóñez and Tornos, 1997; Khalidi *et al.*, 2000). The commonly used diagnostic methods contain pleural fluid cytology obtained through thoracentesis, needle biopsy of pleural tissue using CT guidance, video-assisted thoracoscopic surgery with direct visualisation and biopsy of pleural nodules, and open thoracotomy. Further diagnostic

techniques include oesophageal ultrasound, mediastinoscopy and laparoscopy, which are applied more for the disease staging purposes (Mott, 2012).

Several staging systems for mesothelioma have been developed and put into use over the years. Most of them have been dealing with primary pleural mesothelioma, whereas peritoneal mesothelioma does not have its own staging system. The first staging system, still commonly used for pleural mesothelioma in some parts of the world, is the **Buchart system**. It is based on a simple characterisation of the extent of the disease and disregards its histologic subtype: Stage I refers to pleura-contained tumour, Stage II to chest wall or mediastinal invasion, Stage III to peritoneal or diaphragmatic penetration and Stage IV to distant metastases.

Another staging system, which is not used at present any more, is the **Birgham system**. It specifies surgical resectability and lymph node involvement (Mott, 2012).

The most frequently utilised system for mesothelioma staging is the **International Mesothelioma Interest Group (IMIG) system** (Table 2.4), which has been developed by the International Mesothelioma Interest Group (cancerresearchuk.org). It is based on the **Tumour-Node-Metastasis (TNM) system** (Table 2.5), which has been developed by the American Joint Committee on Cancer (AJCC) and the International Union Against Cancer (UICC). It is used for other cancer types as well, and describes the extent of the primary tumour (T), whether the cancer has spread into lymph nodes close to the tumour (N), and whether the cancer has spread to other parts of the body to form metastases (M). Each cancer type has its own classification system based on the TNM staging system and letters and numbers do not always have to refer to an identical item (American Joint Committee on Cancer, 2010). Once the TNM categories have been decided, this information is grouped together to give the stages. Stage 1 is the earliest stage and stage 4 is the most advanced stage. Most people who are diagnosed with mesothelioma have a more advanced stage of the pathology.

Table 2.4: Tumour-node-metastasis staging for mesothelioma.
(adapted from Edge *et al.*, 2010; Mott, 2012)

Stage I (potentially resectable)	T1a-b, N0, M0 Ipsilateral parietal pleura with or without the involvement of visceral pleura
Stage II (potentially resectable)	T2, N0, M0 Involving <u>each</u> ipsilateral pleural surface (parietal, mediastinal, diaphragmatic, visceral) with ≥ 1 of following: involving diaphragm muscle or extension into lung parenchyma
Stage III (potentially resectable)	T1-2, N1-2, M0 or T3, N0-3, M0 Above (T1-2) with ≥ 1 of following (T3): involve thoracic fascia, mediastinal fat, solitary focus into chest wall, pericardium. Ipsilateral pulmonary/hilar nodes (N1), ipsilateral mediastinal or sub-carinal nodes (N2)
Stage IV (unresectable)	T4, any N, M0 or any T, N3, M0 or any T, any N, M1 Tumour extension into CW with or without rib destruction, transdiaphragmatic extension into peritoneum, contralateral pleura extension, mediastinal organs, spine, into myocardium (T4). Contralateral mediastinal, or any supraclavicular nodes (N3) or distant metastases (M1)

Table 2.5: The TNM staging system for different types of cancer based on the standard criteria developed by the American Joint Committee on Cancer (AJCC) and the International Union Against Cancer (UICC).

(cancerstaging.org, 2010)

T category classifies the primary tumour.	TX	Primary tumour cannot be evaluated.
	T0	No evidence of primary tumour
	Tis	Carcinoma in situ (early cancer that has not spread to neighbouring tissue)
	T1-T4	Size and/or extent of the primary tumour
N category describes whether the cancer has reached nearby lymph nodes.	NX	Regional lymph nodes cannot be evaluated.
	N0	No regional lymph node involvement
	N1-N3	Involvement of regional lymph nodes
M category defines whether there are distant metastases.	M0	No distant metastasis
	M1	Distant metastasis

MM has a very high mortality rate with limited therapeutic options. At present, no standard therapy exists for this aggressive neoplastic disease. The main reason for the low survival periods of MM patients is that the diagnosis is not made until stage III or IV of the pathology. This results directly from the mild, frequently undetectable symptoms in the early phases of the disease development. Early diagnosis of the disease and more importantly prognosis for MM patients have shown insignificant improvement. Currently, the treatment of mesothelioma involves several approaches including chemotherapy, surgery and radiation therapy. While surgical resection and radiotherapy techniques have been refined in recent years, the patient survival compared to untreated controls has not been substantially prolonged. The aggressive multimodal treatment remains still the best alternative for patients with expected poor prognosis. Induction chemotherapy followed by surgical resection and additional radiotherapy is possible. However, this multimodality treatment is applicable only to patients with early (T1-T2) disease with no evidence of nodal participation at the pre-operative evaluation (Tomasetti *et al.*, 2009).

Although not very efficient, chemotherapy is often used in patients with late mesothelioma. Single agents or combinatorial modes are utilised; however, the effect on median survival is low with rates of objective regression of <20% (Steele and Klabatsa, 2005; Tomasetti *et al.*, 2009). Cisplatin has been shown to be the most efficient agent used in chemotherapy of MM (Berghmans *et al.*, 2002; Tomasetti *et al.*, 2009). In case of combinatorial therapy, higher response rates have been achieved while the survival period has not been prolonged. Improved objective response rates of 41% have been reported with the anti-folate pemetrexed in combination with cisplatin, while gencitabine plus cisplatin has shown similar results (Byrne *et al.*, 1999; Vogelzang *et al.*, 2003). Currently, the combination of pemetrexed with a platinum derivate can be regarded as standard in the unresectable disease (Tomasetti *et al.*, 2009).

Surgery is carried out in MM patients with the localised disease. The principal surgical procedures for MM include pleurectomy/decortication (P/D), pleurodesis and extrapleural pneumonectomy (EPP). The cytoreductive procedures EPP and P/D are used for gaining local control over MM. EPP is a radical excision of the entire lung, both visceral and parietal pleura, pericardium, ipsilateral diaphragm, and mediastinal nodes followed by synthetic reconstruction (Cassidy *et al.*, 2010; Mott, 2012). P/D is aggressive to a lesser extent and also less technically invasive than EPP; it can be performed in most hospital facilities. It enables the removal of the visceral, parietal and pericardial pleura but the complete resection in most cases is not possible. This procedure can also be tolerated by patients whose poor health excludes EPP (Tomasetti *et al.*, 2009).

Further therapeutic approach used for MM is radiation therapy (RT). Whether this procedure itself has any effect on survival of MM patients is controversial. Several studies have shown that it can improve symptoms such as alleviating pain, dysphagia, dyspnea and vena cava obstruction (de Graaf-Strukowska *et al.*, 1999; Chapman *et al.*, 2006; Tomasetti *et al.*, 2009). RT is frequently combined with surgery to enhance local control and limit local failure of the treatment (Senan and van de Pol, 2004; Tomasetti *et al.*, 2009). A more intensive type of radiation with greater accuracy is referred to as intensity-modulated radiotherapy (IMRT). It is often used after EPP to restrict the threat of the disease recurrence (Mott, 2012).

Targeted therapy represents another, rather innovative modality for MM treatment. Most of the targeting agents, which are currently used in treatment of other cancer types, have shown only marginal effect on MM though, as none of them has been targeted to molecular modifications specific for MM or for its sub-groups. MM frequently shows high levels in expression of the epidermal growth factor receptor (EGFR) (Destro *et al.*, 2006; Tomasetti *et al.*, 2009). Relevant autocrine growth factors in MM include the vascular endothelial growth factor (VEGF) and the platelet-derived growth factor (PDGF) (Zucali and Giaccone, 2006; Tomasetti *et al.*, 2009). Limited or no efficacy has been achieved with gefitinib and erlotinib, EGFR tyrosine kinase inhibitors, and imatinib mesylate (Govindan *et al.*, 2005; Garland *et al.*, 2004; Mathy *et al.*, 2005; Tomasetti *et al.*, 2009). Among further tested agents with little effect on MM are PTK787 and thalidomide (Jahan *et al.*, 2005; Baas *et al.*, 2005; Tomasetti *et al.*, 2009), and also SU5416, an inhibitor of the VEGF receptor (VEGFR) Flk-1 (Kindler, 2004). Vorinostat, a histone deacetylase inhibitor, has caused objective responses in some MM patients (Kelly *et al.*, 2005). Bortezomib, a proteasome inhibitor, has been efficient in pre-clinical models of MM. It acts by a mechanism suppressing the transcription factor nuclear factor- κ B (NF κ B) and angiogenesis. Notably, NF κ B has been shown to play an essential role in chemoresistance of MM (Park and Lenz, 2004).

MM is generally characterised by resistance to apoptosis. Thus, overcoming this difficulty is one of the most promising strategies in MM treatment. Processes, which may surpass various types of resistance to apoptosis in cancer cells include direct targeting of anti-apoptotic molecules found in cancer and also the re-sensitisation of cancer cells by re-expression of pro-apoptotic molecules and by suppression of inhibitors of apoptosis (Zangemeister-Wittke and Hopkins-Donaldson, 2005; Tomasetti *et al.*, 2009). The apoptotic signalling pathways are rather complex, and they are described below.

2.3 Apoptosis

Apoptosis, or programmed cell death (PCD), is morphologically and biochemically well-defined form of cell death. It is a genetically regulated process, which is essential for the preservation of tissue homeostasis and elimination of damaged, harmful or excess cells of an organism (Tomasetti *et al.*, 2009). This energy-dependent form of cell suicide plays a

key role in the development as well as in the pathogenesis of diseases including cancer (Green and Reed, 1998; Kroemer, 2002; Breckenridge and Xue, 2004; Green and Kroemer, 2004; Armstrong, 2006). Thus, apoptosis is considered to be a relevant tumour-suppression mechanism (Youle and Strasser, 2008).

The morphological changes that define apoptosis include nuclear condensation and fragmentation of chromosomal DNA - a process known as DNA laddering, limited ultrastructural modifications of cytoplasmic organelles, reduction of cellular volume, rounding-up of the cell, retraction of pseudopodes, plasma membrane blebbing, apoptotic body formation and their engulfment by resident phagocytes (Kroemer *et al.*, 2009). There are two major apoptotic pathways, the extrinsic (or receptor-mediated) and intrinsic (or mitochondrial) (Figure 2.6) pathway, and complex interconnection exists between them, which increases the total apoptotic response (Bredesen, 2000; Igney and Krammer, 2002; Wajant, 2002; Schultz and Harrington, 2003; Armstrong, 2006). The details are discussed below.

Beside these two apoptotic pathways, a supplementary pathway exists concerning largely T-cell mediated cytotoxicity and perforin/granzyme killing of cells. The perforin/granzyme pathway can activate apoptosis by either granzyme A or granzyme B. The extrinsic, intrinsic and granzyme B pathways lead towards an identical execution pathway, which is activated by caspase-3 fission, leading to DNA fragmentation, cleavage of both nuclear and cytoskeletal proteins followed by apoptotic body formation, ligand expression for phagocytic cell receptors, culminating in their engulfment and digestion by phagocytic cells. Unlike granzyme B, granzyme A pathway triggers a caspase-independent cell death pathway mediated by single strand DNA cleavage (Figure 2.6) (Martinvalet *et al.*, 2005; Elmore, 2007).

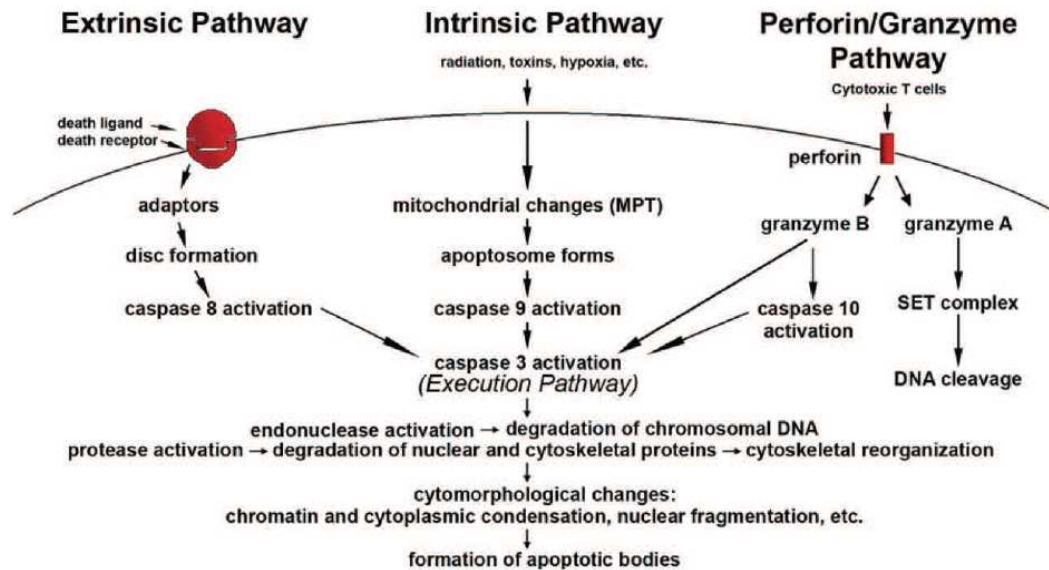


Figure 2.6: Schematic overview of apoptotic events occurring during extrinsic and intrinsic apoptotic pathways and the perforin/granzyme pathway.
(Elmore, 2007)

2.3.1 The extrinsic pathway of apoptosis

The extrinsic pathway is activated upon ligand binding to cognate cell surface death receptors (DRs) or upon cell-to-cell contacts (Zapata *et al.*, 2001). These DRs belong to the tumour necrosis factor receptor (TNFR) gene superfamily (Locksley *et al.*, 2001; Elmore, 2007). They contain extracellular cysteine-rich domains (CRD) and an intracellular death domain (DD), which consists of approximately 80 amino acids (Ashkenazi and Dixit, 1998; Elmore, 2007). This DD is essential for the transfer of death signals from the cell surface to the intracellular signalling cascades. The well-defined ligands are the DR complexes TNF- α /TNFR1, FasL/Fas, Apo3L/DR3, TRAIL/DR4 and TRAIL/DR5 (Chicheportiche *et al.*, 1997; Ashkenazi and Dixit, 1998; Peter and Kramer, 1998; Suliman *et al.*, 2001; Rubio-Moscardo *et al.*, 2005; Elmore, 2007).

The best characterised extrinsic apoptotic signalling pathways are the FasL/Fas (Figure 2.7) and the TNF- α /TNFR1 systems. In both cases, receptors form clusters and bind to a cognate trimeric ligand. This is followed by the recruitment of the intracellular adapter proteins and leads to the exhibition of appropriate DDs, which bind to the receptors. The interaction of the Fas ligand with the Fas receptor (also known as CD95, a prototypic death receptor) causes binding of the Fas-associated protein with a death domain (FADD), and

the interaction of the TNF ligand with the TNF receptor leads to the association with the adapter protein TRADD with recruitment of the adapter protein FADD and RIP (Hsu *et al.*, 1995; Wajant, 2002; Elmore, 2007). Besides its DD, FADD comprises an N-terminal death effector domain (DED), which allows the protein to dimerise with other DED-containing proteins such as pro-caspases 8 (or -10) (Boldin *et al.*, 1996; Muzio *et al.*, 1996; Medema *et al.*, 1997; Kischkel *et al.*, 2001; Ashe and Berry, 2003). The assembly of FasL, Fas, FADD and pro-caspase 8 (or -10) results in the formation of a supramolecular platform termed the 'death-inducing signalling complex' (DISC) (Kischkel *et al.*, 1995; Wajant, 2002; Ashe and Berry, 2003; Galluzi *et al.*, 2012b). This is followed by the auto-catalytic activation of the initiator-caspases 8 (or -10), and the execution phase of apoptosis is initiated. (Kischkel *et al.*, 1995; Elmore, 2007). The DR-activated apoptotic pathway can be blocked by the cellular FLICE inhibitory proteins (c-FLIPs) and cellular inhibitor of apoptosis proteins (cIAPs), which exert their anti-apoptotic functions within the DISC by association with FADD and caspase-8 (or -10), keeping them inactive (Kataoka *et al.*, 1998; Scaffidi *et al.*, 1999; Elmore, 2007; Galluzi *et al.*, 2012b).

There are two types of cells using different Fas signalling pathways upon DISC aggregation (Scaffidy *et al.*, 1998; Ashe and Berry, 2003). Thymocytes and peripheral T-cells belong to Type I cells and liver cells and neurons represent Type II cells (Algeciras-Schimmich *et al.*, 2002; Plesnila *et al.* 2001, Yin *et al.*, 2002; Ashe and Berry, 2003). In Type I cells activated caspase-8 directly initiates the caspase cascade by directly cleaving the effector caspase-3, whereas in Type II cells caspase-8 stimulates the mitochondrial outer membrane permeabilisation (MOMP) via processing the BH3-only protein Bid followed by cytochrome c (Cyt c) release (Galluzi *et al.*, 2012b). In the latter case, the extrinsic pathway of apoptosis actually activates the intrinsic pathway (Ashe and Berry, 2003).

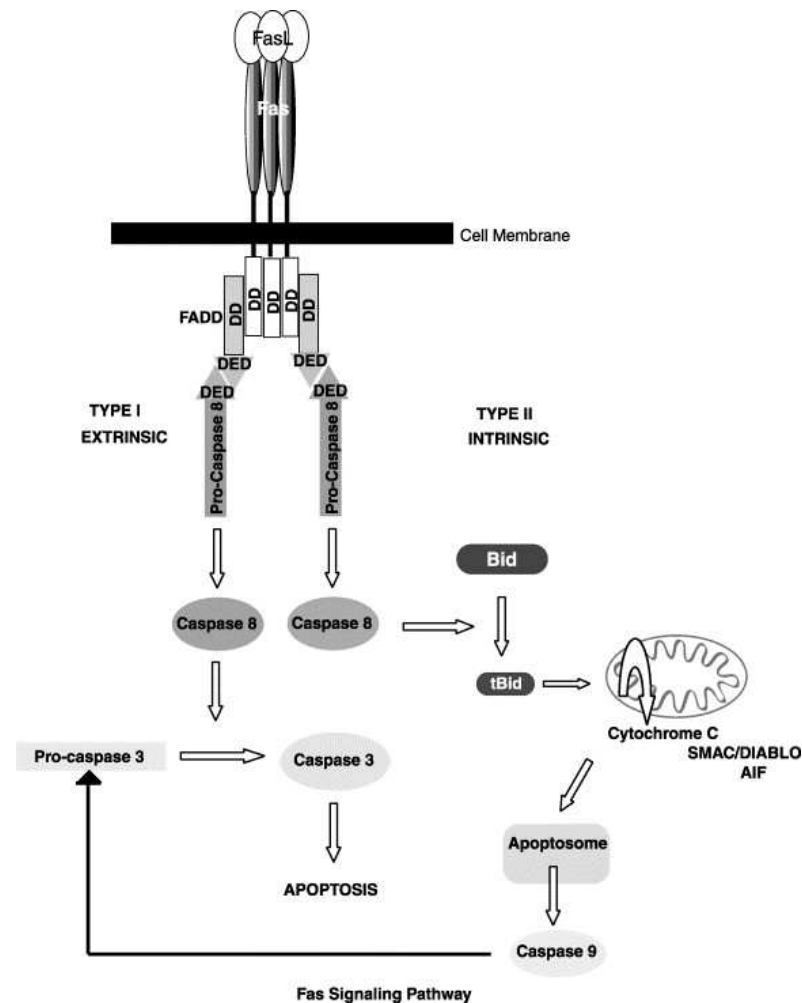


Figure 2.7: A schematic representation of Fas-mediated extrinsic apoptotic pathway. (Ashe and Berry, 2003)

The apoptotic cascade triggered by FasL is shown for Type I (direct activation of caspase-3 by caspase-8) and Type II cells (involvement of the MOMP via cleavage of the BH3-only protein Bid).

TRAIL signalling

Tumour necrosis factor- α -related apoptosis-inducing ligand (TRAIL)-mediated process represents a key extrinsic apoptotic pathway (Figure 2.8). TRAIL (or Apo2L) is known to play an important role in the anti-cancer immunity. It belongs to Type II trans-membrane death ligands presented on the surface of immune cells as membrane proteins or in a soluble form in most human tissues (Wiley *et al.*, 1995; Pitti *et al.*, 1996; Ashe and Berry, 2003). TRAIL binds specifically to four DRs: DR4, DR5, DcR1 (TRAIL-R3) and DcR2 (TRAIL-R4) (Chaudhary *et al.*, 1997; Marsters *et al.*, 1997; Pan *et al.*, 1997a,b; Sheridan *et al.*, 1997; Ashe and Berry, 2003). It triggers apoptosis by binding to DR4 and/or DR5 by a mechanism similar to that of FasL. Binding to the DRs causes their trimerisation followed by the recruitment of apoptotic down-stream mediators through the interaction of

FADD with pro-caspase-8 (-10) which results in the formation of DISC (Curtin and Cotter, 2003). Thus, the initiator caspases-8 (-10) can become activated by an auto-catalytic processing, and cleave and activate effector caspases. Effector caspases in turn cleave the cellular substrates resulting in a subsequent degradation of the cell. As described in the FasL/Fas apoptotic signalling, caspase-8 can process and activate the Bcl-2 homology-3 (BH3)-only protein Bid, which in turn induces Bax and/or Bak pore formation in the mitochondrial outer membrane (MOM), which is followed by Cyt c efflux from mitochondria to the cytosol. Therefore, there is a crosstalk between the extrinsic and the intrinsic pathway as described for the FasL/Fas signalling, providing a greater apoptotic response. Another important player in the process is Smac/DIABLO. It has been documented that its cytosolic translocation is crucial for the TRAIL-induced apoptosis, since in the absence of Smac/DIABLO, XIAP can prevent caspase-3 activation, even under condition of activated caspase-9 (Ashe and Berry, 2003).

It has been shown that TRAIL induces selective toxicity to cancer cells. This selectivity most likely depends on different expression levels of the so-called decoy receptors (DcRs), DcR1 and DcR2 in normal and cancer cells (Ashe and Berry, 2003). DcRs inhibit TRAIL-mediated apoptotic signalling by binding TRAIL, thus inhibiting the FADD recruitment to DR4/DR5 and the subsequent down-stream signalling events of apoptosis (Merino *et al.*, 2006). Normal cells often express both DcRs in contrast to cancer cells, which do not produce these receptors, thus showing higher sensitivity to TRAIL-mediated apoptosis.

Some cancers are able to develop different resistance strategies against TRAIL-induced apoptosis. Cancer resistance to TRAIL-mediated apoptosis is sometimes induced by increased levels of DcRs (Merino *et al.*, 2006). It has been reported that the selectivity of cancer cells to TRAIL-mediated apoptosis can be provided by p53-induced transcription of DR5. In contrast to normal cells, cancer cells are more susceptible to TRAIL, which leads to expression of p53 that then induces the expression of DR5. It has been shown that p53 also up-regulates DR4 (Kim *et al.*, 2001; Ashe and Berry, 2003). Further activity of p53 in the regulation of TRAIL-mediated apoptosis regards the suppression of FLIP by its increased proteasome degradation, a condition under which FLIP can no longer inhibit caspase-8 (Fukazawa *et al.*, 2001; Ashe and Berry, 2003). Increased FLIP expression levels have been found in a variety of tumours as a resistance strategy against TRAIL-mediated

cell death (Irmeler *et al.*, 1997; Kim *et al.*, 2000; Rippo *et al.*, 2004; Wicha *et al.*, 2006; Geseric *et al.*, 2008; Zobalova *et al.*, 2008).

Apart from apoptosis, binding of TRAIL to DR4 and DR5 can trigger the JNK and NF κ B signalling in a similar way to the TNF-mediated apoptotic pathway (Chaudhary *et al.*, 1997; Schneider *et al.*, 1997; Mühlenbeck *et al.*, 1998; Ashe and Berry, 2003). Activation of NF κ B signalling is proposed as a pro-survival pathway, protecting the cells from TRAIL-induced apoptosis (Ashe and Berry, 2003).

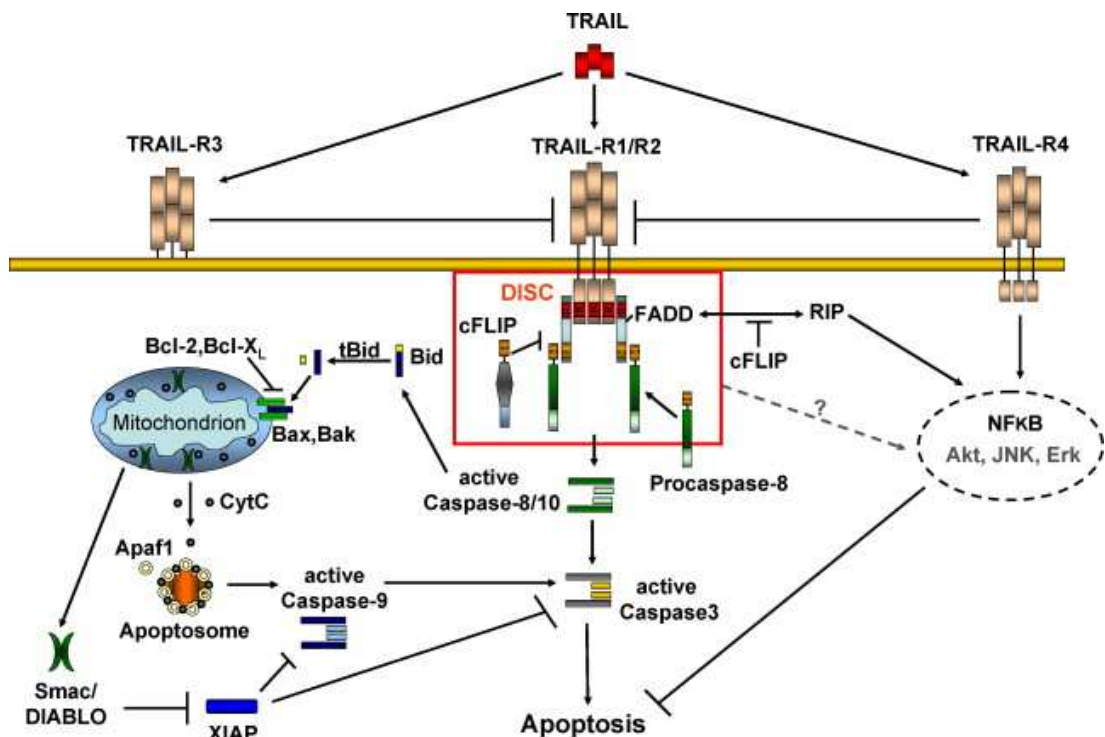


Figure 2.8: TRAIL mediated apoptotic pathway.

(Falschlehner *et al.*, 2007)

The apoptotic pathways triggered by TRAIL via its interaction with TRAIL-R1 (DR4) and TRAIL-R2 (DR5) are shown. Indicated is also the role of TRAIL-R3 (Dcr1) and TRAIL-R4 (Dcr2) that compete with TRAIL-R1/R2 to protect cells from TRAIL-mediated apoptosis.

2.3.2 The intrinsic pathway of apoptosis

The intrinsic pathway is mitochondria-dependent. It is often activated in response to multiple intracellular stress conditions (e.g. DNA damage, activation of oncogenes, cytosolic Ca^{2+} overload or other internal stimuli such as generation of ROS that affect mitochondria). The barrier to apoptosis induction depends on intact MOM, which sequesters a variety of apoptotic proteins in an inactive form in the mitochondrial inter-

membrane space (IMS). When lethal signals prevail, the compactness of this membrane is disrupted and leads to mitochondrial trans-membrane potential ($\Delta\Psi_{m,i}$) dissipation, arrest of mitochondrial ATP synthesis and $\Delta\Psi_{m,i}$ -dependent transport activities. Furthermore, the electron transport chain becomes uncoupled, leading to electron leakage and reactive oxygen species (ROS) generation. Consequently, apoptosis-promoting proteins, localised in the IMS, are released into the cytosol (Galluzzi *et al.*, 2012b). These proteins either activate caspases (enzymes of the cysteine protease family, see below) (Figure 2.9) or act in a caspase-independent manner to induce cell death (Armstrong, 2006).

Caspase-dependent factors contain Cyt c, Smac (second mitochondria-derived activator of caspases) also known as DIABLO (direct inhibitors of apoptosis protein-binding protein with low pI), and possibly HtrA2/Omi (high-temperature requirement protein A2). Caspase-independent factors contain endonuclease G (Endo G) and the apoptosis-inducing factor (AIF) (van Loo *et al.*, 2002; Kuwana and Newmeyer, 2003; Armstrong, 2006).

Cyt c, a small heme-containing protein, is tethered to the outer face of the MIM and is involved in the electron transport. Once released into the cytosol, it drives, together with the cytoplasmic adaptor protein APAF1 (apoptotic protease-activating factor-1) the energy-dependent (ATP/dATP) assembly of an oligomeric complex called apoptosome. Apoptosome is equipped with a caspase recruitment domain (CARD), which binds and processes caspase-9. This results in formation of a holoenzyme complex, that then activates the ‘executioner’ caspase-3 (Cain *et al.*, 2002; Armstrong, 2006). Targeted proteolysis of numerous cellular substrates is initiated and the cell is gradually degraded.

To prevent the cell from unintentional apoptosis (or, in cancer cells as a defense mechanism), the system is negatively regulated by IAPs (inhibitors of apoptosis proteins). With their baculoviral IAP repeat (BIR) domains, IAPs can bind to specific caspases and inhibit their activation or activity (Shi, 2002, Armstrong, 2006). IAPs are under the control of IAP-inhibitor proteins HtrA2/Omi and Smac/DIABLO. They contain an IAP-binding motif, which allows them to specifically interact with the BIR domains of IAPs. In this way they inhibit the formation of the IAP/caspase complex and reinstate the caspase function (Wu *et al.*, 2000; Shi, 2002; Armstrong, 2006).

HtrA2/Omi, a mitochondrial serine specific protease, cleaves and inactivates cellular inhibitor of apoptosis protein (cIAP-1). It participates in caspase-dependent as well as -independent cell death (Suzuki *et al.*, 2001; Jin *et al.*, 2003; Armstrong, 2006). Smac/DIABLO contains an N-terminal 4 amino acid IAP-binding motif, which interacts with the surface groove of BIR3 domain of X-linked inhibitor of apoptosis protein (XIAP) and thus restores caspase 9-activity (Chai *et al.*, 2000; Armstrong, 2006).

AIF is a mitochondrial flavoprotein oxidoreductase, responsible for chromatin condensation and high molecular weight DNA fragmentation without DNA laddering (Susin *et al.*, 1999; Armstrong, 2006). It comprises three distinct domains, the FAD-binding domain, the NADH-binding domain and the C-terminal domain (Susin *et al.*, 1999; Mate *et al.*, 2002; Armstrong, 2006). The FAD-binding domain is necessary for the oxidoreductase activity of AIF but is edundant for its pro-apoptotic function, suggesting that AIF might have a function in mitochondrial electron transfer as well as during apoptosis (Miramar *et al.*, 2001; Armstrong, 2006). AIF has also been found to be involved in the respiratory complex I formation, indicating that its true function may be related to oxidative posphorylation (Vahsen *et al.*, 2004).

EndoG is a mitochondrial intermembrane space protein, which is responsible for the internucleosomal DNA fragmentation together with other proteins like AIF (Widlak and Garrard, 2005; Armstrong, 2006). Collectively, these examples of the regulation of apoptosis induction and execution via mitochondria, both positive and negative, document the complexity of the process. Further, they document how tightly this process is controlled and how many options the cancer cell has to overcome its elimination for example as a consequence of therapeutic intervention.

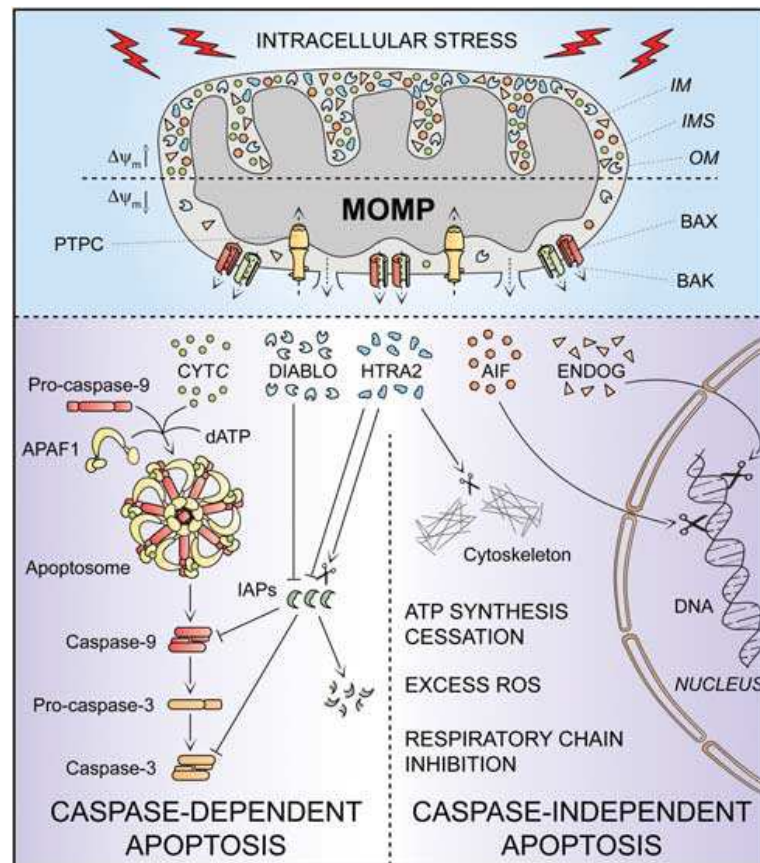


Figure 2.9: Intrinsic apoptotic pathway.

(Galluzzi, *et al.*, 2012b)

The intrinsic pathway involves mitochondria as organelles central to this process. The triggers are multiple and usually result in the formation of the MOMP followed by cytosolic translocation of a variety of apoptosis-mediating proteins. Both caspase-dependent and -independent processes follow, culminating in the final demise of the cell. More detailed explanation of these pathways in the context of this project is above and also below.

2.3.3 Caspases

With some exceptions, all apoptotic pathways converge on the activation of caspases (cysteine aspartic acid-specific proteases), a family of intracellular cysteine rich proteases that cleave their substrates after specific tetrapeptide motifs (P4-P3-P2-P1) where P1 represents an Asp residue. Most of the caspases are involved in the initiation as well as in the execution of apoptosis. There are several caspases (caspase-1, -4, -5 and -12) that instead of apoptosis regulate innate immune responses and control the processing of inflammatory cytokines. Caspase-12 is considered to be an inflammatory caspase but also participates in endoplasmic reticulum stress-induced apoptosis (Nakagawa *et al.*, 2000; Ashe and Berry, 2003).

The caspase family can be divided into initiator and executioner (effector) caspases. Initiator caspases possess the ability to auto-activate and subsequently initiate the cleavage of effector caspases that, in turn, cleave numerous down-stream death substrates (Taylor *et al.*, 2008). To date, there are at least 14 known mammalian caspases (Figure 2.10) (Ashe and Berry, 2003). All caspases contain a pro-peptide, a large (~20 kDa) and a small (~10 kDa) subunit. The pro-peptide is of variable length, depending on the caspase type, and exists in two different but structurally cognate forms, the DED and the CARD. DEDs can be found in caspase-8 and -10 whereas CARDS are present in caspase-1, -2, -4, -5, -9, -11, -12, -13 and -14 (Ashe and Berry, 2003). These domains enable the association of procaspases with various adaptor molecules comprising identical motifs.

Caspases are synthesised as zymogens (inactive precursor proteins) with poor or no enzymatic activity (Stennicke and Salvesen, 1998; Stennicke *et al.*, 1998; Nicholson, 1999; Creagh *et al.*, 2003; Taylor *et al.*, 2008). Upon different apoptotic stimuli, procaspases are proteolytically processed between the large and small subunits, at the decisive aspartate residues, to their active forms. This leads to the formation of heterodimers. Two heterodimers dimerise thereafter, forming a heterotetramer, which results in the active caspase form (Walker *et al.*, 1994; Ashe and Berry, 2003). In healthy cells, initiator caspases are present as monomers in contrast to the effector caspases, which exist as pre-formed dimers.

Initiation of both intrinsic and/or extrinsic apoptotic pathways leads to the activation of initiator caspases. Intrinsic apoptotic stimuli lead to activation of initiator caspase-9, whereas DR-mediated apoptosis activates initiator caspases-2, -8 and/or -10. Activated initiator caspases can subsequently proteolytically process procaspases and thus activate effector caspases-3, -6 and -7 or enhance the activation of initiator caspases by their cleavage (Ashe and Berry, 2003). Caspases are able to cleave their target substrates, which participate in cell death, cell cycle regulation, maintenance of cell morphology, DNA metabolism and signal transduction. This so called death substrate is a large group of proteins that is essential for the life of the cell and, upon their cleavage, the cell enters the final stages of apoptosis. In fact, it is thought that upon activation of the effector caspases, the cell enters the commitment phase, at which stage it will progress into complete death.

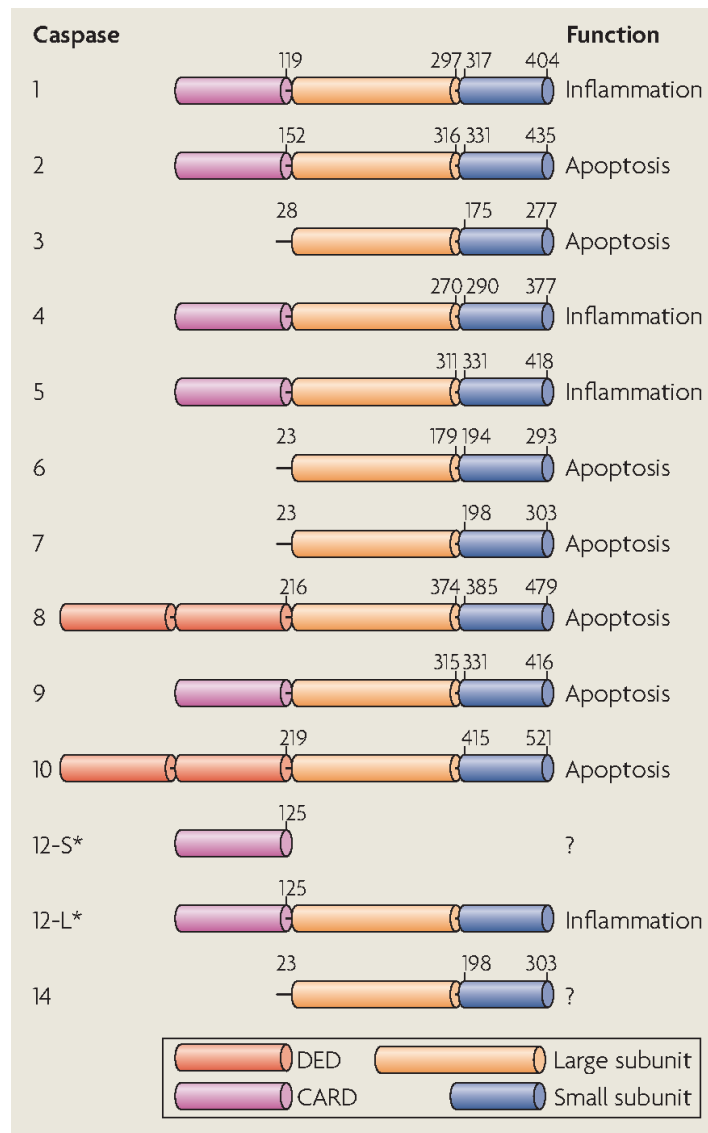


Figure 2.10: The caspase family.

(Taylor *et al.*, 2008)

The various caspases are shown, including the members involved in inflammation and apoptosis. The initiator caspases include the DED sequence (pink) or the CARD sequence (orange).

2.3.4 The Bcl-2 family of proteins and their role in apoptosis

Beyond their role in cancer, the B-cell lymphoma-2 (Bcl-2) family proteins are essential for the regulation of apoptosis since they are able to control the MOMP (Armstrong, 2006). Members of the Bcl-2 family can either inhibit or promote apoptosis. The Bcl-2 family can be divided into three groups, the anti-apoptotic, the pro-apoptotic and the BH3-only proteins (Figure 2.11) (Taylor *et al.*, 2008; Youle and Strasser, 2008). Bcl-2 proteins comprise between one and four conserved regions of amino acid sequence resemblance

known as the BH domains (Taylor *et al.*, 2008). At least 12 core Bcl-2 family members known in mammals show similarities to the prototypic Bcl-2 protein in their predicted secondary structures or in three-dimensional structures (Youle and Strasser, 2008).

BH3-only proteins are homologous among each other, whereas with the Bcl-2 core members they share only the BH3 motif indicating that a distant evolutionary relationship between them exists (Figure 2.11) (Zha *et al.*, 1996a; Aouacheria *et al.*, 2005; Youle and Strasser, 2008). Proteins from the anti-apoptotic group including Bcl-2, Bcl-x_L, Bcl-w, Mcl-1, Bcl-2A1 and Bcl-B consist of four BH domains, and most of them comprise also trans-membrane domains (TM) responsible for their membrane incorporation (Taylor *et al.*, 2008). The anti-apoptotic Bcl-2 proteins such as Bcl-2 and Bcl-x_L prevent the release of several apoptogenic proteins from mitochondrial IMS by inhibiting Bax and/or Bak oligomerisation in the MOM. The interaction between the anti-apoptotic Bcl-2 proteins and the BH3-only proteins is diverse; some BH3-only proteins (Noxa and Bad) bind only to specific anti-apoptotic Bcl-2 proteins, whereas other BH3-only proteins (Puma, Bim and Bid) bind to all anti-apoptotic Bcl-2 family members (Taylor *et al.*, 2008; Youle and Strasser, 2008). The structurally diverse BH3-only group includes the proteins Puma (also known as BBC3), Noxa, Bid (also known as 2Bid), Bik (also known as BLK or NBK), Bim (also known as Bod), Bad, Bmf and Hrk (also known as death protein-5, DP5) (Youle and Strasser, 2008). By overexpression, all of these BH3-only proteins trigger apoptosis through their conserved BH3 domain, which can directly interact with the anti-apoptotic Bcl-2 proteins and inhibit them. In doing so, the BH3-only proteins shift the balance between the pro- and anti-apoptotic Bcl-2 proteins in favour of the former.

The pro-apoptotic BH3-only proteins are initial sensors of apoptotic signalling that arises from diverse cellular events. They are regulated in a different manner. Puma, and to some extent Noxa and Bid are under the transcriptional control of the tumour suppressor protein p53, being transcriptionally upregulated in response to DNA damage (Oda *et al.*, 2000; Nakano and Vousden, 2001; Yu *et al.*, 2001; Youle and Strasser, 2008). Noxa can also be activated in response to stress via the Mst1 kinase/FoxO-1-dependent pathway (Valis *et al.*, 2011). Bid is in some cases activated by caspase-8 through proteolytically processing. Bim can be activated either by the transcription factors CCAAT-enhancer binding protein- α (CEBP α) or the CEBP-homologous protein (CHOP) in response to endoplasmic reticulum

stress or FoxO3A-dependent manner in response to growth factor deprivation (Puthalakath *et al.*, 2007; Youle and Strasser, 2008). Growth factor deprivation also leads to the dephosphorylation of Bad, which leads to its activation. Inactivation of Bad is ensured by its phosphorylation and subsequently sequestration mediated by the 14-3-3 proteins. Bim is associated with the dynein motor complex and Bmf with the actin-myosin motor complex. Dismantling of the cytoskeleton can release this BH3-only proteins and activate them to induce apoptosis.

The pro-apoptotic Bcl-2 family members Bax and Bak promote apoptosis by permeabilisation of the MOM followed by the efflux of pro-apoptotic molecules including Cyt c and Smac/Diablo and other soluble proteins from mitochondrial IMS into the cytosol. This results in caspase activation and the final demise of the cell. The third pro-apoptotic protein capable of forming a pore in the MOM is Bok. As the name of the protein indicates (Bok stands for Bcl-2-related ovarian killer), its occurrence is rather restricted to ovarian cancer, although it has been also identified in several other types of cancer, including lung carcinoma (Rodriguez *et al.*, 2006). The mechanism by which the Bax or Bak proteins are activated to form oligomeric structures and thereafter pores in the MOM are multiple. They include direct activation, such as that of Bax via initial ROS-dependent dimerisation (D'Alessio *et al.*, 2005), or the better studied initial conformational change of Bax monomers by interaction with the Bid protein, followed by Bax oligomerisation via association of the individual BH3 domains.

The major difference between the activation of Bax and Bak is that the former is resting in the cytosol of cells and, upon a trigger, it mobilises to the MOM. Bak, however, resides in the MOM all the time. It is prevented from forming oligomeric structures in the resting state of a cell by interaction with one of the anti-apoptotic proteins, such as Mcl-1. Upon an apoptogenic signal, the anti-apoptotic protein departs, such as by the interaction with the BH3-only protein Noxa, as found for Mcl-1, which is followed by its proteosomal degradation. We have found that exposure of cancer cells (Jurkat cells or non-small cell lung cancer cells) to α -TOS caused FoxO1-dependent upregulation of Noxa followed by dissociation of Mcl-1 from Bak and formation of Bak channels (Valis *et al.*, 2011). This is one of several proposed mechanisms of Bak-dependent pore formation in the MOM.

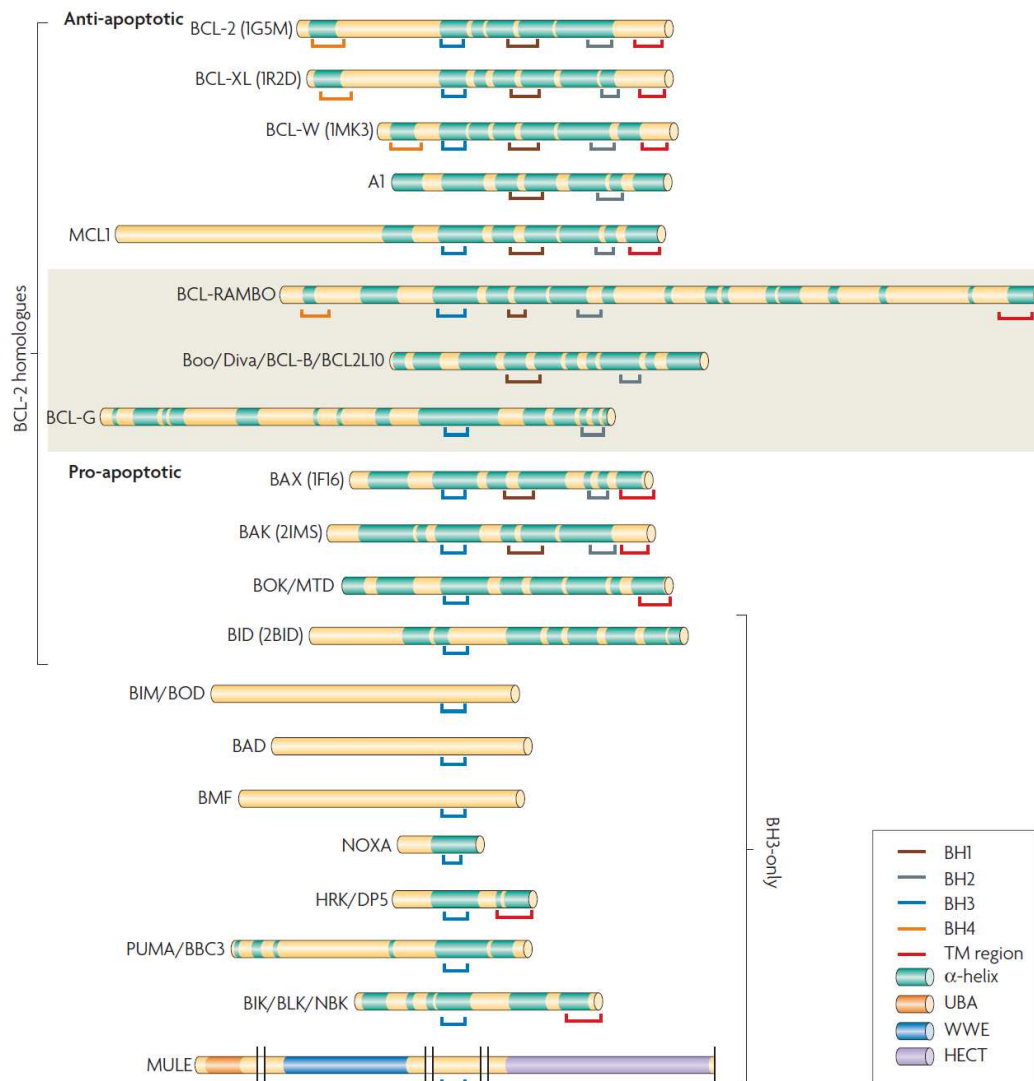


Figure 2.11: The Bcl-2 family - sequence alignment of core Bcl-2 family and BH3-only proteins.

(Youle and Strasser, 2008)

This illustrates the several groups of Bcl-2 family of proteins and the presence of the domains that dictate their function.

2.3.5 p53 and its role in apoptosis control

The transcription factor p53 is a tumour suppressor protein which plays a key role in carcinogenesis. In humans, it is encoded by the *TP53* gene. p53 is responsible for controlling cellular stress responses and is therefore often called ‘the cellular gatekeeper’ (Levine, 1997). The activation of p53 results in various responses depending on the cellular stress and cell type (Figure 2.12) (Chène, 2003). Under normal physiological conditions, the level of the p53 protein is kept low by the E3 ubiquitin ligase MDM2, which acts as a negative regulator by targeting p53, which is then degraded via the proteasome in a

ubiquitin-dependent way. MDM2 and p53 constitute a regulatory feedback loop (Figure 2.13) (Picksley and Lane, 1993; Wu *et al.*, 1993; Chène, 2003). Cellular stress such as DNA damage, hypoxia, nucleotide depletion and overexpression of oncogenes lead to the disruption of the p53-MDM2 association and very fast p53-stabilisation (Yu and Zhang, 2005; Horn and Vousden, 2007; Carjaval and Manfredi, 2013). p53 accumulates in the cell and undergoes complex post-translational modifications (Toledo and Wahl, 2006; Meek and Anderson, 2009; Carjaval and Manfredi, 2013). Subsequently, p53 translocates to the nucleus where it binds to DNA in a sequence-specific manner and exerts its activity as transcription factor. The transcription of a large and highly diverse group of target genes can be either activated or repressed by p53.

Many of the p53-regulated genes play essential roles in the control of cell cycle arrest, DNA repair, apoptosis, cellular senescence and necrosis. Further roles of p53 have been documented by many studies including regulation of cell survival and oxidative stress (Bensaad and Vousden, 2005), angiogenesis (Toeodoro *et al.*, 2006), differentiation (Murray-Zmijewski *et al.*, 2006), regulation of glycolysis (Bensaad *et al.*, 2006; Matoba *et al.*, 2006) and autophagy (Crichton *et al.*, 2006), invasion and motility (Roger *et al.*, 2006), as well as bone remodelling (Wang *et al.*, 2006). Most of these functions indicate that p53 plays an essential role as a tumour suppressor by preventing improper cell proliferation and maintaining the genome integrity upon genotoxic stress induction (Vogelstein *et al.*, 2000; Vousden and Lu, 2002; Yu and Zhang, 2005). Many types of human cancer are characterised by mutated *TP53* gene, ensuring a selective advantage in cancer progression. Acquired mutations in *TP53* can occur in all major types of human cancer (Hussain and Harris, 1999; Yu and Zhang, 2005), while germline mutations are found in the Li-Fraumeni syndrome, which is an infrequent hereditary cancer predisposition condition (Diller and Friend, 1992; Yu and Zhang, 2005). Tumours lacking *P53* mutations often show inactivation of the p53 pathway by defective upstream signals or by oncoproteins (Vogelstein *et al.*, 2000; Vousden and Lu, 2002; Yu and Zhang, 2005).

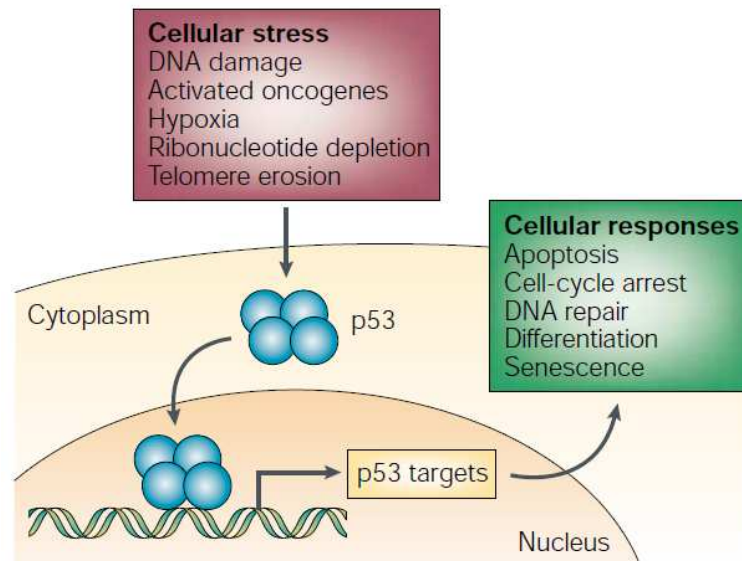


Figure 2.12: Activation and the function of p53.

Cellular stress promotes the p53 protein to accumulate in the cell and translocate into the nucleus where it can bind in its tetrameric form to its response-elements and activate or repress the transcription of a variety of genes participating in the regulation of cell cycle, DNA repair, apoptosis, differentiation and senescence (Chène, 2003).

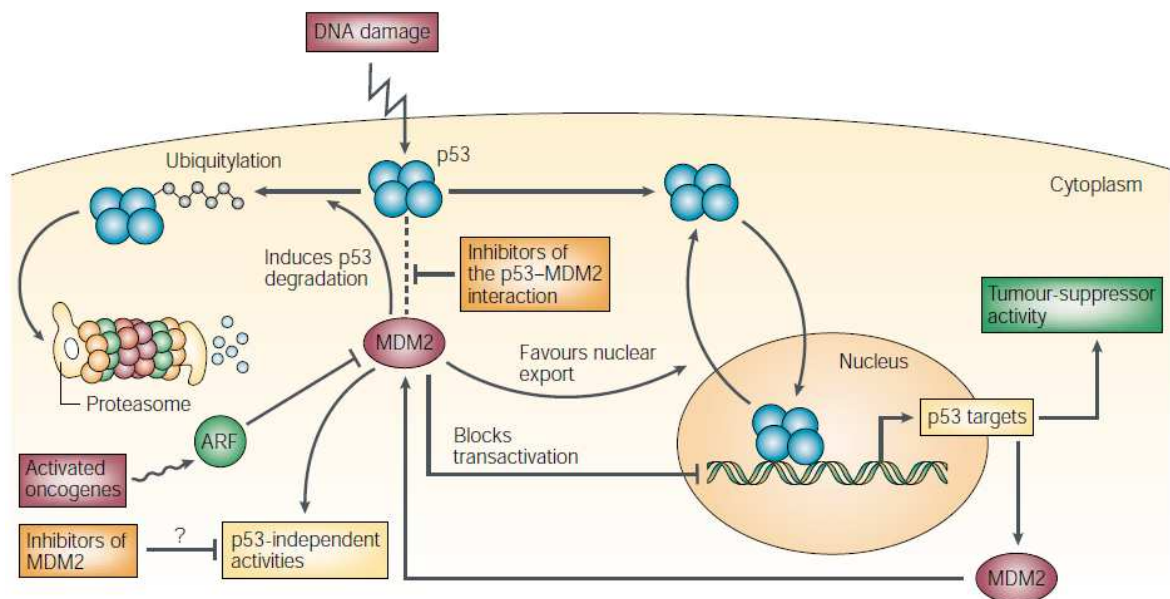


Figure 2.13: Regulation of p53 by MDM2.

MDM2 and p53 create an auto-regulatory feedback loop. p53 activates the expression of MDM2. MDM2 favours the export of p53 from the nucleus and provokes its degradation. p53 activation is triggered by diverse cellular signals including DNA-damage and oncogene activation. DNA damage leads to phosphorylation of p53 and prevents its interaction with MDM2. Activated oncogenes suppress the MDM2-mediated degradation of p53 via activation of the ARF protein (Chène, 2003).

While it is obvious that p53-mediated transcriptional regulation of specific genes is essential for the decision of the cellular fate, the molecular mechanism of target gene selectivity remains obscure. At present, there are three basic models which have been suggested by published literature. The p53-dependent selectivity for target genes and the appropriate cell fate decisions can be explained by 1) dissimilarities in p53 affinity to specific target gene promoters, 2) the effect of interacting proteins on target gene selectivity and 3) the influence of post-translational modifications of p53 (Carvajal and Manfredi, 2013).

Induction of apoptosis is one of the most dramatic responses to p53 activation. Regulation of apoptosis is a fundamental and evolutionary conserved function of p53 as a tumour suppressor. p53 modulates both the extrinsic and intrinsic apoptotic pathways upon a stress signal induction (Figure 2.14) (Green, 1998). In response to acute stress, p53 regulates the expression of genes that encode proteins acting in both apoptotic pathways. Many of them are components of the elementary apoptotic apparatus and can function independent of p53 (Yu and Zhang, 2005). However, it appears that the tumour suppressor protein mainly regulates proteins of the intrinsic apoptotic pathway while the extrinsic pathway is utilised to increase the apoptotic response (Jeffers *et al.*, 2003; Fridman and Lowe, 2003; Villunger *et al.*, 2003).

p53 activates or represses a variety of genes that are involved in the intrinsic apoptotic signalling. Among these are several mitochondrial and Bcl-2 family protein members. Bax was the first described pro-apoptotic Bcl-2 family protein controlled by p53 (Miyashita and Reed, 1995). However, it has been reported that Bax is not exclusively regulated by p53 in many tissues (Knudson *et al.*, 1995; Bouvard, *et al.*, 2000; Yu and Zhang, 2005). p53 controls also the expression of some BH3 domain-only proteins, which exert their apoptotic functions upstream of Bax/Bak. The genes encoding the BH3-only proteins Puma and Noxa are regulated by p53 upon DNA damage (Oda *et al.*, 2000; Han *et al.*, 2001; Nakano and Vousden, 2001; Yu J, *et al.*, 2001; Yu and Zhang, 2005). In colorectal carcinoma, Puma has been shown to be involved in apoptosis triggered by p53, hypoxia, ER-stress and DNA damaging compounds (Yu J *et al.*, 2003; Reimertz *et al.*, 2003; Yu and Zhang, 2005). However, the regulation of Noxa by p53 is not exclusive (Valis *et al.*, 2011). Another BH3-only protein, which can be regulated by p53, is Bid (Sax *et al.*, 2002; Yu and

Zhang, 2005). Bid represents the link between the intrinsic and extrinsic apoptotic pathways. On the other hand, *Bcl-2*, *Bcl-x_L*, and *survivin* have been identified to be negatively regulated by p53 (Vousden and Lu, 2002; Yu and Zhang, 2005). Caspase-6 and Apaf-1 are further apoptotic mediators, which can be activated upon DNA damage in a p53 dependent manner (Kannan *et al.*, 2001; Moroni *et al.*, 2001; Robles *et al.*, 2001; MacLachlan and El-Deiry, 2002; Yu and Zhang, 2005).

p53 can trigger extrinsic apoptotic pathway by activation of DRs, including DR4, DR5 and Fas. DR4 and DR5 have been reported to be under the control of p53. They can initiate apoptosis or amplify the process activated by TRAIL or chemotherapeutic agents (Sheikh *et al.*, 1998; Liu *et al.*, 2004; Yu and Zhang, 2005). p53 can activate Fas upon DNA damage in specific tissues, however, it is usually not needed in Fas-mediated apoptosis (Müller *et al.*, 1998; Bouvard *et al.*, 2000; Yu and Zhang, 2005).

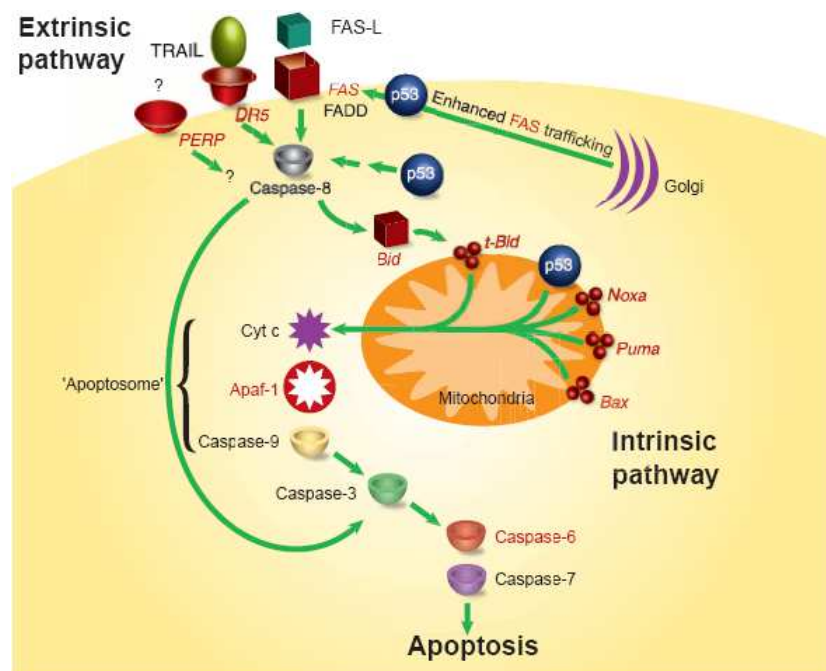


Figure 2.14: A scheme of p53-mediated apoptosis.

p53 exerts its functions in both the extrinsic and intrinsic apoptotic pathways. The two pathways converge by means of the Bid protein. p53 target genes are shown in red (Haupt *et al.*, 2003).

2.4 Mitochondria

Mitochondria (Figure 2.15), the so-called ‘power-houses’ of cells (Siekevitz, 1957), are organelles, essential for the survival of higher eucaryotic cells, including cancer cells. At the same time, these organelles play a crucial role in controlling cell death. Originally, mitochondria have been described as ‘bioplasts’ (Altman, 1890). Very soon they have been re-named due to their threadlike appearance during spermatogenesis (mitos = thread, chondros = granule) (Benda, 1898).

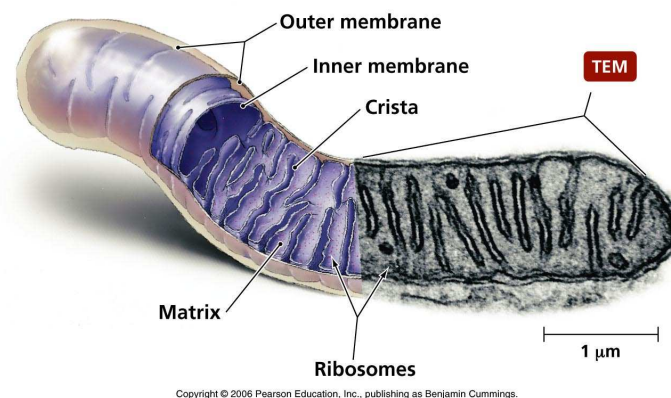


Figure 2.15: Schematic structure of mitochondria.

(<http://academic.pgcc.edu/~kroberts/Lecture/Chapter%203/membranous.html>)

Mitochondria are enclosed by a double membrane, which is composed of an outer membrane surrounding an inner membrane. The mitochondrial inner membrane (MIM) is folded into cristae that increase the surface area for the placement of membrane-bound molecules involved in energy transfer. The inner fluid of mitochondria is called mitochondrial matrix and contains enzymes, mitochondrial own circular DNA and ribosomes.

Mitochondria divide independently of the cell cycle by simple fission, which means that mitochondrial replication is not coupled to cell division. Mitochondrial division is stimulated by energy demand, so that cells with an increased need for energy contain greater numbers of these organelles than cells with lower energetic requirements.

Important mitochondrial metabolic pathways include the Krebs cycle, oxidative phosphorylation, heme biosynthesis, β -oxidation of fatty acids, steroidogenesis, the metabolism of certain amino acids and reactions belonging to gluconeogenesis and ketogenesis (Michal, 1999; Galluzzi *et al.*, 2010) (Figure 2.16).

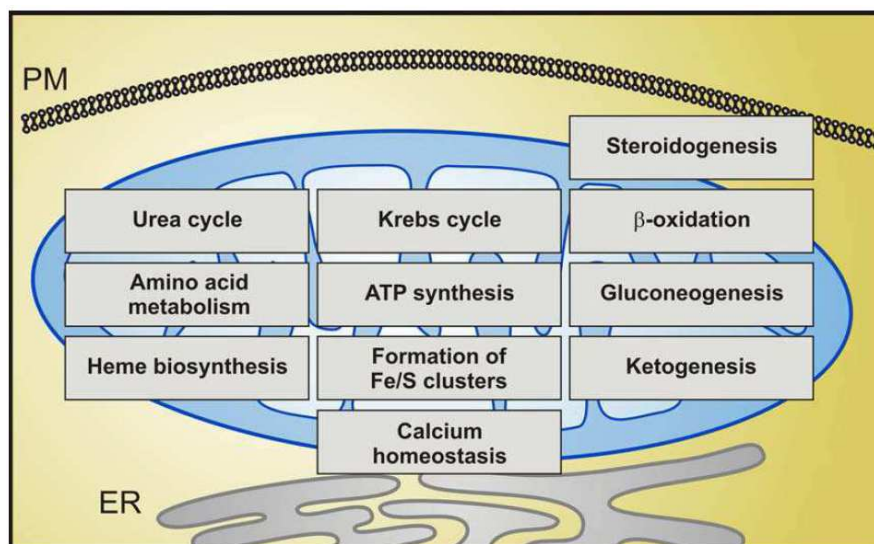


Figure 2.16: Mitochondrial metabolic pathways.
(Galluzzi *et al.*, 2010).

ER = endoplasmic reticulum; PM = plasma membrane

The most important biochemical process carried out by mitochondria is oxidative phosphorylation (OXPHOS), which is a reaction that couples mitochondrial electron transport chain with ATP synthesis (Figure 2.17). Energy resources are first metabolised in the cytoplasm. The products are then imported into mitochondria, the place where these substrates are subsequently catabolised by metabolic processes such as the tricarboxylic acid (TCA) cycle (or Krebs cycle), fatty acid and amino acid oxidation resulting in the generation of two energy-rich electron donors, NADH and FADH₂. Energy production is attained by oxidation of these reducing equivalents (mainly NADH) and transport of the retained electrons through an electron transport chain (ETC) (or respiratory chain) to the terminal acceptor, molecular oxygen (O₂), which is finally reduced to water (Figure 2.17) (Lenaz and Genova, 2010; Grimm, 2013).

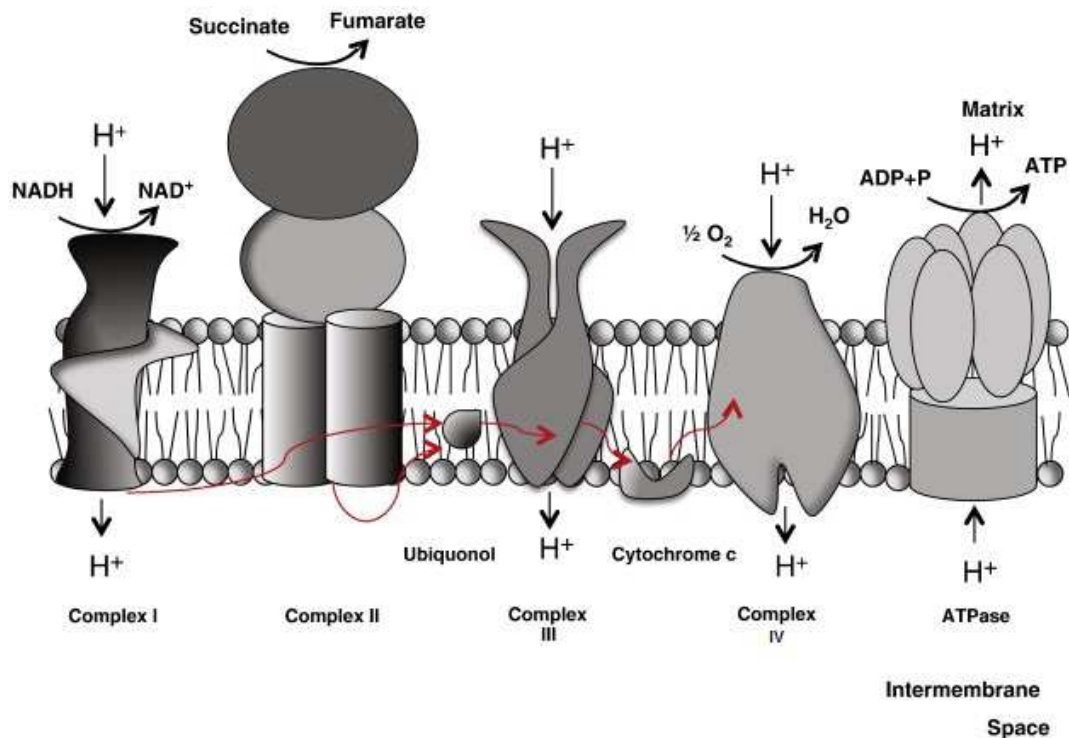


Figure 2.17: Schematic representation of the mitochondrial respiratory chain complexes and the OXPHOS system.

Depicted are the enzyme complexes I-IV and the ATP synthase (CV) as well as the electron/proton flows along these complexes. CI and CII are responsible for the main entrance of reducing equivalents into the ETC. CI (NADH UbQ reductase) accepts electrons from the TCA cycle electron carrier NADH and passes them by a flavin mononucleotide (FMN) cofactor to iron-sulfur (Fe-S) clusters to reduce the UbQ to ubiquinol, which is coupled to the translocation of protons from the matrix into the intermembrane space. CII (succinate UbQ oxidoreductase) also transfers electrons from succinate (a TCA cycle intermediate) to its cofactor FAD and subsequently via Fe-S clusters resulting in the reduction of UbQ. Ubiquinol passes its electrons to CIII (ubiquinol Cyt c oxidoreductase) via two heme centers (cytochromes b and c1) and one Fe-S cluster. CIII transfers its electrons to Cyt c that delivers them to CIV (Cyt c oxidase), which uses the electrons and hydrogen ions to reduce molecular oxygen to water. The final complex of ATP synthase (CV) uses the electrochemical proton gradient to generate ATP from ADP and Pi (inorganic phosphate). Red arrows represent the electron transfer, whereas black arrows depict the proton flow (Grimm, 2013).

The mitochondrial respiratory chain contains five macromolecular protein complexes (complex I-V; CI-CV), which are all embedded in the MIM. These enzyme complexes together with two soluble factors, coenzyme Q₁₀ (ubiquinone Q, UbQ) and Cyt c, act as electron shuttles within the mitochondrial inter-membrane space by transferring electrons from CI and CII via CIII to CIV, resulting in energy production preserved as electrochemical proton gradient across the MIM (Galluzi *et al.*, 2010; Neuzil *et al.*, 2013). The energy from the electrochemical proton gradient is then used in a regulated manner by the F₁F₀-ATP synthase (CV), which pumps protons back into the mitochondrial matrix and thus produces the majority of intracellular ATP reserves from ADP and inorganic phosphate (Mitchell, 1961; Reichert and Neupert, 2004; Galluzzi *et al.*, 2010). Around

2,000 mitochondria generate more than 90% of intracellular ATP in a healthy eukaryotic cell (Voet and Voet, 1995; Pedersen, 2007).

Mitochondria are central to the life and death of a cell. Thus, it is not surprising that mitochondrial dysfunction contributes to a wide range of diseases including defective apoptosis in cancer and degenerative diseases (Kroemer *et al.*, 1998), diabetes (Nishikawa *et al.*, 2000), disorders associated with mitochondrial DNA mutations (Pitkanen and Robinson, 1996; Wallace, 1999), Friedreich's ataxia (Puccio *et al.*, 2001; Kaplan, 1999), Huntington's disease (Tabrizi *et al.*, 2000), Parkinson's disease (Schapira *et al.*, 1992) and the pathophysiology of aging (Beckman and Ames, 1998; Raha and Robinson, 2000; Ames *et al.*, 1995; Smith *et al.*, 2003).

Mitochondria are the cells main energy producers and are therefore essential for cellular survival, but their role is not limited to ATP production for maintaining the metabolic processes. They are also important for the production of ROS, which are involved in the regulation of many physiological processes and which might also be detrimental to the cell if produced excessively. Moreover, they are essential for keeping the intracellular Ca^{2+} in homeostasis, and they are key players in the regulation of cell death pathways. These mitochondrial activities are of fundamental importance for tumour cell physiology, growth and survival (Gogvadze *et al.*, 2008). For these reasons, mitochondria have recently emerged as a new promising target for cancer therapy and prevention that are yet to be fully exploited and that offer thus far unprecedented possibilities to trigger apoptosis in cancer cells in an efficient and selective manner.

Many of the compounds with anti-cancer activity that act on mitochondria, mitocans (an acronym standing for mitochondria and cancer), hold a considerable promise to be developed into efficient anti-cancer drugs, based on their selectivity for cancer cells (Fulda *et al.*, 2010; Ralph *et al.*, 2010a,b; Wallace *et al.*, 2010; Wang *et al.*, 2010; Fulda and Kroemer, 2011; Kepp *et al.*, 2011; Lemarie and Grimm, 2011; Shoshan-Barmatz and Ben-Hail, 2012; Neuzil *et al.*, 2013).

2.5 Mitocans as promising anti-cancer agents

Mitochondria have recently emerged as effective targets for novel anti-cancer drugs referred to as ‘mitocans’ (Ralph *et al.*, 2006). The term ‘mitocans’ has been recently proposed for small compounds with anti-cancer activity that destabilise mitochondria, resulting in apoptosis induction, often selectively affecting cancer cells. These compounds have been classified into several groups based on their molecular targets and mechanism of action (Neuzil *et al.*, 2012). Table 2.6 contains the individual classes of mitocans, epitomised by several examples, in particular those that are considered as potentially clinically applicable anti-cancer agents. The classification begins with targets at the surface of mitochondria and ends up with those in the mitochondrial matrix. Molecular targets of the individual classes of mitocans are depicted in Figure 2.18. Some of the listed compounds are being tested in pre-clinical/phase I/II trials as potential anti-cancer drugs, others are reported in various publications (Neuzil *et al.*, 2012).

The individual classes of mitocans include hexokinase inhibitors, compounds targeting Bcl-2 family proteins (including BH3 mimetics), thiol redox inhibitors, VDAC/ANT targeting drugs, electron redox chain targeting drugs, Lipophilic cations targeting inner membrane, drugs targeting the TCA cycle and drugs targeting mtDNA. Of these classes, our laboratory has been focusing on the class 5 compounds, epitomised by α -TOS and MitoVES (see below). These agents act via blocking the electron transport within OXPHOS, resulting in generation of ROS and induction of apoptosis. Since they hold a substantial promise to be developed into clinically relevant drugs, they are a subject of our ongoing studies and are also a focus of this project.

Table 2.6: Classification of mitocans and examples of compounds in individual classes.(Neuzil *et al.*, 2013)

Class number	Type	Examples	References (for detail see Neuzil <i>et al.</i>, 2013)
1	Hexokinase inhibitors	3BP, 2DG	
2	Compounds targeting Bcl-2 family proteins	Gossypol, ABT-737, antimycin A, α -TOS	
3	Thiol redox inhibitors	Isothiocyanates, arsenic trioxide	
4	VDAC/ANT targeting drugs	Lonidamide, arsenites, steroid analogues such as CD437	
5	Electron redox chain targeting drugs	α -TOS, MitoVES, tamoxifen, adaphostin, 3BP	
6	Lipophilic cations targeting inner membrane	Rhodamine-123, F16, (KLAKKLAK) ₂	
7	Drugs targeting the TCA cycle	DCA, 3BP	
8	Drugs targeting mtDNA	Vitamin K3, fialuridine, 1-methyl-4-phenyl-pyridinium, MitoVES	
9	Drugs targeting other (unknown) sites	Betulinic acid	

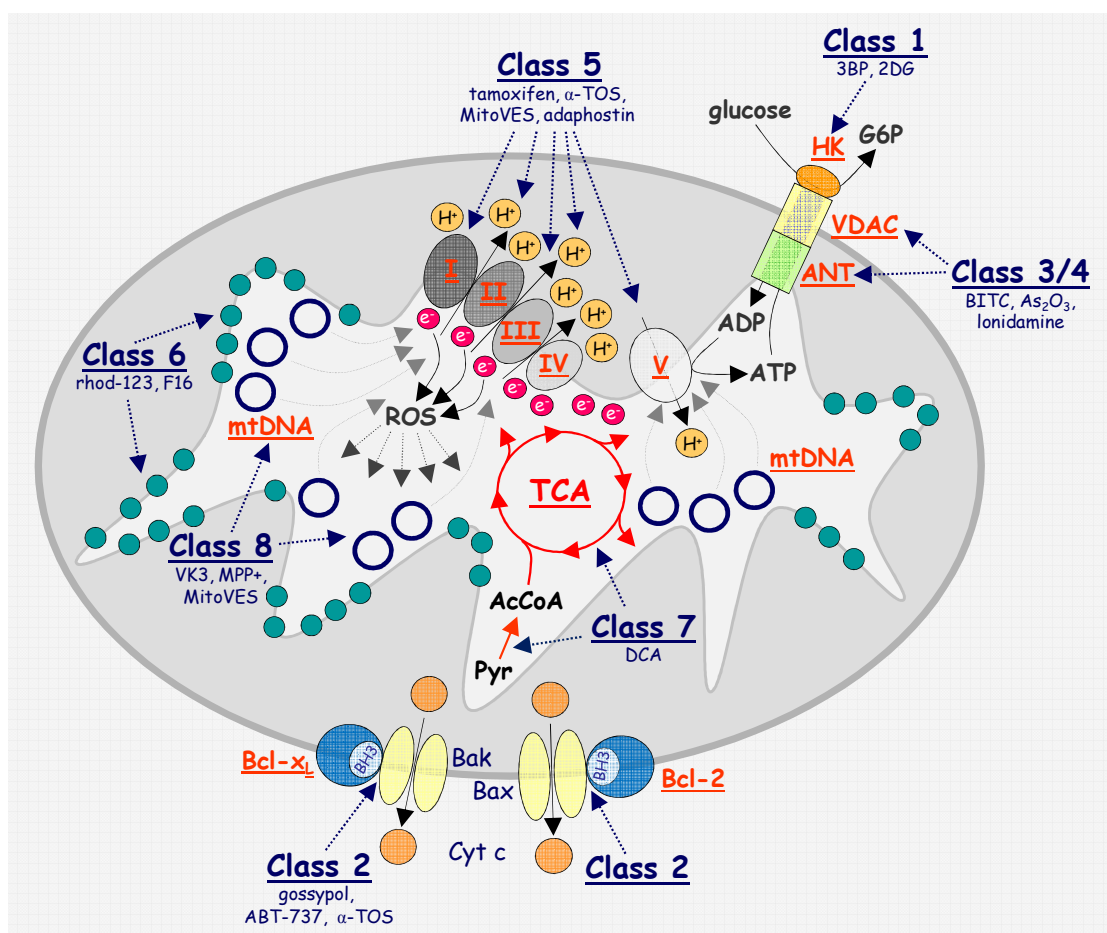


Figure 2.18: Schematic illustration of the molecular targets of individual classes of mitocans.

(Neuzil *et al.*, 2013)

The cartoon indicates the major classes of mitocans and depicts their molecular targets. Several representative molecules for each class are included.

2.5.1 Class 5 mitocans: electron redox chain targeting drugs

Each class of mitocans represents a potential for novel anti-cancer therapy. However, mitocans that belong to Class 5 targeting mitochondrial complexes of the electron redox chain, particularly CII, are of great interest, due to their molecular mode of action and due to the interference with the major pathway of energy production in the cancer cell. Furthermore, since cancer cells utilise glycolysis as an alternative/additional process to generate energy to meet the high demands, it is obvious, that interference with OXPHOS will not necessarily deprive the cancer cell of its ATP (which is needed for the execution of the apoptotic caspase and the ensuing demise of the cell). Rather, targeting the ETC will result in the generation of ROS that, consequently, trigger apoptosis (Neuzil *et al.*, 2012,

2013). The reason why we particularly became interested in CII stems from the notion that it is a molecular target for the VE analogues α -TOS and MitoVES (see below) (Neuzil *et al.*, 2012, 2013).

Complex II (CII) is the smallest mitochondrial respiratory chain complex. It comprises two enzymatic activities, the succinate dehydrogenase (SDH) activity and the succinate UbQ reductase activity (SQR), and consists of four subunits (SDHA, SDHB, SDHC and SDHD). It is the only respiratory complex that is completely encoded by nuclear DNA. The transmembrane proteins SDHC and SDHD are responsible for the anchorage of CII to the MIM (Yankovskaya *et al.*, 2003; Sun *et al.*, 2005; Grimm, 2013). In eukaryotes, SDHC and SDHD are interconnected with the subunit SDHB. SDHB forms together with SDHA the hydrophilic part of the protein complex, which also represents the catalytic core of the complex. The catalytic core is able to convert succinate to fumarate within the TCA cycle. Within the SDHA/B subcomplex, electrons are passed to the FAD cofactor buried in the SDHA subunit and subsequently to three Fe-S clusters of SDHB. The SDHC/SDHD part of CII contains two sites that bind UbQ, Q_p (proximal to the [3Fe-4S] cluster) and Q_d (distal from the [3Fe-4S] cluster) (Sun *et al.*, 2005; Rutter *et al.*, 2010; Grimm, 2013). UbQ reduction at the Q_p site proceeds in two steps protecting CII against excessive escape of electrons. The first electron leads to the formation of a partially reduced semiquinone, and the second electron then reduces it to ubiquinol (Guo and Lemire, 2003; Rutter *et al.*, 2010; Grimm, 2013). The SDH activity includes the complete electron translocation from succinate at the catalytic site of SDHA to UbQ at the Q_p site at the SDHC/SDHD interface (Grimm, 2013).

2.5.2 Vitamin E analogues as prototypic members of Class 5 mitocans

α -TOS, a redox-silent compound from the family of vitamin E analogues is one of the most extensively studied mitocans (Neuzil *et al.*, 2001a,b; Weber *et al.*, 2002). The pro-vitamin α -TOS behaves very differently to its redox-active counterpart, α -tocopherol (α -TOH) (Figure 2.19). Thus, instead of protecting cells from ROS-induced apoptosis, α -TOS asserts cell death by exerting pleiotrophic responses in malignant cells that result in cell cycle arrest, differentiation, and apoptosis, in most if not all cases due to the initial ROS

generation in response to α -TOS exposure (Turley *et al.*, 1997; Neuzil *et al.*, 2001a,c; You *et al.*, 2002; Birringer *et al.*, 2003; Ni *et al.*, 2003; Tomasetti and Neuzil, 2007).

α -TOS is an analogue of VE. The term VE constitutes lipid-soluble micronutrients comprising two groups, tocopherols (α -, β -, γ - and δ -TOH) and tocotrienols (α -, β -, γ - and δ -T3H) (Tomasetti and Neuzil, 2007). α -TOH and its analogue α -TOS consist of three structural domains (Domain I, II and III) (Neuzil *et al.*, 2004) (Figure 2.19). Domain I, which is also known as the functional domain, provides the compounds with their major biological activity. In case of α -TOS, the redox-active hydroxyl group within Domain I makes the compound a redox-active, antioxidant substance, while in α -TOS this hydroxyl group is esterified with a succinyl moiety, which makes the analogue redox-silent and endows it with strong apoptogenic efficacy. The other two domains do not differ between VE and α -TOS. Domain II (signalling domain) is involved in modulation of signalling pathways, such as the protein phosphatase 2A/protein kinase C pathway. Domain III (hydrophobic domain) allows the agents to dock in biological membranes and circulating lipoproteins (Tomasetti and Neuzil, 2007).

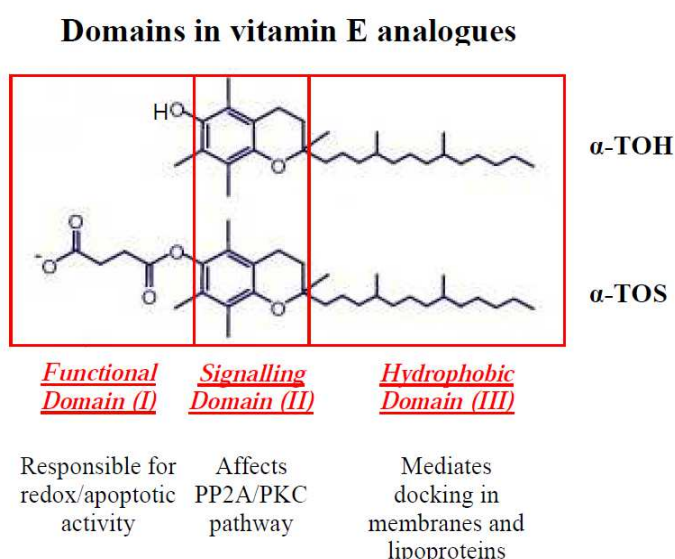


Figure 2.19: Scheme of major domains in vitamin E (α -tocopherol; α -TOH) and its esterified analogue α -tocopheryl succinate (α -TOS).

(adapted from Birringer *et al.*, 2003)

α -TOS is a potent apoptosis inducer highly selective for malignant cells while it is not harmful toward normal cells and tissues with apoptotic rates less than 5% compared to that

of malignant cells (Neuzil *et al.*, 2001a,b). To date, α -TOS has been shown to induce high levels of apoptosis in no less than 50 types of cancer cells (human, murine and avian) and tissue type (breast, colon, lung, lymphoma, mesothelium, skin, ovary, pancreas, prostate and stomach) (Israel *et al.*, 2000; Neuzil *et al.*, 2001a; Yu W *et al.*, 2001, Weber *et al.*, 2002; Wu *et al.*, 2002; Neuzil, 2003; Yu W *et al.*, 2003; Anderson *et al.*, 2004; Kline *et al.*, 2004; Neuzil *et al.*, 2004; Tomasetti *et al.* 2004a,b; Stapelberg *et al.*, 2005; Tomasetti and Neuzil, 2007). α -TOS has proved to be an efficient suppressor of tumour progression in animal models, too. This has been shown for malignancies such as colorectal carcinomas (Neuzil *et al.*, 2001a), melanomas (Malafa *et al.*, 2002), mesotheliomas (Tomasetti *et al.*, 2004b), and breast cancer (including HER2^{high} mammary carcinomas) (Dong *et al.*, 2007). It activates apoptosis in cancer cells via both intrinsic and extrinsic pathways. Typical morphological and biochemical alterations, characterised by chromatin condensation, chromatin crescent shaping and/or margination, DNA fragmentation and apoptotic body formation, appear after the activation of apoptosis by α -TOS in a variety of types of tumour cells (Tomasetti and Neuzil, 2007).

The molecular mechanism of apoptosis induced by α -TOS, the prototypic redox-silent VE analogue, is depicted in Figure 2.20, encompassing several reports on the mode of action of the VE analogue (Weber *et al.*, 2003, Neuzil *et al.*, 2004). α -TOS translocates to the cell, activates sphingomyelinase (SMase), giving rise to the formation of the lipid second messenger ceramide, possibly causing also destabilisation of lysosomes. These events converge on destabilisation of the mitochondrial membrane, which is amplified by ROS generation during this process. Mitochondrial membrane destabilisation, likely promoted by leakage by lysosomal proteases, leads to the cytosolic relocation of pro-apoptotic factors (such as cytochrome *c*, Smac/Diablo or the apoptosis-inducing factor, AIF) that can be regulated by the Bcl-2 family of proteins (including the anti-apoptotic proteins Bcl-2, Bcl-x_L or Mcl-1, which can be compromised by the pro-apoptotic Bax, probably mobilised to mitochondria after cleavage of Bid to its pro-apoptotic form tBid). Cyt *c*, Apaf-1 and pro-caspase-9 form the apoptosome, a ternary complex resulting in activation of the initiator caspase-9 that, in turn, activates the effector caspases. Smac/Diablo may amplify this process by suppressing the caspase-inhibitory activity of the inhibitor of apoptosis protein (IAP) family members, while AIF transmits the mitochondrial destabilisation directly to nuclear apoptotic events, bypassing the caspase cascade (Neuzil *et al.*, 2007).

membrane (MOM), forming a channel. ROS also oxidise cardiolipin (CL) within the mitochondrial inner membrane (MIM). This leads to dissociation of cytochrome c (Cyt c) from the inter-membrane face of CL. Free Cyt c then escapes via the Bax channel in the MOM into the cytoplasm. α -TOS, occupying the BH3 domains of Bcl-2 and Bcl-x_L, prevents Bax from forming inactive oligomers with Bcl-2 and Bcl-x_L, thereby increases the pool of available Bax dimers that form MOM channels promoting induction of apoptosis. Further, the VE analogue transcriptionally activates the protein Noxa (see below for detail).

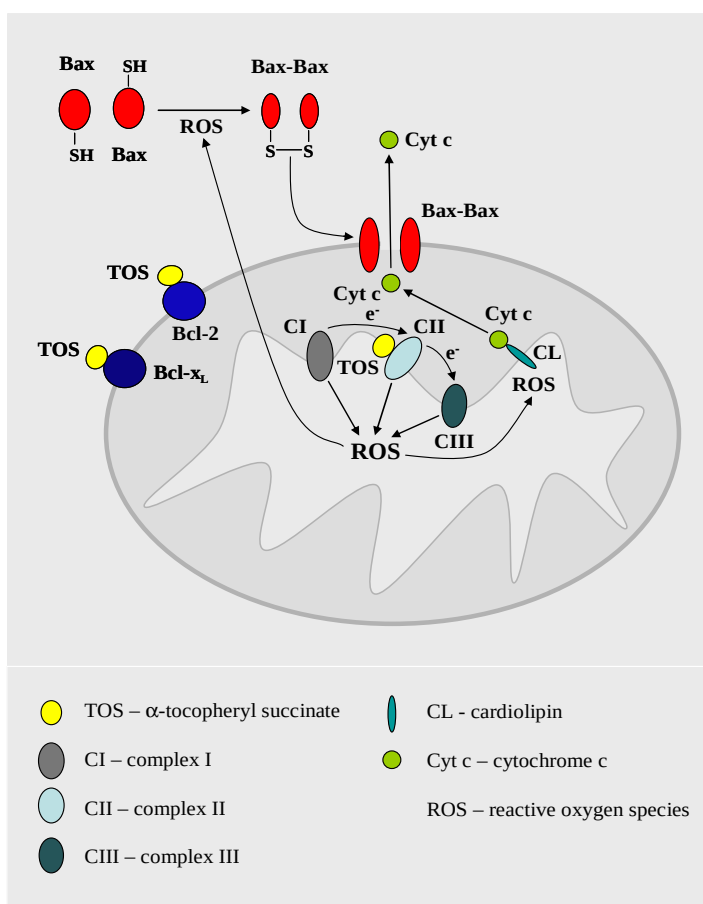


Figure 2.21: A working model of apoptosis induced by VE analogues (Neuzil *et al.*, 2006).

The ROS formed in the response of cancer cells to α -TOS activate the transcription of the Noxa gene by means of phosphorylation of the transcription factor FoxO1 by the Mst1 kinase, which itself is autophosphorylated as a response to the ROS stress (Valis *et al.*, 2011). The BH3-only protein Noxa is increased and then interacts with the anti-apoptotic protein Mcl-1, diverting it from the pro-apoptotic Bak. Free monomers of Bak then

oligomerise to form a pore in the MOM, ensuing in the promotion of the apoptotic cascade down-stream of mitochondria (Prochazka *et al.*, 2010).

The VE analogues, thus trigger apoptosis by initial generation of ROS. In search for the molecular target of α -TOS, we tested the possibility that the agent interacts with the mitochondrial CII. It was found that it does so in several types of cancer cells, including the T-lymphoma Jurkat cells and the breast cancer MCF7 cells. Direct evidence came from the use of Chinese hamster lung fibroblasts with mutations in CII and transformed with oncogenic H-Ras (Dong *et al.*, 2008). Additional experiments, including molecular modelling indicated a strong interaction of α -TOS with the Q_p and Q_d site in CII (Dong *et al.*, 2008). Importantly, too, CII was also found relevant for the anti-cancer activity of α -TOS in a mouse model (Dong *et al.*, 2009).

2.5.3 Mitochondrial targeting enhances the apoptogenic activity of VE analogues

There is insurmountable evidence that α -TOS induces apoptosis via mitochondria (Neuzil *et al.*, 1999) in a cancer cell-selective manner (Neuzil *et al.*, 2001b). However, it does not seem to distinguish the different membrane compartments within the cell, associating indiscriminately with all hydrophobic structures. In order to enhance the activity of α -TOS, it was decided to modify the compound by its tagging with a delocalised cationic group. The approach used for modification and mitochondrial targeting of antioxidants with the positively charged TPP⁺ group, as has been utilised for a variety of redox-active compounds (Smith *et al.*, 2003; Murphy and Smith, 2007; Biassutto *et al.*, 2010), was applied to VE analogues. The principle of mitochondrial targeting is described in Figure 2.22. The charge on the phosphonium group is delocalised, therefore the compound can move across biological membranes. The first hurdle is the plasma membrane, with negative potential on the cytosolic face, causing an increase of the compound across the membrane of 5-10-fold. The high negative potential ($\Delta\Psi_{m,i}$), across the MIM then causes up to 500-fold increase in the concentration of the compound at the interface of the mitochondrial matrix and the MIM (Biassutto *et al.*, 2010).

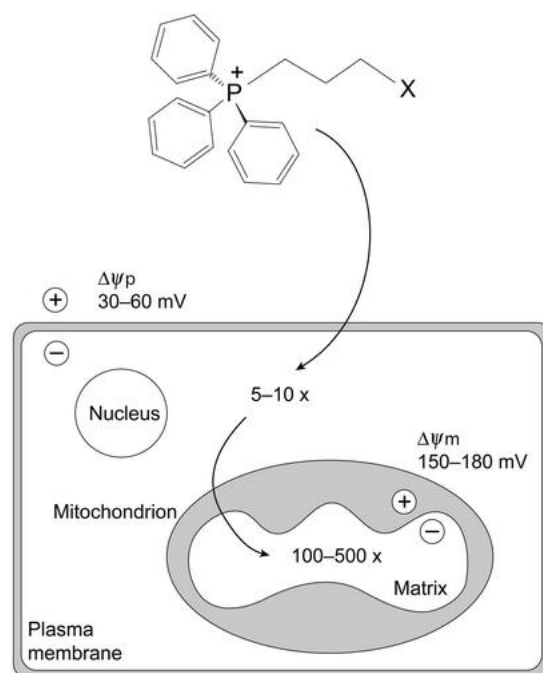


Figure 2.22: The uptake of alkyltriphenylphosphonium cations by mitochondria within cells.
(Smith *et al.*, 2003)

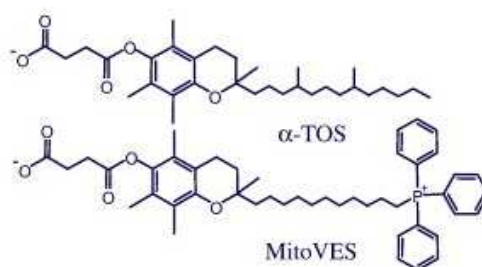


Figure 2.23: Chemical structures of α -tocopheryl succinate (α -TOS) and its modified form, the mitochondrially targeted vitamin E succinate (MitoVES).

The product of tagging of VE succinate with the TPP⁺ group, MitoVES (see Figure 2.23 for its structure), proved to be some 20-50-fold more efficient in killing cancer cells while preserving the selectivity for cancer cells (Dong *et al.*, 2011a,b). Fluorescently labelled MitoVES (MitoVES-F) proved almost exclusive mitochondrial localisation of the agent. Mutation of the UbQ-binding Ser68 of the Q_p site (Sun *et al.*, 2005) (replacement of Ser with Ala or Leu) caused a decrease in MitoVES-induced apoptosis, as well as lower activity to suppress the CII enzymatic activity and generation of ROS. Another piece of evidence that MitoVES interacts with the Q_p site stems from experiments, in which Jurkat cells were exposed to homologues of MitoVES with shorter aliphatic chain spanning the TPP⁺ group and the tocopheryl succinyl moiety. The shorter chain homologues were less

active to induce apoptosis and generate ROS, and in suppressing CII enzymatic activity. This is supported by molecular modelling. MitoVES is positioned such that the TPP⁺ part is at the matrix face of the MIM and the succinyl part of the agent should be able to reach the UbQ-binding Ser68 in the Q_p site. The length of the aliphatic chain of 11 carbons is ideal for the optimum activity of MitoVES (Dong *et al.*, 2011a,b).

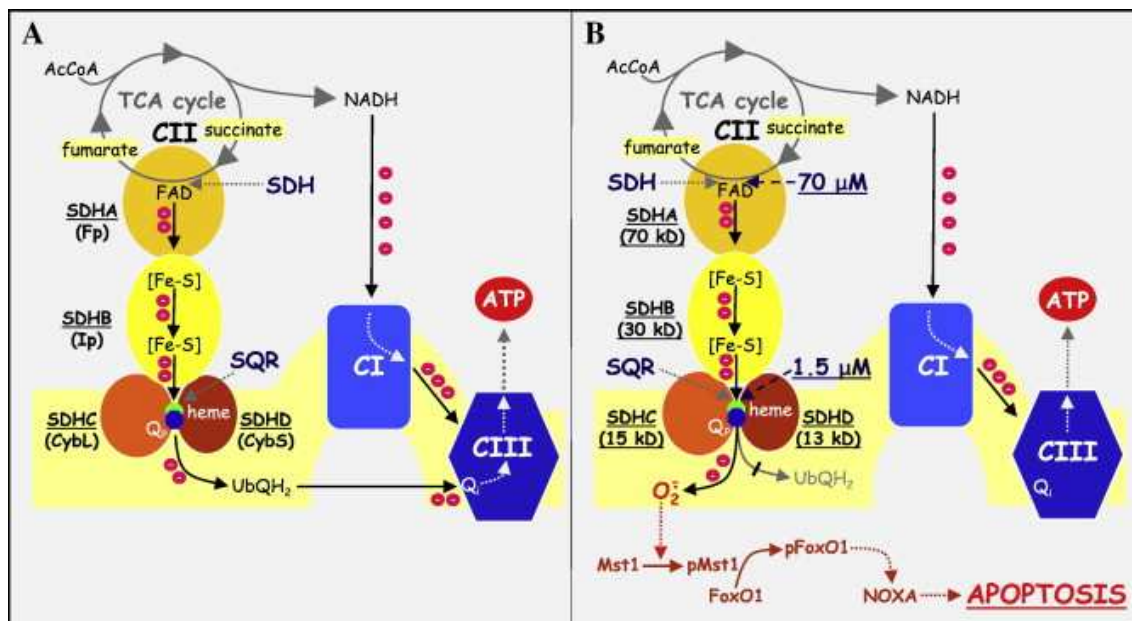


Figure 2.24: Molecular mechanism for the generation of ROS and the induction of apoptosis by vitamin E analogues via targeting of CII. (Kluckova *et al.*, 2013; Neuzil *et al.*, 2013)

Of particular interest are the findings of the effect of MitoVES on the two enzymatic activities of CII, viz. the SDH and SQR activity. While the IC₅₀ value for the inhibition of SDH by MitoVES is about 70 μM, that for the inhibition of the SQR activity is 1.5 μM (Dong *et al.*, 2011a,b; Rodríguez-Enríquez *et al.*, 2012). Therefore, a model was proposed as depicted in Figure 2.24. In the absence of MitoVES, the electrons generated during conversion of succinate to fumarate by SDH at the SDHA (Fp) subunit are relayed to the [Fe-S] clusters in the SDHB (Ip) subunit, which direct them to the Q_p (and/or, possibly, Q_d) site made up by residues from the SDHC (CybL) and SDHD (CybS) subunits. This results in a two electron reduction of the oxidised form of UbQ to ubiquinol, which has low affinity for its binding site(s) in CII. Ubiquinol is then released from CII to bind to CIII, where it gives up the two electrons to CIII at the Q_i site to be transferred to UbQ at the Q_o site. Hence, the incoming ubiquinol is re-oxidised and released to return as UbQ to

CII (A). This 'electron shuttle' activity of UbQ becomes disrupted in the presence of agents like the VE analogues, epitomised by MitoVES (B). In this case, MitoVES interferes with the function of UbQ, most likely by displacing it. Therefore, the electrons generated during conversion of succinate to fumarate cannot be intercepted by their natural acceptor. As a consequence, they interact with molecular oxygen to yield superoxide. The IC_{50} values for inhibition of the SDH activity and the CII→CIII electron transfer (represented by the SQR activity) are also indicated. The superoxide generated as indicated then causes (auto) phosphorylation of the Mst1 kinase, which phosphorylates the transcription factor FoxO1. The pFoxO1 protein translocates to the nucleus, where it triggers expression of the NOXA gene. The resulting increased level of the Noxa protein then causes the formation of the Bak and/or Bax channel in the MOM, ultimately resulting in the entry of the cell into the commitment phase of apoptosis.

2.6 Conclusion and relation to the work

Mitocans, anti-cancer agents targeting mitochondria, epitomised by VE analogues, are of considerable interest as potential clinically relevant agents. This is particularly important for cancers with high level of resistance to established therapies. An excellent example is mesothelioma that is thus far untreatable in all patients diagnosed with the disease. It is therefore important to understand whether VE analogues, represented by the highly efficient MitoVES, induce apoptosis in MM cells and whether they are also efficient in pre-clinical models of MM. If so, the molecular mechanism of the activity of the agent should be studied from the point of view of the mechanism of apoptosis as well as modulatory activity towards important signalling pathways. These notions can be reconciled with a recent application of α -TOS to an MM patient. The agent was applied transdermally and extended the life of the patient by four years. Further studies are needed and they require better understanding of the mechanism of action of α -TOS and, in particular, the highly efficient MitoVES.

3 Experimental part

3.1 Introduction

Malignant mesothelioma is characterised as a very aggressive neoplasm of serosal cavities, which is resistant to standard therapy. The incidence of MM is increasing in many countries all over the world. It is expected to rise in the following 10-20 years in the consequence of extensive exposure to asbestos in the past decades (Robinson *et al.*, 2005; Tomasetti *et al.*, 2009). To date, the treatment options for MM are very limited and the mean life-expectancy for someone with the disease is 1-12 month from diagnosis (Fennell *et al.*, 2008; Tomasetti *et al.*, 2009; Hollevoet *et al.*, 2012). The development of novel anti-cancer drugs and anti-cancer strategies that would improve the treatment of the disease is of great importance. Agents from the recently defined group of mitocans that selectively induce apoptosis in cancer cells by mitochondrial destabilisation have been shown to be very promising anti-cancer drugs, suppressing the growth of various tumour cells and many types of carcinomas in preclinical models.

3.2 Aims

The experimental part comprises two main aims:

The **first, major aim** of this study was to test the pro-apoptotic effect of a novel promising anti-tumour agent termed MitoVES (mitochondrially targeted vitamin E succinate) (Dong *et al.*, 2011a; Dong *et al.*, 2011b; Rohlena *et al.*, 2011) from the group of mitocans in various human and murine mesothelioma cell lines and in an experimental MM mouse model, and to compare it with the effect of its parental compound α -TOS (α -tocopheryl succinate). This arised from previous studies where the VE analogue α -TOS suppressed tumours in different models of cancer (Neuzil *et al.*, 2001a,b; Yin Y *et al.*, 2007; Zhao Y *et al.*, 2009), including MM (Tomasetti *et al.*, 2004a; Stapelberg *et al.*, 2005). Earlier work also refers to the interaction of α -TOS with ubiquinone-binding sites in mitochondrial respiratory complex II (Dong *et al.*, 2008; Dong *et al.*, 2009). Considerably stronger killing efficacy was achieved when the parental compound was modified by the addition of a delocalised cationic TPP⁺ group (Dong *et al.*, 2011a; Dong *et al.*, 2011b; Rohlena *et al.*,

2011). Since the therapeutic options for MM are very poor, MitoVES was tested here as an auspicious anti-MM agent.

Bellow is a more detailed list of aims that were tried to achieve in this part of the project:

1. To estimate the killing efficacy of α -TOS and MitoVES in various MM cell lines and to determine their IC₅₀ values;
2. To compare the propensity of both VE analogues to suppress MM cell viability and to induce apoptosis;
3. To assess and compare the generation of reactive oxygen species (ROS) and dissipation of mitochondrial membrane potential, induced by the two mitocans in MM cells;
4. To test, whether the mitochondrially targeted compound MitoVES accumulates in mitochondria of MM cells and to compare it with α -TOS using their fluorescently labelled analogues and confocal microscopy;
5. To study whether MitoVES targets mitochondrial respiratory complex II (CII) in MM and whether CII and the structure of the compound are important for its killing activity;
6. To estimate, whether MitoVES and α -TOS affect the formation of supercomplexes of the mitochondrial respiratory chain;
7. To test and compare the anti-cancer activity of MitoVES and α -TOS in an MM mouse model.

All the aims listed here have been achieved. All data and results presented in this part will be published by **Kovarova J et al.**, 2013 (under review).

In the **second, minor part** of the project, we tried to find out, which proteins/signalling pathways are involved in the induction of apoptosis mediated by the vitamin E analogue α -TOS in MM cells. MitoVES has not been tested here, since it was designed and synthesised later than the parental compound. This study involved proteomic as well as genomic approach.

Aims that were attempted to be achieved in the second part of the work are listed below:

1. To identify proteins which are either up-regulated or down-regulated after treatment of MM cells with α -TOS by 2-dimensional gel electrophoresis (2-D GE) and mass spectrometry (MS) analysis;
2. To confirm the changed expression levels of the identified proteins by western blot analysis;
3. To determinate, how the different expression of the identified proteins is regulated after exposure to α -TOS, whether this depends on transcriptional level of genes that code for the identified proteins or on post-transcriptional mechanisms;
4. To confirm the role of the identified genes and proteins by functional studies (using gene silencing).

Regrettably, the goals of the second part of this work were achieved only partially, since there was a need during this work to focus more on the project described in the first aim, i.e. the mechanisms of killing of MM cells by MitoVES, due to the oncoming clinical trials (planned by our laboratory). Despite this slight change of focus, it is worth to complete the second part of this project, as the identified proteins have been described by other previous studies to play important roles in a variety of functions of (cancer) cells, including regulation of apoptosis, cell proliferation, carcinogenesis and metastasis. It is planned that this part of the project will be completed in a follow up study in our laboratory.

The second part of the work contains unpublished data.

3.3 Methodology

A complete list of materials used for the here described methodology is provided in Appendix A.

3.3.1 Methodology used in Aim I

3.3.1.1 Cell culture and treatment

Human adherent Ist-Mes-2 cells (epitheloid phenotype), MM-BI cells (mixed phenotype), Meso-2 and H28 cells (sarcomatoid phenotype) (Pass *et al.*, 1995), and murine adherent AE17 MM cells (Jackaman *et al.*, 2003) were cultivated in cell culture flasks (TPP) in the DMEM or RPMI medium supplemented with 10% foetal bovine serum (FBS) and antibiotics/antimycotics (further referred to as complete medium) at 37°C in a humidified atmosphere of 5% CO₂.

For treatment, the cells were trypsinised using the trypsin/EDTA solution (1:1 ratio), harvested and seeded as required. They were allowed to re-attach and to reach 60-70% confluency before the addition of vitamin E (VE) analogues (*cf* Figure D.1). VE analogues included α -tocopheryl succinate (α -TOS) and, unless specified otherwise, the full length MitoVES homologue, which contains an 11-C aliphatic chain. α -TOS, a powdery substance, was purchased from Sigma. MitoVES was synthesised as a solid crystalline or powdery substance as described earlier (Dong *et al.*, 2011a,b). Both agents were found to be soluble in ethanol (EtOH for UV spectroscopy), prepared as 50 mM stock solutions and stored at -20°C; working 10 mM solutions were made and stored for shorter time at 4°C.

In some experiments, different MitoVES derivatives with shortened aliphatic chains were tested as well as an analogue of α -TOS with the TPP⁺ group esterified at the free carboxylate of its succinyl moiety via a 4-C spacer. Additionally, fluorescently labelled analogues of MitoVES and α -TOS were tested, which were synthesised as described earlier (Dong *et al.*, 2011a,b) (Figure D.1). The agents were dissolved in EtOH and added to the cell culture at v/v <0.1 %. Controls were treated with the same volume of EtOH only.

3.3.1.2 Viability assay

Toxicity of the VE analogues towards malignant cells was assessed on the basis of the IC₅₀ values using the crystal violet method as follows. Adherent MM cells were seeded in 96-well plates (TPP) at 10⁴ cells per well and allowed to adhere. On the next day, the cells were treated with increasing concentrations of MitoVES (0.5 to 10 µM) and α-TOS (25 to 100 µM) in triplicates for a period of 24 h. The cells were then washed with PBS and fixed with 3% paraformaldehyde for 30 min. The fixative was removed and the cells were washed three times with PBS, followed by staining with 0.05% crystal violet for 1 h at room temperature. The dye was then removed and the cells were washed again three times with PBS, and subsequently solubilised in 1% SDS by gentle agitation for 1 h. The final absorbance was determined at 595 nm using the Tecan Infinity plate reader and the obtained absorbance values analysed using Excell, before being transferred to GraphPad Prism 5 for graphing of the killing curves and evaluation.

3.3.1.3 Cell death detection

Cell death level was assessed by flow cytometry using the annexin V-FITC/propidium iodide (PI) method. Annexin V is a specific marker for cells in early stages of apoptosis, binding to phosphatidyl serine that translocates from the inner leaflet of the plasma membrane to its outer leaflet in the early phases of apoptosis. PI staining was used to detect necrosis and/or secondary, post-apoptotic necrosis. To characterise the extent and nature of cell death, the 'dual' staining method was applied. Adherent MM cells were placed into 12-well plates (TPP) at 10⁵ cells per well and maintained as mentioned in section 3.3.1.1. On the next day, the cells were exposed to increasing concentrations of α-TOS and MitoVES for 24 h. After the drug treatment, the cells were harvested, centrifuged (300 x g, 5 min) and washed twice with PBS. The supernatant was removed and the pellet resuspended in the annexin V detection reaction mix, containing annexin V-FITC, annexin V-binding buffer and PBS. The samples were incubated for 20 min at 4°C in the dark, followed by addition of PI and additional incubation for 10 min. Cell death was assessed by flow cytometry, using the BD FACS Calibur instrument. The obtained data were analysed using the FlowJo 9.6.2 software before the values were graphed and assessed for statistical significance using Student's t-test.

3.3.1.4 *Assessment of superoxide generation using MitoSOX™ Red*

The MitoSOX™ Red reagent is a fluorogenic dye which is specifically targeted to mitochondria in live cells. It is rapidly oxidised by superoxide but not by other reactive oxygen species (ROS) and reactive nitrogen species (RNS). The oxidised product is highly fluorescent and can be detected by flow cytometry. To assess the generation of superoxide in response to VE analogues, MM cells were seeded into 12-well plates at 10^5 cells per well and allowed to attach. On the next day, the cells were treated with 100 μ M α -TOS and 5 μ M MitoVES for 0, 15, 30, 45 and 60 min. MitoSOX was added to each sample 15 min before each time point at a final concentration of 5 μ M. After 15 min incubation in the dark, the cells were harvested, spun down (300 x g, 5 min), resuspended in 100 μ l PBS and assessed for superoxide generation by flow cytometry using the BD FACS Calibur instrument. The data were analysed using the FlowJo 9.6.2 software before the values were graphed and determined for statistical significance using Student's t-test.

3.3.1.5 *Assessment of ROS generation using the DHE probe*

Dihydroethidium (DHE), a cell permeable dye, was used to evaluate the intracellular levels of reactive oxygen species (ROS). In response to ROS generation, DHE is oxidised to the fluorescent ethidium, which can be detected by flow cytometry. To assess the generation of ROS in response to VE analogues, MM cells were placed into 12-well plates at 10^5 cells per well and cultivated under normal culture conditions as mentioned in section 3.3.1.1. On the next day, the cells were exposed to 100 μ M α -TOS and 5 μ M MitoVES for 0, 15, 30, 45 and 60 min. 15 min before each time point, each sample was supplemented with DHE at 5 μ M and incubated with the cells in dark. The cells were then harvested, spun down (300 x g, 5 min), resuspended in 100 μ l PBS and assessed for ROS generation by flow cytometry using the BD FACS Calibur instrument. The data were analysed using the FlowJo 9.6.2 software before the values were graphed and determined for statistical significance using Student's t-test.

3.3.1.6 *Assessment of ROS generation using the DCF-DA assay*

To estimate the cellular generation of ROS, the cell permeable probe dichlorodihydrofluorescein diacetate (DCF-DA) was used. DCF-DA is deacetylated by cellular esterases to

a non-fluorescent compound, which is then oxidised by ROS to 2'7'-dichlorodihydro-fluorescein (DCF). DCF is a highly fluorescent compound that can be detected by flow cytometry. To determine ROS generation in response to VE analogues, MM cells were placed into 12-well plates at 10^5 cells per well and cultivated as mentioned in section 3.3.1.1. On the next day, the cells were exposed to 100 μ M α -TOS and 5 μ M MitoVES for 0, 15, 30, 45 and 60 min. 15 min before each time point, DCF-DA was added to each sample to a final concentration of 5 μ M and the cells incubated in the dark. Thereafter, the cells were harvested, spun down (300 x g, 5 min), resuspended in 100 μ l PBS and assessed for ROS generation by flow cytometry using the BD FACS Calibur instrument. The data were analysed using the FlowJo 9.6.2 software before the values were graphed and determined for statistical significance using Student's t-test.

In some experiments, the DCF-DA probe was used in combination with the TMRM assay (see bellow).

3.3.1.7 *Assessment of mitochondrial inner trans-membrane potential*

Tetramethylrhodamine methyl ester perchlorate (TMRM), a cationic red-orange fluorescent dye, which is readily sequestered by active mitochondria, was used to assess mitochondrial inner trans-membrane potential ($\Delta\Psi_{m,i}$). To determine non-specific TMRM-loading, the mitochondrial uncoupler carbonyl cyanide 4-(trifluoromethoxy)phenyl-hydrazone (CCCP) was used as a control. Adherent MM cells were seeded into 12 well plates at 10^5 cells per well and maintained as mentioned in section 3.3.1.1. On the next day, the cells were exposed to 100 μ M α -TOS and 5 μ M MitoVES for 0, 15, 30, 45 and 60 min. 15 min before each time point, TMRM was added at 50 nM to each sample, and the cells incubated at 37°C in the dark. As a control, one sample was pre-treated for 15 min with CCCP at 100 μ M as well. Thereafter, the cells were harvested, spun down, washed with PBS and resuspended in 100 μ l PBS containing 50 nM TMRM or in PBS with 50 nM TMRM and 100 μ M CCCP. Mean fluorescent intensity of all samples was assessed by the BD FACS Calibur instrument and analysed using the FlowJo 9.6.2 software to estimate the $\Delta\Psi_{m,i}$ dissipation. The aquired values were graphed and determined for statistical significance using Student's t-test.

3.3.1.8 *Confocal microscopy*

The cell lines (MM-BI, Meso-2, Ist-Mes-2 and H28) were seeded at 10^5 cells per Lab-tek chamber cover glass slide (Nunc) and allowed to attach overnight. The cells were then stained with 100 nM TMRM and 5 $\mu\text{g/ml}$ Hoechst 33342 for 1 h prior to the addition of the fluorescent agent. The cells were incubated with either MitoVES-F or α -TOS-F at 5 μM for 20 min, and then returned to DMEM supplemented with 10% FBS and antibiotics and antimycotics containing TMRM and Hoechst. Imaging was performed using the Olympus FV1000 fluorescent confocal microscope. In some cases, the cells were pre-treated with 1 μM CCCP for 10 min, which was included in the original incubation with Hoechst and TMRM and in all steps of the sample processing until imaging with an Olympus FV1000 laser scanning confocal microscope, using the 62x objective with digital zoom, with pixel size of 640x640 and the standard lasers and filters.

3.3.1.9 *Succinate-ubiquinone Q oxidoreductase activity*

Succinate-ubiquinone Q oxidoreductase (SQR) activity was estimated according to Barrientos *et al.*, 2009. SQR activity assay measures the electron transfer from succinate to UbQ. The formation of ubiquinol is assessed by the ubiquinol-dependent reduction of 2',6'-dichloroindophenol (DCPIP) which serves as an electron acceptor. This assay involves succinate as the electron donor.

Ist-Mes-2 cells were seeded, and allowed to attach and to reach 60-70% confluency. The cells were then exposed to 5 μM of various analogues of MitoVES (for detail see Results 3.4.1.2). After 60-min incubation with the agents, the cells were evaluated for SQR activity. Total protein was extracted using a Cell Signaling extraction buffer containing a protease inhibitor cocktail, and freeze-thawed to disrupt the MIM. Protein quantitation was estimated using the Pierce Coomassie Plus Protein Assay Reagent (Thermo Scientific). 40 μg of protein lysate was added to 1 ml of the SQR assay buffer (Appendix B). 80 μM DCPIP, 4 μM rotenone, 0.2 mM ATP and 10 mM succinate were added and the samples incubated at 30°C for 10 min. The reaction was started by the addition of 80 μM decylubiquinone, and absorbance evaluated every minute for 5 minutes at 600 nm using the TECAN Infinity plate reader.

3.3.1.10 Mitochondrial respiration and complex II activity

Cellular respiration was estimated using the Oxygraph-2k instrument (Oroboros Instruments), basically as published (Pesta and Gnaiger, 2012). AE17, MM-BI, H28, Ist-Mes-2 and Meso-2 cells were seeded and allowed to reach 60-70% confluency. Experiments evaluating intact cell respiration were performed with MM cells suspended in the complete RPMI medium. The advantage of this setup is that it closely resembles the *in vitro* conditions used in most experiments. The cells were harvested by trypsinisation, spun down at 300 x *g* for 5 min, resuspended in complete RPMI medium at 10^6 cells/ml and placed in the Oxygraph chamber. Increasing concentrations of α -TOS (10-100 μ M) or MitoVES (1-100 μ M) were added and oxygen consumption was recorded.

Experiments assessing digitonin-permeabilised cell oxygen consumption were performed using the aforementioned MM cells to investigate the involvement and contribution of individual mitochondrial respiratory complexes in cellular respiration. The cells were harvested as mentioned above, permeabilised with digitonin (10 μ g/ 10^6 cells), suspended in the special mitochondrial respiration medium (MiR05) (see Appendix B) at 2×10^6 per ml and placed in the Oxygraph chamber. To investigate, whether complex I (CI) or complex II (CII) of the mitochondrial electron transport chain contribute to the respiration, the cell suspension in the chamber was supplemented with the appropriate biochemical substrates and inhibitors of the other complex. Rotenone at 0.5 μ M was added to inhibit CI and malonate at 5 μ M was used to inhibit CII activity. Further, for assessment of CI activity the substrates ADP at 3 mM, glutamate at 10 mM and malate at 2 mM were added, and for determination of CII activity the cell suspension was supplemented with the substrates ADP at 3 mM and succinate at 10 mM. Increasing concentrations of α -TOS and MitoVES (see above) were added to the Oxygraph chamber and the total oxygen concentration consumption was assessed. The results were related to the cell number.

3.3.1.11 Native blue electrophoresis and Western blotting

Native blue electrophoresis (NBE) was performed according to Schagger and von Jagow, 1991.

Isolation of mitochondria (equal for Hr-CNE)

MM cells (Ist-Mes-2 and H28) were allowed to reach 60-70% confluency. The cells were trypsinised, harvested, spun down (500 x *g*, 5 min), washed with PBS and spun down again. The supernatant was removed and the cell pellet kept at -80°C until use. Mitochondria were isolated by differential centrifugation. Frozen cell pellets were allowed to thaw on ice and resuspended in the sucrose-Tris buffer (STE) (Appendix B) containing the protease inhibitor cocktail (PIC). The cell suspension was homogenised on ice with glass-Teflon homogenizer for cca 15 min. The homogenate was spun down at 600 x *g* for 10 min at 4°C to remove the nuclei and cell debris. Mitochondria floating in the supernatant were pelleted by centrifugation at 10,000 x *g* at 4°C for 20 min. The supernatant was discarded and the pellet resuspended in the STE followed by further centrifugation under the same conditions. The pellet was resuspended in the remaining STE and mixed to obtain homogenous samples. Aliquots were prepared and kept at -80°C. The mitochondrial protein content was determined using the BCA protein assay kit (Thermo Scientific).

Solubilisation of mitochondria

500 µg isolated mitochondria were centrifuged at 10⁴ x *g* at 4°C for 10 min. The pellet was resuspended in 150 µl extraction buffer (EB) (Appendix B), solubilised by the addition of 30 µl digitonin (digitonin/protein ratios ~10 g/g) and incubated for 40 min on ice; the mixture was then vortexed every 10 min. This was followed by centrifugation at 23,000 x *g* for 40 min at 4°C.

Preparation of samples for electrophoresis

The samples were diluted for a constant loading volume in the EB, and the BNE sample buffer (Appendix B) was added at the 1:10 ratio. The protein extracts were diluted at the 1:1 ratio with the BNE sample buffer.

Electrophoresis conditions (equal for Hr-CNE)

The samples were loaded on pre-cast NativePAGE Novex 4-16% and 3-12% Bis-Tris gels. The gels were run at 4-7°C. The initial voltage was set to 100 V. When the samples entered the gel, the voltage was increased to 300 V, with the current limited to 15 mA. Electrophoresis was stopped after 2-3 h, when the sharp leading line of Ponceau S dye approached the gel front or when the native marker reached the appropriate size for native proteins.

Semi-wet electroblotting of BNE gels

After electrophoresis, the gels were equilibrated in the native gel transfer buffer containing 0.1% SDS for 10 min. Separated proteins were transferred to the PVDF membrane using the Transblot Invitrogen system. Prior to the transfer, PVDF membranes (GE Healthcare) were activated by 15 s washing with 100% methanol, followed by deionised water and finally with the transfer buffer. PVDF membranes were blotted at 30 V for 1-2 h at the starting current of approximately 100 mA. Within the transfer, the current dropped to about 50 mA. After the transfer, membranes were fixed in 5% acetic acid for 20 min and washed with TBS/0.1% Tween20. The membranes were then blocked with 5% low fat milk (Sigma) in TBS/0.1% Tween20 for 1 h.

Immunodetection

Individual mitochondrial complexes were detected using the following antibodies from Abcam: CI, anti-NDUFS1 (79 kDa); CII, anti-SDHA (70 kDa); CIII, anti-UQCRC2 (48 kDa); CIV, anti-MTCO2 (26 kDa); CV, anti-ATP5A (60 kDa). Anti-VDAC IgG (Abcam) was used as a loading control. All antibodies were dissolved 1:10,000 in 1% low fat milk in TBS/0.1% Tween20 and incubated with the membranes overnight. Specific proteins were visualised using the SuperSignal[®] West Femto Maximum Sensitivity Substrate (Thermo scientific) and the multipurpose CCD camera system (ImageQuant LAS 4000, GE Healthcare).

3.3.1.12 *In-gel catalytic activity assay for complex I and II*

The in-gel assay was performed according to Wittig *et al.*, 2007. Isolated mitochondria were lysed (as described above) and subjected to high resolution clear native electrophoresis (HrCNE), run basically as described above but without the addition of the blue dye. The assays were performed at room temperature, the samples fixed after analysis using the fixation solution. Gel strips were loaded with 60 µg of extracted mitochondria from MM cells. CI and CII assay were both performed in the activity assay buffer (Appendix B). CI activity was assessed on 3-12% gel strips using the NADH:NTB reductase assay. This gel strip was evaluated in freshly prepared NADH:NTB reductase assay buffer (Appendix B), after 15 min transferred to the activity assay buffer and scanned. CII activity was analysed on 4-16% gel strips using the succinate:NTB reductase assay. The gel strip was incubated in the freshly prepared succinate:NTB reductase assay buffer (Appendix B) for 10-20 min, immediately transferred to the activity assay buffer and scanned.

3.3.1.13 *Preparation of SDHC-deficient cells*

Ist-Mes-2 cells were seeded in 24-well plates at 2×10^4 cells/well in antibiotic-free DMEM complemented with 10% FBS and allowed to attach over night. The SureSilencing human SDHC shRNA or non-silencing (NS) plasmid (SuperArray Biosciences) was mixed with Eugene HD transfection reagent in a 3:1 ratio and added to the cells, which were left for 48 h to recover. The cells were then incubated with 4 µg/ml puromycin for 2 weeks for selection of transfected cells. To estimate the successful transfection in selected clones of cells, SDHC protein level was assessed via western blot using anti-SDHC IgG (Santa Cruz). Protein concentration was normalised with HRP conjugated anti β-Actin (Cell Signalling).

3.3.1.14 *SDH activity*

Transfected Ist-Mes-2 cells with human SDHC shRNA or NS shRNA (see above) were seeded in phenol free DMEM supplemented with 10% FBS and penicilline/streptomycine at 10^4 cells/well in 96 well plates and allowed to attach overnight. The cells were then incubated with 10 mM succinate for 1 h followed by the addition of 10 µl MTT (5 mg/ml)

to each well for 4 h at 37°C and 5% CO₂. After the incubation, the cells were washed in PBS and then the MTT crystals were dissolved using 200 µl DMSO. The absorbance at 570 nm was estimated using the TECAN Infinite 200 Pro plate reader (Tecan).

3.3.1.15 *Animal experiments*

For *in vivo* evaluation, we used immunodeficient, athymic Balb/c *nu/nu* mice. The animals were injected subcutaneously with 2×10^6 Ist-Mes-2 cells freshly harvested at 50% confluency and suspended in 100 µl PBS. Tumours formed within about two weeks depending on the number of injected cells. The animals were regularly checked by ultrasound imaging using the Vevo770 ultrasound imaging apparatus (VisualSonics) fitted with the 80 Mhz scan-head with the resolution of 30 µm. As soon as tumours reached $\sim 30 \text{ mm}^3$, the animals were injected into the peritoneum with 1 µM MitoVES or 15 µM α -TOS every 3-4 days. Both agents were first solubilised in EtOH (the final concentration of EtOH was 4% v/v), and then dissolved in corn oil. Control mice were injected with an equal volume of the excipient only. Tumours were visualised and quantified non-invasively by the ultrasound imaging apparatus. Threedimensional reconstruction of tumours enabled precise quantification of tumour volume.

3.3.1.16 *Statistical analysis*

Experimental data are presented as mean $\pm$ SD. Mann-Whitney-U-test for unpaired samples and Kruskal-Wallis analysis for multiple comparisons were used to perform comparisons between groups. Statistical calculations were carried out using the SPSS statistical package version 12.0F. Statistical differences of at least $p < 0.05$ were considered statistically significant.

3.3.2 Methodology used in Aim II

3.3.2.1 *Cell culture and treatment*

Human adherent Ist-Mes-2 cells (epitheloid phenotype) were maintained in the complete DMEM medium at 37°C in a humidified atmosphere of 5% CO₂ as described in section 3.3.1.1. For treatment, the cells were trypsinised using trypsin/EDTA solution (1:1 ratio),

harvested and seeded as required. They were allowed to re-attach and to reach 60-70% confluency before addition of the vitamin E (VE) analogue α -tocopheryl succinate (α -TOS) (cf Figure D.1) which was prepared and stored as described in section 3.3.1.1. α -TOS was dissolved in EtOH for UV spectroscopy and added to the cells grown in the complete DMEM medium. Controls were treated with an equal volume of EtOH only.

3.3.2.2 2-D gel electrophoresis and mass spectrometry/HPLC analysis

Preparation of whole cell lysates

Adherent MM cells were seeded and cultivated in T150 cell culture flasks (TPP) as described in section 3.3.1.1. The cells were allowed to reach the 60-70% confluency and were then exposed to 25 μ M α -TOS for 24 h. Control cells were treated with an equal volume of EtOH only. After the treatment, the cells were harvested (with the medium in which they were maintained), centrifuged (300 x g , 10 min, 4°C), washed twice with PBS and spun down again. The supernatant was removed and the pellet resuspended in the lysis buffer containing 1:100 protease inhibitor mix (100x) (GE Healthcare) (10⁶ cells/1 ml lysis buffer). The cells were lysed in the lysis buffer for 30 min at room temperature. The DNA in lysates was digested by the addition of benzonase (1 μ l/150 μ l lysis buffer) (Novagen, 25 U/ μ l) for 1 h on ice. Protein lysates were stored at -80°C.

Estimation of protein concentration from the whole cell lysates

The lysates were allowed to thaw on ice and were then centrifugated for 15 min at 15,000 x g at room temperature. The pellet was removed. Protein concentration of the supernatant was estimated using the EZQ Protein Quantitation Kit (Molecular Probes) (according to the manufacturer's instruction). EZQ-stained paper stripes were imaged using the laser-based scanner Molecular Imager FX. The protein concentrations were calculated from a standard curve using the Quantity One Software (version 4.6.0).

Preparation of the protein samples for isoelectric focusing – rehydration of IPG strips

300 µg protein in 340 µl rehydration buffer was used per one 18 cm IPG strip (GE Healthcare Life Sciences). The protein samples were centrifuged prior to loading (20,000 x *g* for 15 min at room temperature) to remove the unsolubilised debris; excess salts were removed from the samples using the 0.5 ml Zeba spin desalt columns (Thermo Scientific) (according to the manufacturer's instruction). The samples were then pipetted into the Immobiline re-swelling tray (GE Healthcare Life Sciences). For rehydration, IPG strips (pH 4-7, 18 cm, non-linear) were positioned in the tray gel-side down onto the protein samples and finally covered with the Immobiline dry strip cover fluid (mineral oil) (GE Healthcare Life Sciences). The rehydration occurred over night.

Isoelectric focusing – First dimension

Proteins were separated according to their isoelectric point (pI) by preparative, liquid-phase isoelectric focusing (IEF). IEF was performed on Ettan IPGphor II IEF system (GE Healthcare Life Sciences). The strips were positioned gel side-up in the ceramic Ettan IPGphor cup loading manifold. Electrode paper pads wetted with water were positioned on both sides of a strip. A further electrode paper was wetted with 15 mM DTT and positioned onto the basic side of the strip close to the basic electrode filter pad on the minus pole. The IPGphor manifold was placed into the IPGphor unit. The electrodes were then positioned precisely on both paper pads wetted with water. Each strip was covered with cca 5 ml dry strip cover fluid (mineral oil) and IPGphor was run. The current did not exceed 80 µA.

The programme used for isoelectring focusing (set for a 18 cm gel strip) is below:

Step	Voltage (V)	Current (µA)	Vh	Time
1	150	80	150	1 h
2	300	80	600	2 h
3	300-1000	80	108	10 min
4	1000	80	1000	2 h
5	1000-8000	80	4000	1 h
6	8000	80	95000	12 h
7	8000	80	25000	3 h
Total			125000	cca 20 h

Equilibration of IPG strips prior to SDS-PAGE

The focused IPGstrips were first equilibrated in the equilibration solution 1 containing 2% DTT for 10 min. Free thiol groups were alkylated for 10 min in the equilibration solution 2 containing 2.5% iodoacetamide (IAA) and bromphenol blue.

SDS-PAGE – Second dimension

Proteins were separated according to their molecular mass by SDS PAGE. Equilibrated strips were positioned between two glass plates with pre-cast 10% separating and 4% stacking acrylamide/polyacrylamide gel and overlayed with 0.5% agarose in 1x running buffer. SDS-PAGE was performed using the Protean II XL apparatus (BioRad) with 1 mm spacer and external water cooling and 1 x running buffer at 4°C. The gels were run at 35 mA per 2 gels for about 60 min until the bromphenol blue line entered the separating gel, and further at 65 mA for about 240 min until the dye line was about 1 cm from the bottom of the gel.

Protein visualisation

The gels were fixed for 1 hr in the fixation solution, washed briefly with deionized water and stained with the NovexTM Colloidal Blue Stain (Invitrogen) overnight according to the manufacturer's instructions. The gels were destained by several washing steps with deionized water.

Gel imaging and analysis

Stained gels were scanned using a calibrated PDi 420oe scanner/densitometer (Agfa) in the OD range of 0.0-0.4. The images were processed via the PDquest 7.1 2D Gel Analysis Software (BioRad). Gel set triplets were used to detect, match and analyse the protein spots, resulting in the creation of a virtual gel (Master gel). A gel set included a gel with an untreated sample and a gel with an α -TOS-treated sample. The master gel summarised all spot identifications. In total, three master gels were prepared (each from a gel set triplicate) and analysed. Normalisation of spot density was based on the total density in the gel image. Protein spots that were in average more than 1.5-fold differentially expressed after

treatment with α -TOS were considered for protein identification. Statistical significance of the different expression of these proteins was estimated using Student's t-test.

Proteolytic digestion and sample preparation

The preparative gel used for protein identification contained a higher protein amount (485 μ g protein/gel). It was prepared in the same way as analytical gels. Spots of interest were excised from the gel, cut into small pieces and destained in the sonic bath (Ultrasonic Cleaner, VWR) by several washing steps in 0.1 M 4-ethylmorpholine acetate (pH 8.1) in 50% acetonitrile (MeCN). After complete destaining, the gel pieces were washed with deionised water, shrunk by dehydration in acetonitrile and re-swollen again in water. The supernatant was removed and the gel pieces partly dried using a SpeedVac concentrator (Savant). Gel pieces were then reconstituted in the cleavage buffer (see Appendix B) and digested overnight at 37°C. The resulting tryptic peptides were extracted with 10% acetonitrile and 1% acetic acid and dried in the SpeedVac concentrator. For LC-MS/MS or direct MS and MS/MS analysis, the peptide samples were transferred to the Institute of Analytical Chemistry, Brno, Czech Republic.

Mass spectrometry/HPLC analysis and protein identification

Prior to the LC-MS/MS analysis samples were desalted using Varian OMIX C18 ZipTips (10 μ l) and dried. The desalted peptide mixtures were separated by 2D HPLC 3000 Ultimate System (Dionex). A volume of 10 μ l of each sample dissolved in 0.1% reifluoroacetic acid in water was injected onto a strong cation exchange (SCX) column (Dionex), followed by elution with the salt plug injection of 0-2000 mM sodium chloride. The fractions resulting from the salt plug injection onto the SCX column and the flow through fractions were desalted on the 'trapping' column and subsequently eluted onto the RP C18 nano-LC column, where fractions were separated with a linear gradient: 3 min, 6-25% B; 27 min, 25-55% B; 1 min, 55-90% B. A: 0.05 trifluoroacetic acid in water, B: 0.05% trifluoroacetic acid in acetonitrile. The eluting peptides were mixed with the CHCA solution (3 mg/ml in 66% acetonitrile) and fractionated every 30 s directly on a MALDI plate using the Probot TM Micro Fraction Collector (Dionex).

Direct MS and MS/MS analysis: Prior to the MS measurement, the samples of the protein digests were desalted by Varian ZipTip C₁₈, dried and re-dissolved in 2 µl 0.05% trifluoroacetic acid in water. Each sample at 1 µl was mixed with 1 µl of the matrix solution (saturated CHCA in 80% MeCN with 0.05% trifluoroacetic acid) and spotted on a MALDI plate.

All spectra were acquired using the Applied Biosystems 4700 Proteomics Analyser (Applied Biosystems) equipped with an Nd: YAG laser (355 nm) of 3-7 ns pulse and 200-Hz firing rate. The TOF/TOF analyser operates at 200 Hz repetition rate in both MS and MS/MS modes. The reflectron mode was used for peptides analysis. MS and MS/MS data were further processed using the DataExplorer 4.5 software (Applied Biosystems).

Database searching for protein identification was performed using the Mascot programme package (local installation, version 1.9.05) against the NCBI nr sequence database (version 22.3.2006) narrowed to mammalian species (cleavage by trypsin, variable modification: carbamidomethyl (C), oxidation (M) and oxidation (HW), one miscleavage possible).

3.3.2.3 Reverse Transcription Polymerase Chain Reaction

RNA isolation

Ist-Mes-2 cells were seeded and maintained in T75 cell culture flasks (TPP) as described in section 3.3.1.1. The cells were allowed to reach 60-70% confluency, and were then exposed to 25 µM α -TOS for 24 h. Control cells were treated with an equal volume of EtOH for spectroscopy only. Total RNA was obtained from cells which were washed with PBS prior to RNA extraction. The cells were lysed in 1 ml of the TRIzol Reagent (Invitrogen) for 5 min at room temperature, scraped off with a cell scraper and transferred into 2 ml centrifuge tube. 200 µl of chloroform was added and the lysate was vortexed for 15 s, incubated for 3 min at room temperature and centrifuged for 10 min at 12,000 x *g* and 4°C. The upper aqueous phase was retained, transferred into a new tube and the volume was estimated. RNA was purified from the aqueous phase by the addition of 1 volume of 70% EtOH, the content was mixed and purified using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. The eluted total RNA was stored at -80°C.

Assessment of total RNA concentration

Concentration of total RNA was estimated using the ND1000 Spectrophotometer (NanoDrop) and the nucleic acid programme. 1 µl of total RNA was pipetted on the sensor. Absorption of total RNA was assessed at 260 nm. 1 µl of nuclease-free water was used as blank. The absorption ratio at 260:280 nm should be 2.10.

Reverse transcription

The purified RNA was used for reverse transcription employing the ProtoScript First Strand Synthesis Kit (New England Biolabs). 0.5 µl total RNA was pipetted into a sterile microfuge tube, 2 µl primer dT₂₃VN, 4 µl dNTP mix and nuclease-free water to the final volume of 16 µl was added. The reaction was heated in the PCR cycler (Mastercycler epgradient S, Eppendorf) for 5 min at 70°C (denaturation step), briefly spun down and placed on ice. After this, 2 µl 10x RT Buffer, 1 µl RNase inhibitor and 1 µl M-MuLV reverse transcriptase were added and the reaction was performed in the PCR cycler at 42°C for 1 h. The enzyme was inactivated at 95°C for 5 min. The reaction was diluted to 50 µl with nuclease-free water. The cDNA samples were stored at -20°C.

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

RT-PCR analysis was performed using the PCR Master Mix (Promega) and the PCR cycler Mastercycler epgradient S (Eppendorf). The RT-PCR reaction contained 2 µl of template cDNA (see above), 1 µl of 10 µM specific forward primer, 1 µl of 10 µM specific reverse primer, 12.5 µl PCR master mix (2x) and 8.5 µl nuclease-free water.

RT-PCR for septin 11, vimentin and RuvB-like 1 was performed under the following conditions: 95°C for 2 min (initial denaturation), 28 cycles of 95°C for 30 s each (denaturation), 55°C for 30 s (annealing) and 74°C for 30 s (extension); and the final extension at 74°C for 5 min. For amplification of further tested genes, the annealing-temperatures and the number of cycles varied and were used as follows: for the ARP3 actin-related protein 3 homolog variant (ARP3), glutathione synthetase (GlutSynth), heat shock protein-70 kDa isoform 8 (HSPA8), mortalin and the house-keeping gene (human ribosomal protein large P0): annealing-temperature 55°C, 35 cycles; for ATP5B:

annealing-temperature 58°C, 25 cycles; for RuvB-like 2 annealing-temperature 58°C, 26 cycles; for aldehyde dehydrogenase (AldDeh), 14-3-3 γ and the Tat binding protein-7 (TBP7): annealing-temperature 60°C, 35 cycles; for the PDI related protein-5 (PDIR5) and 2'5'-oligoadenylatesynthetase (2'5'OAS): annealing-temperature 62°C, 35 cycles; for prolyl-4-hydroxylase (P4H): annealing-temperature 66°C, 35 cycles.

The PCR products (25 μ l) were supplemented with 6x blue gel loading dye (New England Biolabs) and adjusted with water to the final volume of 30 μ l. 3 μ l of the PCR marker mix (New England Biolabs) were supplemented with 6x blue gel loading dye (New England Biolabs) and adjusted with water to the final volume of 10 μ l. The samples were separated on a 2% agarose gel containing the gel red dye (Biotinum). Gel electrophoresis was performed at the constant voltage of 90 V using 1x TAE buffer. DNA was visualised and photographed using the Mini BIS Pro camera system (DNR Bio-Imaging Systems).

Databases

Sequences of the tested genes were obtained from Ensembl (www.ensembl.org).

Primer designing

All primers were designed using the GeneRunner software (www.generunner.net/). The length of primers was between 18 and 23 nucleotides, the GC content was between 50 and 65%. All primers were tested for the presence of secondary structures to avoid interference with the primer structures. The used primers are listed in Table C.2.

3.3.2.4 Quantitative real time polymerase chain reaction

RNA isolation

Ist-Mes-2 cells were seeded and cultivated in T75 cell culture flasks as described in section 3.3.1.1. The cells were allowed to reach a 60-70% confluency, and were then exposed to 25 μ M and 50 μ M α -TOS for 2, 4, 6 and 8 h. Control cells were treated with an equal volume of EtOH only. Total RNA was obtained from cells, which were harvested and washed with PBS prior to RNA extraction. RNA isolation was performed using the Aurum Total RNA Mini Kit (BioRad) according to the manufacturer's instructions. The eluted

total RNA was stored at -80°C.

Assessment of total RNA concentration

Concentration of total RNA was estimated using the multifunctional microplate reader (TECAN Infinite M200). 2 µl of total RNA were pipetted on a special NanoQuant plate. Absorption of total RNA was assessed at 260 nm. 2 µl of the elution solution (Aurum Total RNA Mini Kit, BioRad) were used as blank. The absorption ratio at 260:280 nm should be 2.10.

Reverse transcription

Reverse transcription was performed using the RevertAid First Strand cDNA Synthesis Kit (Fermentas). Total RNA was used as the template and oligo (dT)₁₈ as the primer. 1 µl oligo (dT)₁₈ primer was pipetted into 0.2 ml Eppendorf tube. 2.5 µg of total RNA and nuclease-free water were then added into the tube so that the total volume was 11 µl. The content in the tube was mixed, centrifuged briefly and incubated in the PCR cycler (Mastercycler epgradient S, Eppendorf) at 65°C for 5 min (denaturation step) and cooled on ice. 4 µl of 5x reaction buffer, 1 µl of the RiboLock RNase inhibitor (20 U/µl), 2 µl of the 10 mM dNTP mix and 1 µl of RevertAid M-MuLV reverse transcriptase (200 U/µl) were added, mixed, centrifuged briefly and incubated in the PCR cycler at 42°C for 60 min and subsequently at 75°C for 5 min. The cDNA samples were stored at -20°C.

Quantitative real time polymerase chain reaction (qPCR)

qPCR was performed using the real time cycler BioRadCFX96 (BioRad) and SYBRGreen Jump Start Taq Ready Mix (Sigma). cDNA was diluted in nuclease-free water, so that 4.5 µl contained 250 ng cDNA. 4.5 µl of diluted cDNA were then pipetted into each well of a 96 well BioRad low profile plate (BioRad), and the plate was centrifuged at 400 x g for 1 min. Mastermix was prepared as follows: 5 µl of 2x SybrGreen master mix and 0.5 µl of 10 µM specific forward and reverse primers (see Table C.2 for primer sequences). 5.5 µl of master mix were added to each well and the plate was centrifuged at 400 x g for 1 min. Thereafter, the plate was sealed with an adhesive foil (special optic foil, BioRad), the content mixed and centrifuged at 400 x g for 2 min. The qPCR reaction was

performed using the following conditions: 98°C for 30 s (initial denaturation), 35 cycles at 98°C for 5 s (denaturation), 60°C for 15 s (annealing), and 72°C for 33 s (polymerization and evaluation in the SYBR/FAM mode). For melt curve analysis, the temperature increased gradually from 65°C to 95°C by 0.5°C/5 s. The data were analysed via the BioRad Software.

Databases

Sequences of the tested genes were obtained from the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov).

Primer designing

All primers were designed using the Primer-BLAST Primer designing tool (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>). The primers contained between 18 and 23 nucleotides and a GC content of 50-65%. All primers were tested for the presence of secondary structures to avoid interfering with the primer structures. The length of the amplified qPCR product was between 100 and 150 bp. The primers for qPCR are listed in Table C.2.

3.3.2.5 Western blot analysis

Ist-Mes-2 cells were seeded and cultivated in T75 cell culture flasks as described in section 3.3.1.1. The cells were allowed to reach the 60-70% confluency, and were then exposed to 25 μ M α -TOS for the times shown. Control cells were treated with an equal volume of EtOH only. After incubation, the cells were trypsinised, harvested (including the medium in which they were maintained), centrifuged (300 x *g*, 10 min, 4°C), washed twice with PBS and spun down. The cell pellet was lysed in the RIPA lysis buffer (500 μ l RIPA lysis buffer/T75 flask) containing 1:100 protease inhibitor mix (100x) (GE Healthcare) for 30 min on ice, by vortexing every 5 min. The lysate was then centrifuged (20,000 x *g*, 5 min, 4°C) and the supernatant was stored at -80°C until further analysis. Total protein content from the protein lysates was determined using Pierce BCA Protein Assay kit (Thermo Scientific) and the microplate absorbance reader Sunrise (TECAN). Protein samples (50 μ g) were heated for 5 min at 99°C in the sample-loading buffer (4x) and then

quickly cooled on ice. The protein samples were loaded on a 10% polyacrylamide gel (4% stacking gel). For determination of protein mass the Precision Plus Protein Standard – All Blue (BioRad) was used.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli (1970). Samples were run in 1 x running buffer at the constant current at 0.03-0.05 A at 4°C for approximately 1 h using the mini Protean III Electrophoresis Cell (BioRad). Proteins separated by SDS-PAGE were blotted onto 0.2 µm nitrocellulose membranes (Protran nitrocellulose membrane, Whatman) for 2 h at the constant current of 0.3 A at 4°C using the transfer buffer and a Mini Trans-Blot Cell (BioRad). After the protein transfer, the membrane was washed in TBS with 0.05% Tween20 and blocked with 5% non-fat milk in TBS with 0.05% Tween20 for 1 h. The membrane was incubated overnight at 4°C in TBS/0.05% Tween20/1% non-fat milk containing the primary antibody diluted according to the manufacturer's instructions. The membrane was then washed three times with TBS/0.05% Tween20 and incubated at room temperature for 1 h with the secondary antibody diluted in TBS/0.05% Tween20/1% non-fat milk according to the manufacturer's instructions. Thereafter the membrane was washed twice in TBS/0.05% Tween20 and subsequently in TBS. The secondary antibody was detected using the ECL chemiluminescence reagent (SuperSignal West Pico Chemiluminescent Substrate, Pierce) and the multipurpose CCD camera system (ImageQuant LAS 4000, GE Healthcare). Optical density of protein bands was analysed using the Quantity One Software, version 4.6.0 (BioRad).

Dilutions used for primary antibodies: 1:200 for goat polyclonal anti-septin-11 IgG (N12) (Santa Cruz); 1:500 for goat polyclonal anti-Actin IgG (Santa Cruz); 1:500 for rabbit monoclonal anti-ATP5B IgG (ATP synthase subunit β , mitochondrial precursor) (Sigma) of human origin. Used dilutions for secondary antibodies: 1:2000 for Anti-goat IgG and for Anti-rabbit IgG (both from Sigma).

3.4 Results

3.4.1 Results – Aim I

3.4.1.1 *Estimation of the toxicity of VE analogues towards MM cell lines*

Human MM cell lines Ist-Mes-2, MM-BI, Meso-2 and H28, and the murine MM cell line AE17 were exposed to the mitocans α -TOS and MitoVES (*cf* Figure D.1 for structures) and their IC₅₀ values calculated from individual killing curves. The data included in Table 3.1 clearly document that the IC₅₀ values for all tested MM cells were by about two order of magnitude lower for MitoVES than for α -TOS. This documents the superior killing activity of the former compared to its parental counterpart.

Table 3.1: IC₅₀ values for α -TOS and MitoVES for MM cell lines.

Cell line	IC ₅₀	
	α -TOS (μ M)	MitoVES (μ M)
H28	26.6	2
Ist-Mes2	37.7	0.25
Meso-2	32.9	0.9
MM-BI	25.6	0.75
AE17	37.5	0.5

3.4.1.2 *Determination of cell viability and apoptosis in MM cells exposed to α -TOS and MitoVES*

Cell viability for the five tested MM cell lines (Ist-Mes-2, MM-BI, Meso-2, H28 and AE17) treated with MitoVES and α -TOS was determined by crystal violet staining (Figure 3.1 A, B). Apoptosis plus post-apoptotic necrosis was estimated using annexin V-FITC/PI and detected by flow cytometry (Figure 3.1 C, D). The cells were treated as described in section 3.3.1.1, being exposed to α -TOS or MitoVES for 24 h at the concentrations 0, 0.5, 1, 2.5 and 5 μ M for MitoVES and 0, 25, 35, 50 and 100 μ M for α -TOS. Figure 3.1 depicts the ability of both tested mitocans to suppress MM cell viability and to trigger apoptosis. MitoVES eradicated all cells within 24 h at a concentration of approximately 5 μ M, while a similar result was obtained with about 100 μ M α -TOS, again documenting the superior effect of the mitochondrially targeted compound.

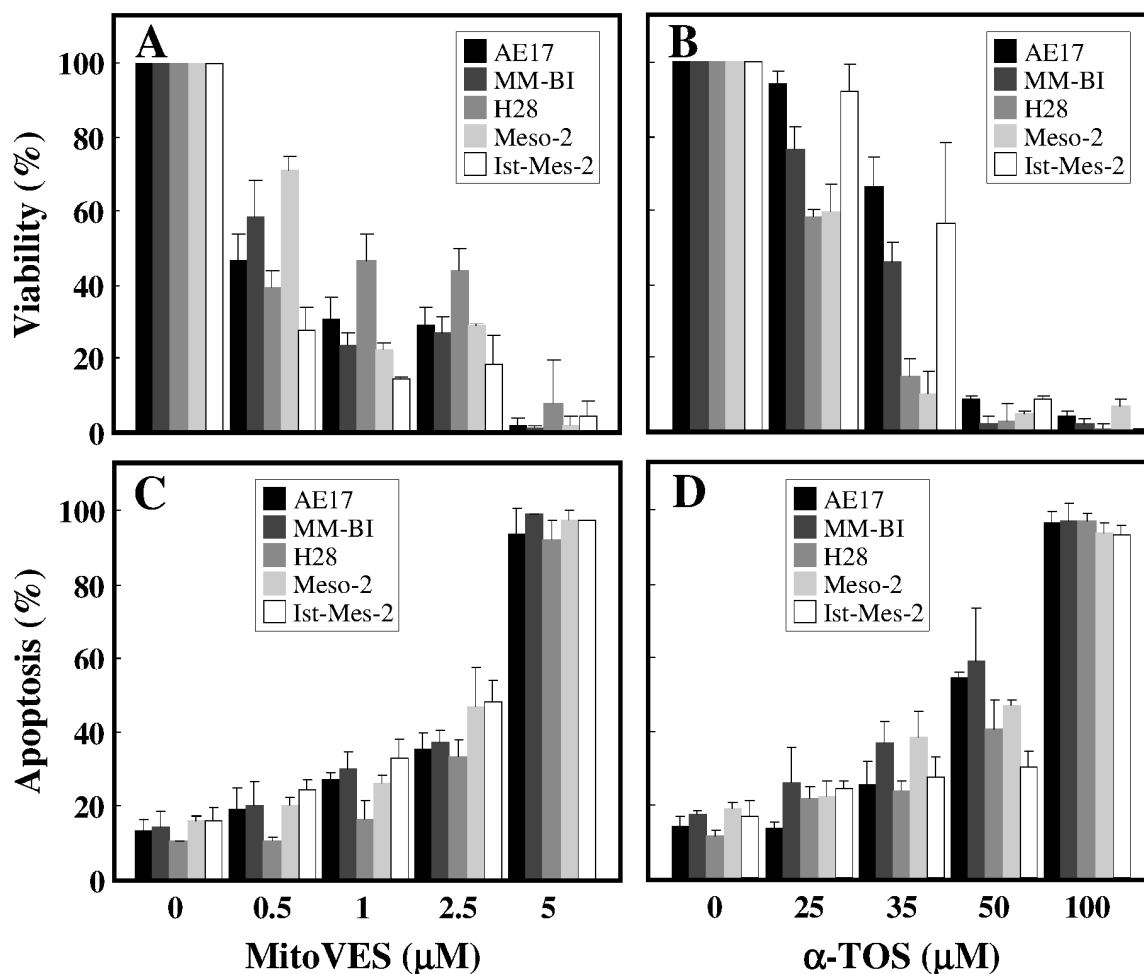


Figure 3.1: Suppression of viability and induction of apoptosis in MM cells exposed to MitoVES and α -TOS.

Ist-Mes-2, MM-BI, Meso-2, H28 and AE17 cells were exposed to MitoVES (A, C) or α -TOS (B, D) for 24 h at the concentrations shown. Cell viability was determined by the crystal violet (A, B) and apoptosis by the annexin V/PI method (C, D). The data are mean values $\pm$ S.D. (n=3).

3.4.1.3 The effect of α -TOS and MitoVES on generation of ROS and $\Delta\psi_{m,i}$

α -TOS as well as its mitochondrially targeted analogue MitoVES are known from previous studies to selectively induce apoptosis in cancer cells and to exert their anti-cancer effect primarily by destabilising mitochondria (Neuzil *et al.*, 1999, 2001a,b; Yu W *et al.*, 2003; Gogvadze *et al.*, 2010; Dong *et al.*, 2011a,b).

The goal of the following experiments was to determine, whether the investigated mitocans induce generation of ROS and cause dissipation of $\Delta\psi_{m,i}$ in MM cell lines, two cellular processes that are known to lead to the activation of the intrinsic apoptotic pathway.

Ist-Mes-2, MM-BI, Meso-2, H28 and AE17 cells were treated as described in section 3.3.1.1 and exposed to 5 μM MitoVES or 100 μM $\alpha\text{-TOS}$ for the times 0, 15, 30, 45 and 60 min. ROS generation was assessed using three different probes including DHE, DCF and MitoSOX. DHE was utilised to determine the generation of superoxide, while DCF was used to assess the levels of hydrogen peroxide. MitoSOX interacts specifically with superoxide that is generated in mitochondria. Data represented in Figure 3.2 A, C, E document that at 5 μM , MitoVES caused significant increase in ROS levels within 10 min when using all three ROS-detecting probes. This result corresponds with previously published data regarding neuroblastoma cells which were transfected with a redox-sensitive plasmid and treated with MitoVES (Dong *et al.*, 2011b). In contrast to MitoVES, $\alpha\text{-TOS}$ induced only marginal increase in ROS levels in MM cells at 100 μM and at 60 min (Figure 3.2 B, D, F). These results indicate that the mitochondrially targeted VE analogue MitoVES is significantly more efficient in ROS generation than its parental compound $\alpha\text{-TOS}$, suggesting that $\alpha\text{-TOS}$ may act in a different way than MitoVES.

Figure 3.2 G documents that MitoVES also triggered a rapid decline in $\Delta\Psi_{\text{m,i}}$ in most tested MM cell lines. However, in MM-BI cells only a modest dissipation of $\Delta\Psi_{\text{m,i}}$ was observed (Figure 3.2 G) and in Meso-2 cells the $\Delta\Psi_{\text{m,i}}$ even increased (data not shown) when treated with MitoVES. Furthermore it was shown that the uncoupler CCCP reduced the $\Delta\Psi_{\text{m,i}}$ values in AE17 cells from the basal relative level of 1 to 0.34, for MM-BI, H28 and Ist-Mes-2 cells to 0.76, 0.24 and 0.31, respectively. This finding is in accordance with the maximum dissipation of $\Delta\Psi_{\text{m,i}}$ after the exposure of the individual cell lines to MitoVES. The exposure of Meso-2 cells to CCCP resulted in a slight increase in $\Delta\Psi_{\text{m,i}}$ from the initial relative level 1 to 1.09. $\alpha\text{-TOS}$ was shown to induce only a marginal and transitory drop of $\Delta\Psi_{\text{m,i}}$ in the different cell lines (Figure 3.2 H).

The differences in generation of ROS levels and in dissipation of $\Delta\Psi_{\text{m,i}}$ in response to MitoVES and $\alpha\text{-TOS}$ are indicative of potentially two different mechanisms of action of the two VE analogues, on top of the much higher efficacy of the former agent.

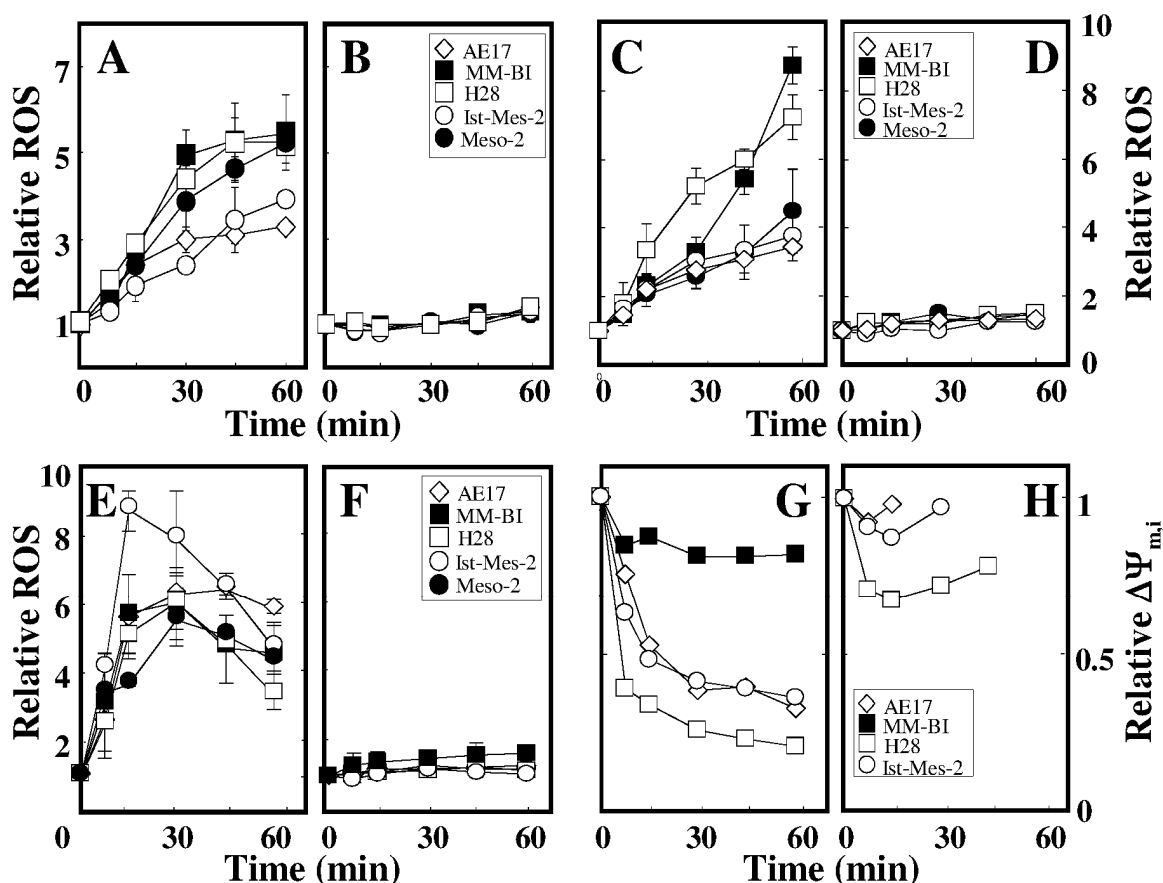


Figure 3.2: MitoVES causes ROS generation and dissipation of $\Delta\Psi_{m,i}$.

AE17, MM-BI, H28, Ist-Mes-2 and Meso-2 cells were exposed to MitoVES at 5 μ M (A, C, E, G) and α -TOS at 100 μ M (B, D, F, H) for the times shown. ROS generation was assessed using the following probes: DHE (A, B), DCF (C, D) and MitoSOX (E, F). The level of ROS was evaluated by flow cytometry and is expressed as relative to the initial value set at 1. Dissipation of $\Delta\Psi_{m,i}$ was determined using the probe TMRM and flow cytometry, and is related to the initial value set at 1 (G, H). The data are mean values $\pm$ S.D. (n=3).

3.4.1.4 Targeting of VE analogues to mitochondria documented by confocal microscopy

Targeting agents with the triphenylphosphonium (TPP⁺) group leads to their directed accumulation in mitochondria (Murphy and Smith, 2007; Biasutto *et al.*, 2010; Dong *et al.*, 2011a,b). Thus, using confocal microscopy, we tried to visualise, whether MitoVES, a compound that is modified with a TPP⁺ group, associates specifically with mitochondria in MM cells. For this purpose, fluorescently tagged analogues of MitoVES (MitoVES-F) and α -TOS (α -TOS-F) were synthesised (*cf* Figure D.1) (Dong *et al.*, 2011a,b). Four different MM cell lines (MM-BI, Meso-2, Ist-Mes-2 and H28 cells) were allowed to attach to cover slips and then incubated for 30 min with either MitoVES-F or α -TOS-F (both at 5 μ M).

Figure 3.3 A-D clearly documents the nearly exclusive localisation of MitoVES in mitochondria, whereas α -TOS, giving highly diffuse pattern of staining can be detected mainly in non-mitochondrial compartments and partially in mitochondria.

Mitochondrial uptake of MitoVES is dependent on $\Delta\Psi_{m,i}$

To determine whether the mitochondrial uptake of MitoVES is $\Delta\Psi_{m,i}$ -dependent, $\Delta\Psi_{m,i}$ was dissipated with CCCP 10 min prior to the supplementation of Ist-Mes-2 cells with MitoVES-F. This resulted in a failure of exclusive localisation of the compound in mitochondria (Figure 3.3 E), suggesting that MitoVES is directed to mitochondria and that its uptake is dependent on high $\Delta\Psi_{m,i}$. Figure 3.3 F shows that high $\Delta\Psi_{m,i}$ is essential for induction of apoptosis in Ist-Mes-2 cells in response to MitoVES but not α -TOS. This follows from the finding that cells pre-treated with CCCP did not undergo efficient apoptosis when compared to cells which were not pre-incubated with the uncoupler. In case of α -TOS, no change in the apoptotic response was observed when the cells were pre-incubated with CCCP.

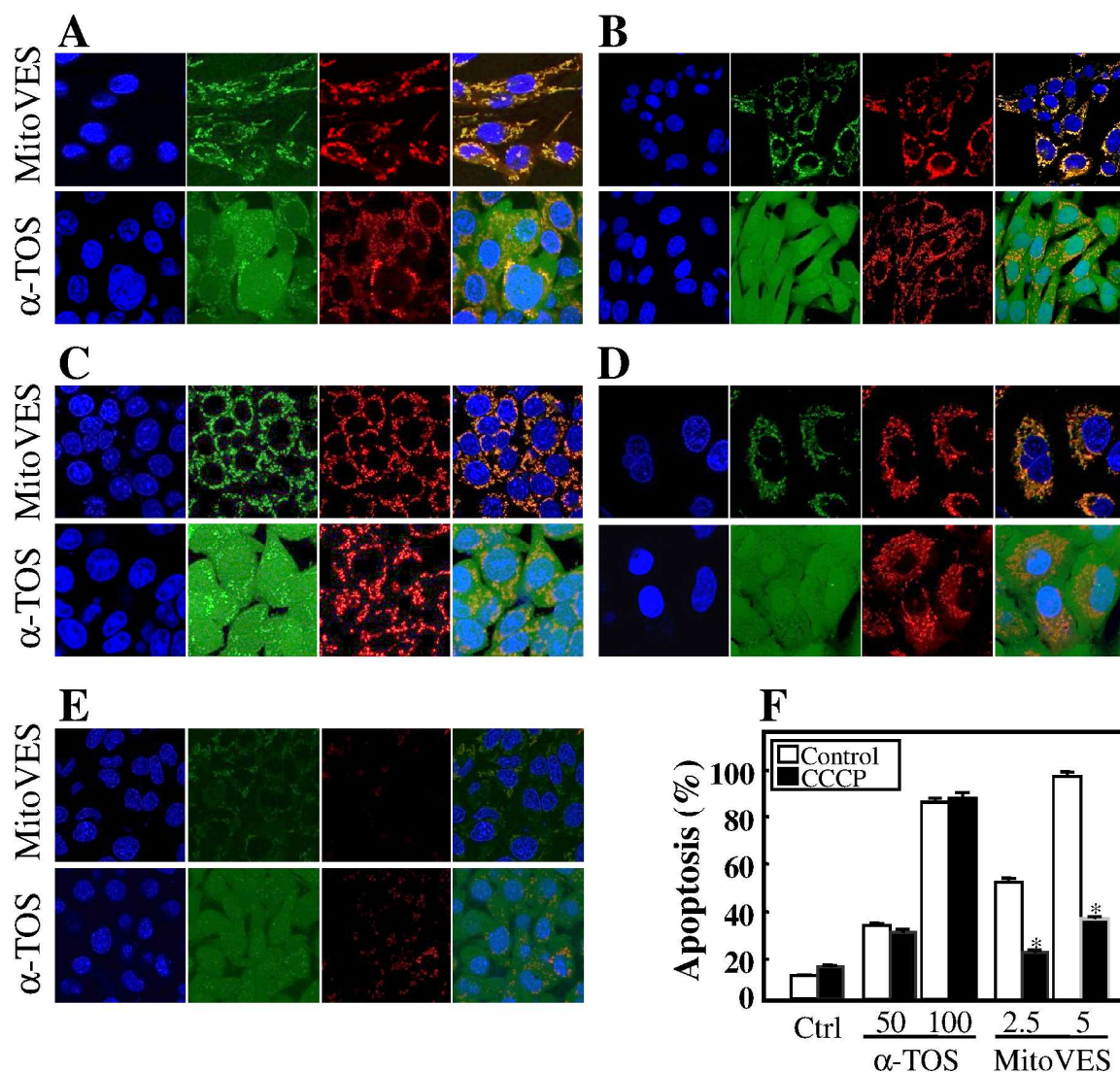


Figure 3.3: Association of MitoVES with mitochondria and its killing activity in MM cells depend on their $\Delta\Psi_{m,i}$.

MM-BI (A), Meso-2 (B), Ist-Mes-2 (C) and H28 cells (D) were seeded on cover slips, stained with 100 nM TMRM and 5 μ g/ml Hoechst 33342 for 1 h prior to the addition of MitoVES-F or α -TOS-F (both 5 μ M) for 20 min. Ist-Mes-2 cells were pre-treated for 10 min with 2 μ M CCCP before the addition of VE analogues (E). The images were acquired using a confocal microscope. Ist-Mes-2 cells were seeded, allowed to reach 60-70% confluency, and exposed to α -TOS or MitoVES for 24 h at the concentrations shown (μ M). Where indicated, the cells were pre-incubated for 10 min with 1 μ M CCCP (F). The images are representative of three independent experiments, data are mean values $\pm$ S.D. (n=3); the symbol '*' indicates significant differences with $p < 0.05$.

3.4.1.5 Importance of the structure of MitoVES for efficient killing of MM cells

Mammalian respiratory complex II (CII) contains two ubiquinone (UbQ)-binding sites, the proximal (Q_P) and the distal (Q_D) site (Sun *et al.*, 2005). It has been reported that α -TOS can interact with both UbQ-binding sites (Dong *et al.*, 2008), while MitoVES can reach

only the Q_p site (Dong *et al.*, 2011a). This is due to the positioning of MitoVES across mitochondrial matrix and mitochondrial inner membrane (MIM) with the charged TPP^+ group in the matrix and the tocopheryl succinyl group in the MIM. The length of the aliphatic chain of the compound, spanning the TPP^+ and the tocopheryl group, may be important for the bioactive moiety of the agent to reach the molecular target (Q_p) (Dong *et al.*, 2011a). Various homologues of MitoVES were synthesised by shortening the aliphatic chain by two carbons at a time to determine the importance of the length of the aliphatic chain for the efficacy of the activity of MitoVES towards MM cells (*cf* Figure D.1). Further, to confirm the assumption that the free carboxylate group of the succinyl moiety of MitoVES is essential for its efficacy (Neuzil *et al.*, 2001), another derivate of MitoVES (MitoVES4TPP) was synthesised containing the succinyl moiety esterified with the TPP^+ group via a 4-C linked (*cf* Figure D.1).

To understand the above premise, Ist-Mest-2 cells were allowed to attach and to reach 60-70% confluency. The cells were exposed to 5 μ M of the individual analogues of MitoVES. After 60 min incubation with the agents, the cells were evaluated for SQR activity (Figure 3.4 A), ROS generation (Figure 3.4 B) and dissipation of $\Delta\Psi_{m,i}$ (Figure 3.4 C). Furthermore, apoptosis induction was determined using the annexin V/PI method after 24 h incubation with the agents (Figure 3.4 D).

SQR activity

The various derivates of MitoVES were tested for their effect on the SQR activity, which catalyses the transfer of electrons from succinate to UbQ. The reduction of UbQ to UbQH₂ was assessed by the ubiquinol-dependent reduction of DCPIP. Figure 3.4 A documents that the full length MitoVES inhibited the SQR activity with considerable efficacy and that the shortening of its aliphatic chain resulted in gradually reduced effect of the derivates, with the C-9 homologue almost as efficient as the full length (11-C) MitoVES. The C-5 as well as the C-7 homologues were shown to be almost inefficient. As expected and in analogy with our earlier results (Neuzil *et al.*, 2001), Figure 3.4 A documents that MitoVES4TPP was very inefficient in inhibiting the SQR activity indicating that the free succinate group is indispensable for the interaction of MitoVES with the Q_p site (serin 68) (Sun *et al.*,

2005) of CII, as was proposed in previous studies (Dong *et al.*, 2008; Dong *et al.*, 2011a) and now confirmed here for MM cells.

Generation of ROS

The various analogues of MitoVES were next investigated for their ability to generate ROS. ROS generation was assessed using the probe DHE. Figure 3.4 B proves that the length of the aliphatic chain of MitoVES influenced its efficacy to generate ROS. Here, the full length (11-C) MitoVES was again the most efficient homologue in induction of ROS production, followed by the 9-C analogue being almost as efficient as the 11-C variant. The 7-C homologue induced approximately 50% generation of ROS level when compared to the full-length homologue. MitoVE₅S as well as MitoVES4TPP caused only a small increase in the generation of ROS levels, as expected. The low effect of MitoVES4TPP on ROS generation indicates that the free carboxylate of the succinyl moiety of MitoVES is important for efficient induction of ROS, an essential event in the subsequent apoptosis induction.

$\Delta\Psi_{m,i}$ dissipation

The different homologues of MitoVES were also tested for their ability to cause $\Delta\Psi_{m,i}$ reduction. Dissipation of $\Delta\Psi_{m,i}$ was evaluated using TMRM. Figure 3.4 C documents that the length of the aliphatic chain as well as the free carboxyl group of MitoVES play an essential role in the dissipation of $\Delta\Psi_{m,i}$. MitoVES₁₁S was shown again to be the most efficient homologue, closely followed by MitoVE₉S. The homologues MitoVE₇S and MitoVES4TPP showed similar, low activity in reduction of $\Delta\Psi_{m,i}$. MitoVE₅S caused only an insignificant drop of $\Delta\Psi_{m,i}$.

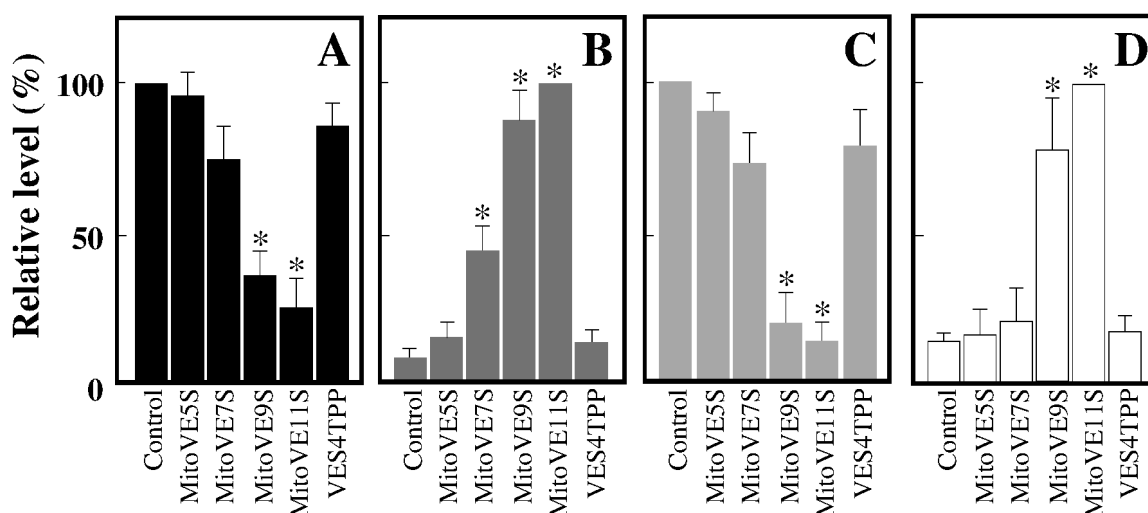


Figure 3.4: Structure of MitoVES is important for efficient killing of MM cells.

Ist-Mest-2 cells were seeded, allowed to reach 60-70% confluency and exposed to 5 μ M analogues of MitoVES as shown. After 60-min incubation with the agents, the cells were evaluated for SQR activity (A), ROS generation using the DHE probe (B) and dissipation of $\Delta\Psi_{m,i}$ using TMRM (C). Apoptosis induction was evaluated using the annexin V/PI method after 24 h incubation (D). The data are related to the highest level (either control cells or cells exposed to MitoVE₁₁S, as relevant), and are derived from three independent experiments; the error bars represent the S.D. The symbol '*' indicates significant differences between control and treated cells, with $p < 0.05$.

Induction of apoptosis

Further evidence for the importance of the length of the aliphatic chain and the free succinate group of MitoVES was obtained by the experiment where the different homologues of MitoVES were tested for their ability to trigger apoptosis in Ist-Mes-2 cells. As expected, the most efficient homologue in apoptosis induction was the full length (11-C) MitoVES, again followed by its (9-C) homologue. The other analogues tested here were largely inefficient (Figure 3.4 D).

3.4.1.6 Cellular respiration in response to α -TOS and MitoVES

Certain anti-cancer agents including mitocans from the group V (agents affecting the electron transfer chain) have been shown to affect mitochondria by suppressing their respiration (Ralph *et al.*, 2010b; Neuzil *et al.*, 2013).

In this experiment, the mitocans α -TOS and MitoVES were tested for their ability to inhibit cellular respiration in different MM cell lines. At first, the effect of the two mitocans on

routine cellular respiration (i.e. total oxygen consumption by whole cells in the cultivation medium without an uncoupler) was tested. For this purpose, AE17, MM-BI, H28, Ist-Mes-2 and Meso-2 cells were seeded and allowed to reach 60-70% confluency. The cells were harvested and placed in the Oxygraph chamber at 10^6 cells/ml in the cultivation medium. Increasing concentrations of α -TOS or MitoVES were added and oxygen consumption was evaluated. Figure 3.5 A documents that MitoVES was in all tested MM cell lines considerably more efficient in the suppression of mitochondrial respiration than its parental compound α -TOS, which proved to be rather inefficient. Furthermore, significant differences were observed in routine respiration between the tested MM cell lines. The AE17 cells respired at 15 pmol O_2 /s/ 10^6 cells whereas up to two-fold higher respiration was observed for Meso-2 cells. At low concentrations, MitoVES induced an initial increase in respiration in all tested MM cell lines, which can be explained by initial and very fast mitochondrial uncoupling. This finding corresponds to recently published data, showing that in intact cells and isolated mitochondria MitoVES at low concentrations has an uncoupling effect and that the agent inhibits oxygen consumption at higher levels (Rodríguez-Enríquez *et al.*, 2012). This suppression in respiration is linked to the generation of ROS induced by MitoVES and is compatible with mitochondrial respiratory CII as a source of ROS (Dong *et al.*, 2011a; Dong *et al.*, 2011b; Quinlan *et al.*, 2012; Moreno-Sánchez *et al.*, 2013).

In a further experiment, the contribution of mitochondrial respiratory CI and CII to mitochondrial respiration in permeabilised whole cells exposed to MitoVES and α -TOS was investigated. The aforementioned MM cell lines were harvested, permeabilised and placed in the mitochondrial respiration medium MiR05 in the Oxygraph chamber at 2×10^6 cells/ml. Oxygen consumption related to CI or CII was assessed after addition of increasing concentration of α -TOS or MitoVES followed by the inhibition of CII or CI, respectively, and using the appropriate substrates as detailed in section 3.3.1.10. Figure 3.5 B documents that all tested MM cells respired primarily via CII. Again a superior effect of MitoVES to α -TOS was observed, which at lower concentrations uncoupled the CII-dependent respiration in MMBI, H28 and Meso-2 cells, followed by its strong suppression. Figure 3.5 B shows, that MitoVES also inhibited the CI-dependent respiration to a somewhat lesser extend, reminiscent of a paper, in which the effect of a redox-silent VE

analogue on CI was reported for leukemic cells (dos Santos *et al.*, 2012). The effect of α -TOS on CI-dependent respiration was insignificant.

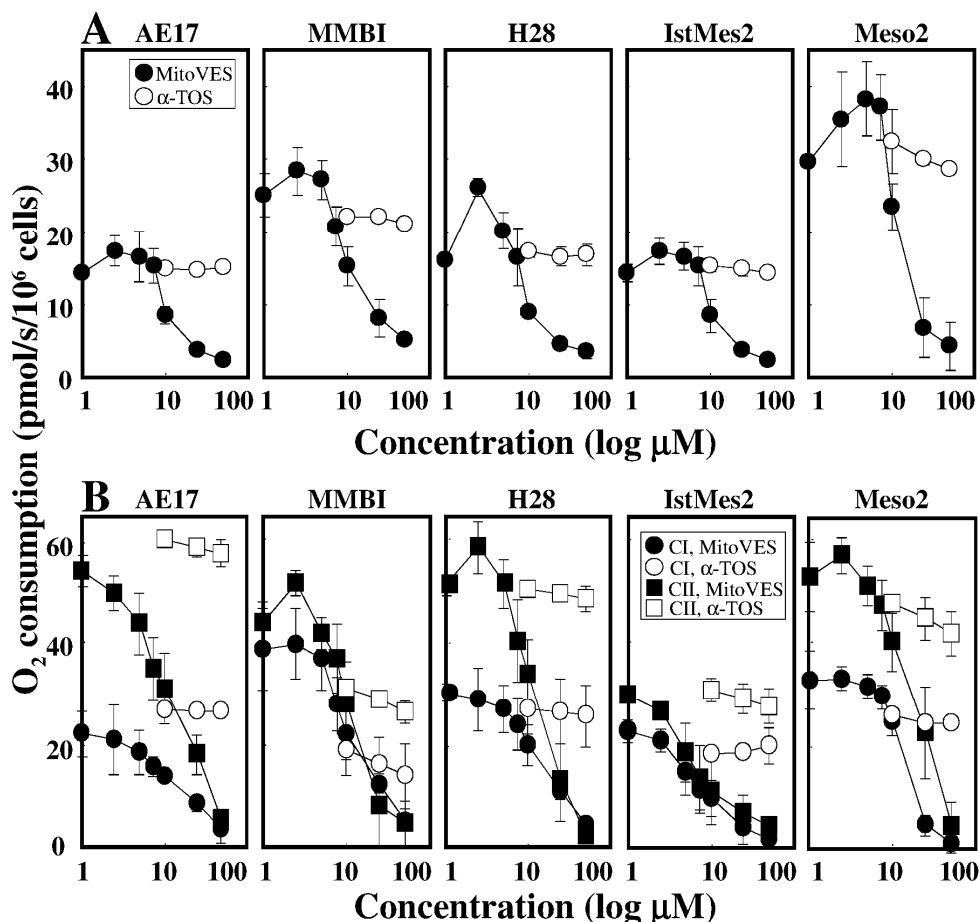


Figure 3.5: MitoVES suppresses mitochondrial respiration.

AE17, MM-BI, H28, Ist-Mes-2 and Meso-2 cells were seeded and allowed to reach 60-70% confluency. The cells were harvested and placed in the Oxygraph chamber at 10^6 per ml in the original medium. Oxygen consumption was assessed following addition of increasing concentration of α -TOS or MitoVES (A). The cells were harvested, permeabilised and placed in the MiR05 medium in the Oxygraph chamber at 2×10^6 per ml. Oxygen consumption linked to CI or CII was evaluated in the presence of increasing concentration of α -TOS or MitoVES following inhibition of CII or CI, respectively, and using the appropriate substrates as detailed in the Experimental part (B). The data are mean values $\pm$ S.D. (n=3).

3.4.1.7 The effect of α -TOS and MitoVES on the assembly of mitochondrial respirasomes

In the next experiment, the effect of α -TOS and MitoVES on the assembly of mitochondrial (super)complexes in MM cells was studied. Ist-Mes-2 and H28 cells were allowed to reach 60-70% confluency, followed by the exposure to α -TOS at 50 μ M for 0.5, 1 or 2 h or at 25 μ M for 24 h, or to MitoVES at 5 μ M for 0.5, 1 or 2 h or at 1 μ M for 24 h.

After exposure to the agents, mitochondrial fractions of the cells were prepared and subjected to NBE as detailed in section 3.3.1.11. Figure 3.6 A documents that in both tested cell lines mitochondrial complexes assemble into supercomplexes, referred to as respirasomes (Acín-Pérez *et al.*, 2008). The effect of MitoVES on the assembly of its target, CII was not proved here. However, Figure 3.6 A shows that at 5 μ M and at 1 h-exposure, the agent destabilised the higher forms of respirasomes, in particular the CI+CIII₂ supercomplex in Ist-Mes-2 cells and the CI+CIII₂+CIV_n supercomplex in H28 cells, which is considered to participate in the direct electron transfer as well as being the most active form of the three complexes (Acín-Pérez *et al.*, 2008). Figure 3.6 A also documents that α -TOS exhibited only mild effects on the formation of the supercomplexes.

In the further experiment, clear native electrophoresis was applied to separate the mitochondrial fraction followed by the CI and CII in-gel assay as detailed in the Experimental part. Figure 3.6 B depicts that MitoVES was quite efficient in inhibiting the CII and, to a lesser extent, the CI activity. This is consistent with the inhibitory effect of MitoVES on CII activity (see above). Taken together, MitoVES showed again a superior effect to α -TOS in MM cells, influencing the formation of mitochondrial respirasomes.

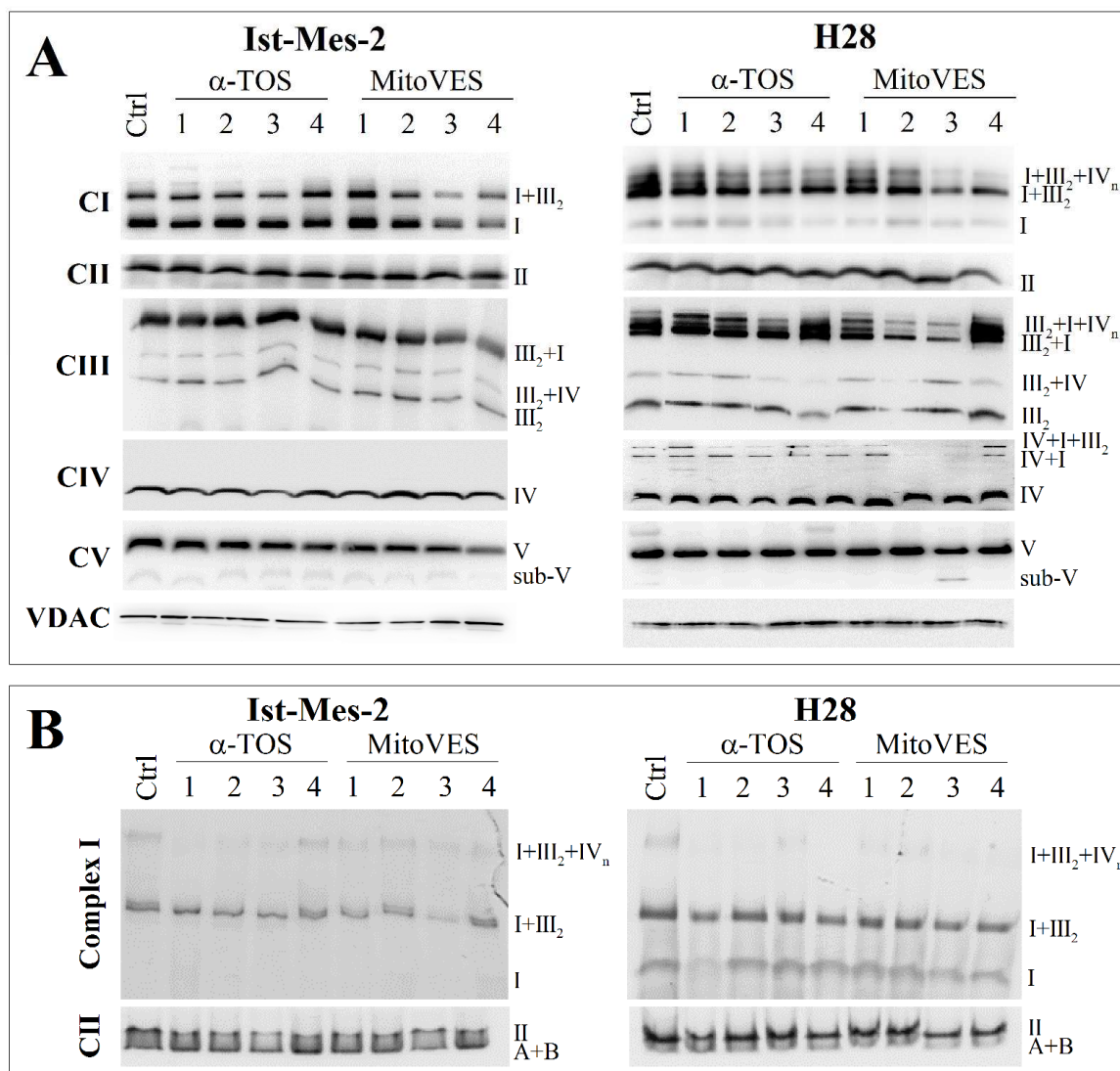


Figure 3.6: MitoVES affects the mitochondrial complexes assembly.

Ist-Mes-2 and H28 cells were allowed to reach 60-70% confluency, after which they were exposed to α -TOS at 50 μ M for 0.5 (1), 1 (2) or 2 h (3) or at 25 μ M for 24 h (4), or to MitoVES at 5 μ M for 0.5 (1), 1 (2) or 2 h (3) or at 1 μ M for 24 h (4), and the mitochondrial fraction prepared. *A*. The mitochondrial fraction was subjected to NBE as detailed in the Experimental part. The text on the right indicates the position of free complexes and supercomplexes as well as partially assembled complexes. *B*. The mitochondrial fraction was separated using clear native electrophoresis and the CI and CII in-gel activity assessed as detailed in the Experimental part. The activity related to the position of individual assembled complexes and supercomplexes, as well as partially assembled complexes is indicated by the text on the right. The images are representative of three independent experiments.

3.4.1.8 The importance of CII for the killing effect of MitoVES in MM cells

To get a definitive evidence for CII as the molecular target of MitoVES, MM cells with suppressed CII function were prepared. For this purpose, Ist-Mes-2 cells were stably

transfected with SDHC shRNA resulting in down-regulation of the SDHC subunit of CII. Control cells were transfected with non-silencing shRNA as described in the Experimental part. The SDHC protein level was estimated by WB and the activity of SQR and SDH.

Figure 3.7 A shows that a successful knock-down of the SDHC protein was achieved, as evidenced by the very low level of the protein and reduced SQR activity. The SDH activity was only marginally reduced when compared with the control cells, transfected with non-silencing shRNA, since the catalytic center of the CII SDH activity is only a weak target for MitoVES (Dong *et al.*, 2011a,b). Further, respiration of the MM cells via CI and CII was assessed using the Oxygraph. As documented in Figure 3.7 B, cells with suppressed SDHC subunit respired to a lesser extent via CII than the corresponding control cells which were transfected with non-silencing shRNA. To show the importance of CII for the activation of apoptosis induced by MitoVES, flow cytometry was performed using the annexin V/PI method. Figure 3.7 C documents low level of apoptosis induced by MitoVES in Ist-Mes-2 cells transfected with SDHC shRNA in contrast to the control cells (Ist-Mes-2 cells transfected with NS shRNA), which showed a considerably higher apoptosis level.

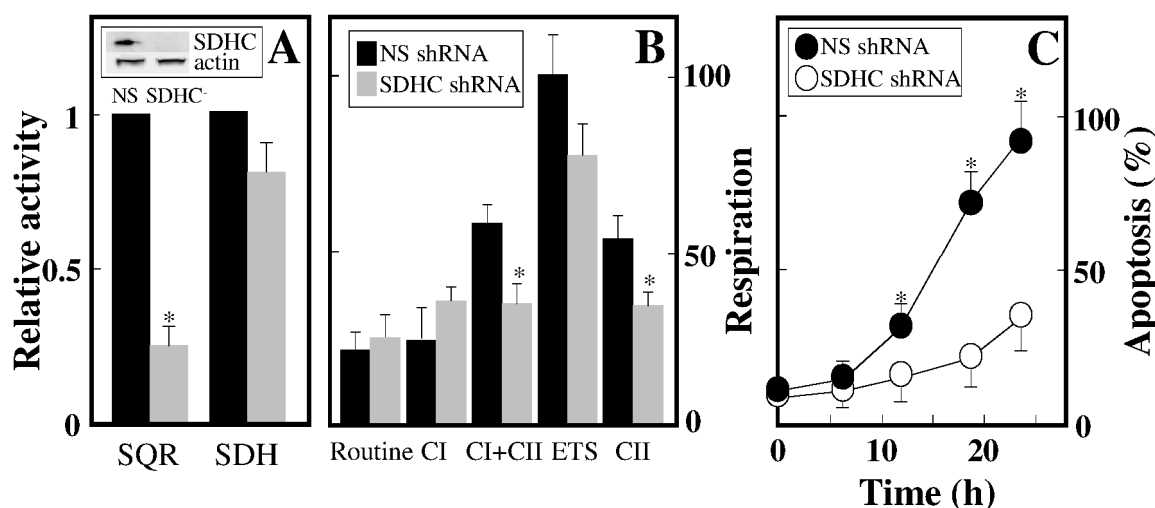


Figure 3.7: CII is relevant for killing of MM cells by MitoVES.

Ist-Mes-2 cells were stably transfected with NS shRNA or SDHC shRNA. The SDHC protein level was estimated by WB and the activity of SQR and SDH (A), respiration rates via CI and CII were assessed using the Oxygraph (B), and the effect of MitoVES (5 μ M) on apoptosis was determined using the annexin V/PI method and flow cytometry (C). The images are representative of three independent experiments, the data in panels A, B and D are mean values $\pm$ S.D. (n=3). The symbol “*” indicates significant differences with $p < 0.05$.

3.4.1.9 The effect of MitoVES and α -TOS on experimental MM

Finally, we tested the effect of the two mitocans, MitoVES and α -TOS, on experimental MM. In this experiment, xenografts in immunodeficient, athymic Balb/c *nu/nu* mice were prepared by their grafting with Ist-Mes-2 cells. The mice were injected subcutaneously with 2×10^6 Ist-Mes-2 cells in PBS per animal. As soon as the tumours reached approximately 30 mm^3 , the animals were treated intraperitoneally either with $100 \mu\text{l}$ $15 \mu\text{mol}$ α -TOS or with $100 \mu\text{l}$ $1 \mu\text{mol}$ MitoVES dissolved in 4% EtOH in corn oil twice per week. Control mice were treated with $100 \mu\text{l}$ of the excipient (4% EtOH in corn oil) only. Tumours were quantified using ultrasound imaging and expressed relative to the initial tumour volume ($\sim 30 \text{ mm}^3$) (for detailed protocol see section 3.3.1.15). Figure 3.8 shows, that MitoVES was significantly more efficient in inhibition of MM *in vivo* than found for α -TOS. Under the above mentioned conditions, MitoVES inhibited tumour growth by $>90\%$ while α -TOS was at a 15-fold higher concentration less efficient, with approx. 80% tumour growth suppression. No toxicity towards the drugs was observed within the experiment. In one mouse, the treatment with MitoVES led to complete tumour disappearance. This finding highlights the anti-cancer effect of the mitochondrially targeted VE analogue and its potential to be used as a promising anti-mesothelioma drug.

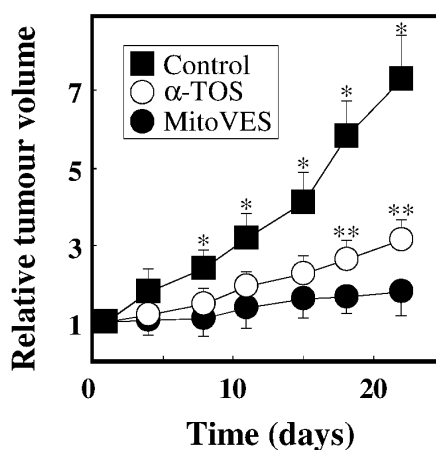


Figure 3.8: MitoVES efficiently suppresses tumours in experimental mice.

Nude mice were injected subcutaneously with 2×10^6 Ist-Mes-2 cells/mouse. The animals were treated intraperitoneally with $15 \mu\text{mol}$ α -TOS or $1 \mu\text{mol}$ MitoVES dissolved in 4% EtOH in corn oil or with $100 \mu\text{l}$ of the excipient (control mice) twice per week. Tumours were quantified using ultrasound imaging and expressed relative to the initial tumour volume ($\sim 20\text{--}30 \text{ mm}^3$). The data are mean values $\pm$ S.D. ($n=5$), the symbol ‘*’ indicates significant differences between values from control and MitoVES-treated animals, ‘**’ between α -TOS- and MitoVES-treated animals, with $p < 0.05$.

3.4.2 Results – Aim II

3.4.2.1 *Identification of proteins with altered expression levels after treatment of MM cells with α -TOS by 2-D GE and MS analysis*

To identify different proteins, which might be involved in the signalling and apoptotic pathways of MM cells exposed to the VE analogue α -TOS, 2-dimensional gel electrophoresis (2-D GE) and mass spectrometry (MS) analysis were performed. Ist-Mes-2 cells were seeded, allowed to attach and to reach a confluency of 60-70% before they were exposed to α -TOS at 25 μ M for 24 h. The control sample was treated with an equal volume of EtOH only. The cells were harvested and whole cell lysates prepared as described in section 3.3.2.1.

Three pairs of 2-D gels from three separate pairs of samples of control cells and cells exposed to α -TOS at 25 μ M for 24 h were prepared per protein identification using 2-D GE and MS analysis. In total, three protein identifications were performed. The 2-D gels were stained with colloidal Coomassie blue (NovexTM Colloidal blue stain kit) and scanned using a densitometer. The gel image triplets were analysed using PDQuest 7.1 2-D Analysis Software (BioRad) in a semi-manual detecting and matching mode. The overall protein expression profiles with pI 4-7 were very similar within the three compared gels of both groups of samples (control and α -TOS-treated). A virtual Master gel (matchset standard) was created from three separate pairs of Raw 2-D gels, and spots representing proteins with significantly changed expression level were determined when comparing the control gel with the α -TOS-treated gel image. In total, three Master gels were generated, each from three pairs of Raw 2-D gels (Figure 3.9). *A, B* represent the first, *C, D* the second and *E, F* the third matchset standard. Red spots in *A, C, E* represent proteins which were found to be more than 1.5-fold up-regulated and in *B, D* and *F* protein spots which were analysed as more than 1.5-fold down-regulated. Blue circles depict protein spots that were identified as statistically significant ($p < 0.05$). These proteins were excised from a preparative gel, digested and analysed by MS as described in section 3.3.2.2. The numbered blue circles represent protein spots, which were identified by MS. Blue circles with no number provided no result.

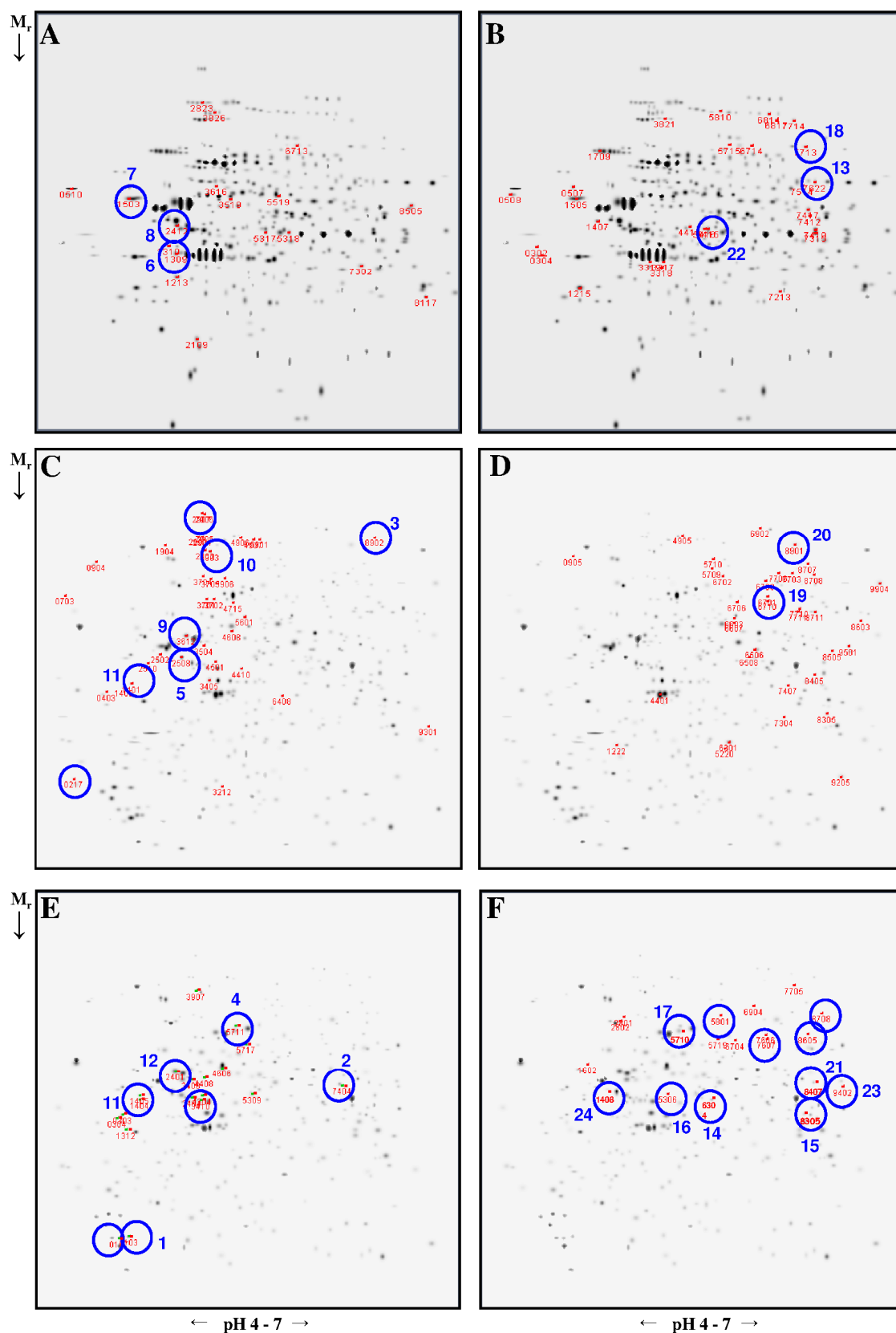


Figure 3.9: Three independent virtual master gel images generated by PDquest 7.1 2-D Analysis Software.
(for detail see concomitant text).

MS analysis enabled the identification of 24 proteins within the pH range 4-7 to be significantly ($\geq 1.5x$) up-regulated or down-regulated after a 24 h exposure of Ist-Mes-2 cells to 25 μM α -TOS (Table 3.2).

Table 3.2: Overview of proteins identified by mass spectrometry.

MM cell line Ist-Mes-2 was treated with 25 μM α -TOS for 24 h. Statistically significant ($p < 0.05$), $\geq 1.5x$ up- or down-regulated proteins from three independent experiments utilising 2-D GE and MS analysis are listed.

Circle number	$\geq 1.5x$ up-regulated proteins	Circle number	$\geq 1.5x$ down-regulated proteins
1	14-3-3 gamma protein	13	2'5'Oligoadenylatesynthetase
2	Aldehyde dehydrogenase	14	ARP3 actin-related protein 3 homolog variant
3	β -subunit (AA 1-312)	15	Elongation factor Tu
4	Heat shock 70 kDa protein 9B precursor variant (Mortalin)	16	Glutathione synthetase
5	mitochondrial ATP synthase, H ⁺ transporting F1 complex beta subunit/ ATP5B protein	17	Heat shock 70 kDa protein 8 isoform 1
6	Mutant β -actin	18	Keratin (type II cytoskeletal 1 epidermal) & Keratin (type II cytoskeletal 2 epidermal)
7	Prolyl-4-hydroxylase	19	Keratin 1
8	Protein disulfid isomerase (PDI)-related protein 5	20	Keratin 9
9	Tubulin, beta	21	Ruv B-like 1
10	Valosin-containing protein	22	Ruv B-like 2
11	Vimentin	23	Septin 11
12	Vimentin/Tubulin alpha – 1B chain	24	Tat binding protein 7

3.4.2.2 *RT-PCR analysis of regulation of the differently expressed proteins after treatment of MM cells with α -TOS*

Most of the proteins identified by mass spectrometry analysis were further tested by RT-PCR to investigate whether their different expression levels found by 2-D GE correspond to their different mRNA levels, i.e. a match between translational and transcriptional data. Ist-Mes-2 cells were seeded, allowed to attach and to reach a confluency of 60-70%. Then the cells were exposed to 25 μ M α -TOS for 24 h. Control cells were treated with an equal volume of EtOH only. The cells were harvested, mRNA was isolated and cDNA synthesised. Primer pairs for the amplification of different genes by RT-PCR were designed as described in section 3.3.2.3 and are listed in Table C.2. The RT-PCR conditions for the different genes are stated in section 3.3.2.3. Primer pairs designed for the synthesis of gene products coding for the proteins β -subunit, elongation factor-Tu, mutant- β -actin, tubulin α -1B chain, tubulin β and valosin-containing protein did not give any PCR-product and are not listed in Table C.2. The different keratin proteins were not tested further. They result most likely from a contamination during the preparation of the samples for MS-analysis. Figure 3.10 clearly shows that the proteins septin 11 and mitochondrial ATP synthase (H^+ transporting F1 complex, β subunit/ATP5B protein) are regulated by α -TOS on a transcriptional level.

Under the RT-PCR conditions mentioned in section 3.3.2.3, septin 11 was down-regulated after the treatment of Ist-Mes-2 cells with 25 μ M α -TOS whereas the mitochondrial ATP synthase was up-regulated after the treatment, which is in agreement with the result from 2-D GE and MS-analysis. Changes in the mRNA expression levels of the other tested genes coding for the proteins identified above by 2-D GE/MS analysis were not confirmed by RT-PCR analysis when using the listed primers.

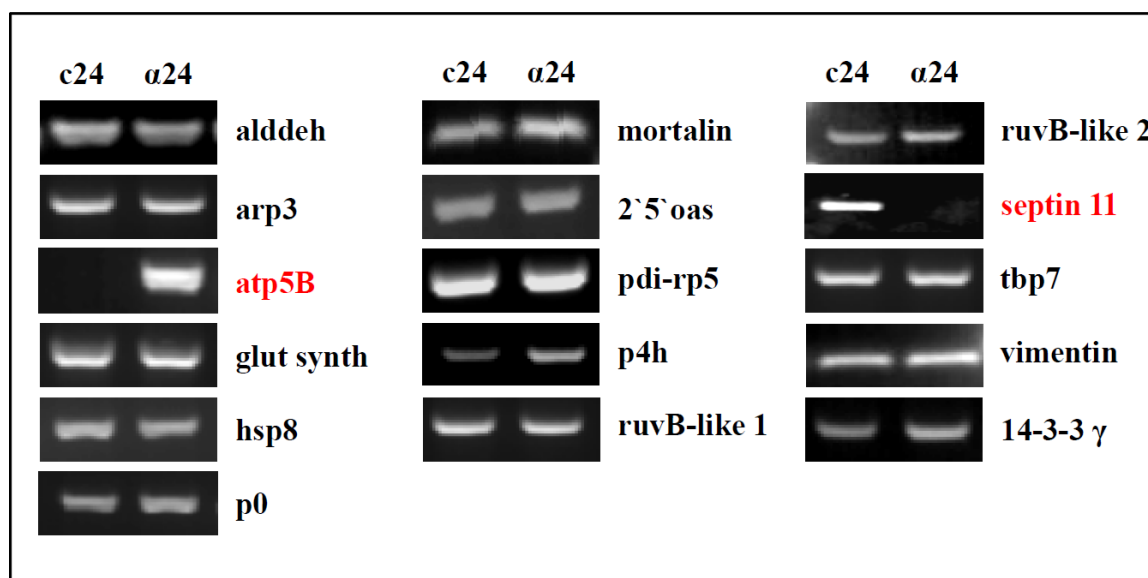


Figure 3.10: The effect of α -TOS on the mRNA level of various proteins in Ist-Mes-2 cells.

The cells were exposed to 25 μ M α -TOS in EtOH for 24 h (α 24) and control cells (C24) were treated with an equal volume of EtOH only. The mRNA levels were determined by RT-PCR analysis. Shown are the PCR-products of genes encoding the proteins aldehyde dehydrogenase (alddeh), ARP3 actin-related protein 3 homolog variant (arp3), mitochondrial ATP synthase, H^+ transporting F1 complex β subunit/ATP5B protein (atp5B), glutathione synthetase (glut synth), heat shock 70 kDa protein 8 isoform 1 (hsp8), mortalin (mortalin), 2'5'-oligoadenylatesynthetase (2'5'oas), protein disulfid isomerase-related protein 5 (pdi-rp5), prolyl-4-hydroxylase (p4h), Ruv B-like 1 (ruv B-like 1), Ruv B-like 2 (ruv B-like 2), septin 11, Tat binding protein 7 (tbp 7), vimentin (vimentin) and 14-3-3 γ protein (14-3-3 γ). PCR products significantly up-/down-regulated after the treatment with α -TOS are shown in red. The mRNA level of a housekeeping gen (p0) is also shown. The PCR products were separated by gel electrophoresis on a 2% agarose gel. The presented are representative of three independent experiments.

3.4.2.3 WB analysis of septin 11 and ATP5B protein after treatment of MM cells with α -TOS

The protein levels of septin 11 and ATP5B after treatment of Ist-Mes-2 cells with α -TOS were further estimated by western blot (WB) analysis. Ist-Mes-2 cells were seeded, allowed to attach and to reach a confluency of 60-70%. The cells were then exposed to 25 μ M α -TOS for 6, 12, 24 and 48 h. Control cells were treated with an equal volume of EtOH only. The cells were harvested and whole cell lysates prepared. Fresh cell lysates were prepared for each time point from the control cells. WB was performed as described in section 3.3.2.5. The used antibodies are listed in Table C.1. Figure 3.11 clearly documents that septin 11 is down-regulated after the treatment of Ist-Mes-2 cells with 25 μ M α -TOS. After 48 h the protein band nearly disappeared whereas the up-regulation of

the ATP5B protein after treating the cells with 25 μ M α -TOS was not confirmed on the protein level.

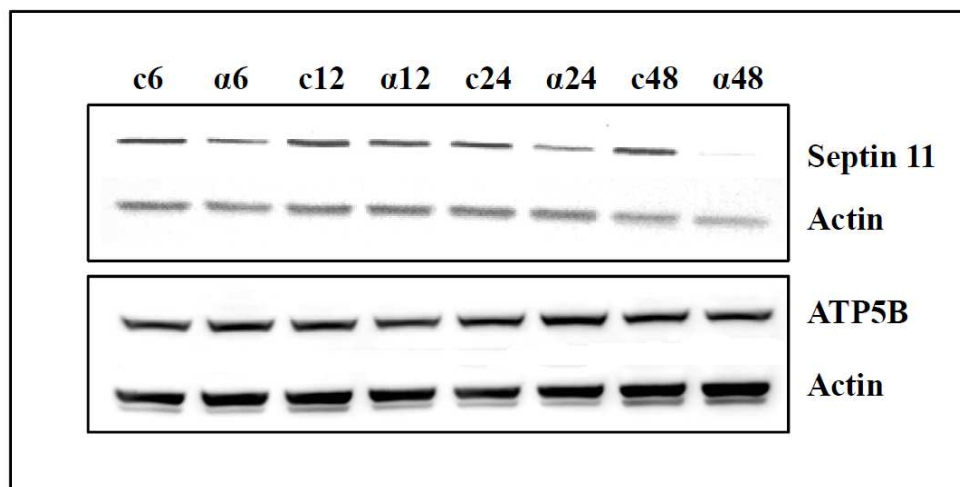


Figure 3.11: The effect of α -TOS on septin 11 and ATP5B expression in Ist-Mes-2 cells.

The cells were exposed to 25 μ M α -TOS in EtOH for 6, 12, 24 and 48 h (α 6, α 12, α 24 and α 48) and control cells (c6, c12, c24 and c48) were treated with an equal volume of EtOH only. Whole cell lysates were isolated and the protein levels were estimated by WB analysis employing. Anti-actin IgG was used as a loading control. The images shown are representative of three independent experiments.

3.4.2.4 qPCR analysis of the regulation of septin 11 by α -TOS in MM cells

Further evidence that septin 11 is down-regulated on the mRNA level in MM cells after exposure to α -TOS comes from qPCR experiments. Ist-Mes-2 cells were seeded, allowed to attach and reach the confluency of 60-70% and treated with either 25 or 50 μ M α -TOS in EtOH for 2, 4, 6 and 8 h. Control cells were treated with an equal volume of EtOH only. Thereafter mRNA was isolated from the cells and transcribed into cDNA. Using the primer pairs listed in Table C.2 qPCR analysis was performed. The methodology is described in section 3.3.2.4. Figure 3.12 documents a successful down-regulation of septin 11 after treatment of Ist-Mes-2 cells with α -TOS. Treating the cells with 50 μ M α -TOS for 8 h resulted in 2.6-fold down-regulation of the mRNA level of septin 11.

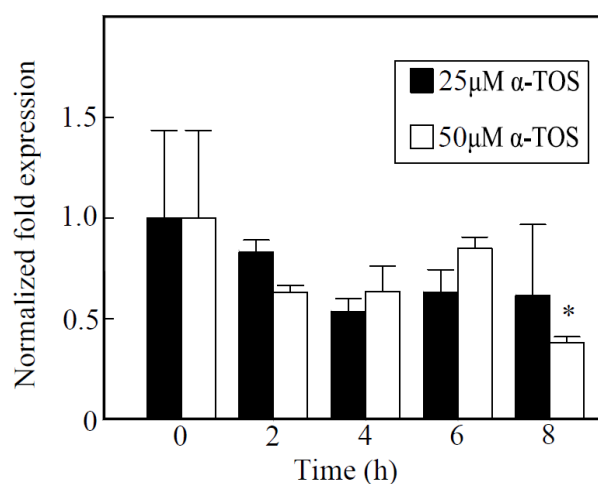


Figure 3.12: The effect of α -TOS on mRNA level of septin 11 in Ist-Mes-2 cells.

The cells were exposed to 25 or 50 μ M α -TOS in EtOH for 2, 4, 6 and 8 h. Control cells (0 h) were treated with an equal volume of EtOH only. The mRNA level for septin 11 was determined by qPCR analysis. The data were normalised to the data of the mRNA level of the housekeeping gene p0. They are expressed relative to the highest level (control cells) set as 1. The data are mean values $\pm$ S.D. (n=3), the symbol ‘*’ indicates significant differences between control and α -TOS-treated cells, with $p < 0.05$.

3.4.2.5 WB analysis of septin 11 after treatment of MM cells with NAC and α -TOS

In the next experiment we attempted to find out, whether septin 11 is down-regulated in Ist-Mes-2 exposed to α -TOS as a consequence of the generation of ROS. To explore this, we used the free radical scavenger N-acetyl-L-cysteine (NAC). The cells were seeded, allowed to attach and reach the confluency of 60-70%. Then they were treated with 25 μ M α -TOS in EtOH and NAC at 1, 5 and 10 mM for 24 h. Control cells were treated with equal concentrations of NAC without α -TOS. After the incubation the cells were harvested and whole cell lysates prepared as described in section 3.3.2.5. WB analysis was performed as described in section 3.3.2.5. The used antibodies are listed in table C.1. Figure 3.13 documents that α -TOS caused no down-regulation of septin 11 in Ist-Mes-2 cells which were exposed to 1, 5 and 10 mM NAC when compared with control cells that were exposed to the different concentrations of NAC but not to α -TOS. Cells which were not treated with NAC were found to be 1.59-fold down-regulated upon exposure to 25 μ M α -TOS for 24 h. This result indicates that the down-regulation of septin 11 might be caused by the generation of ROS induced by α -TOS.

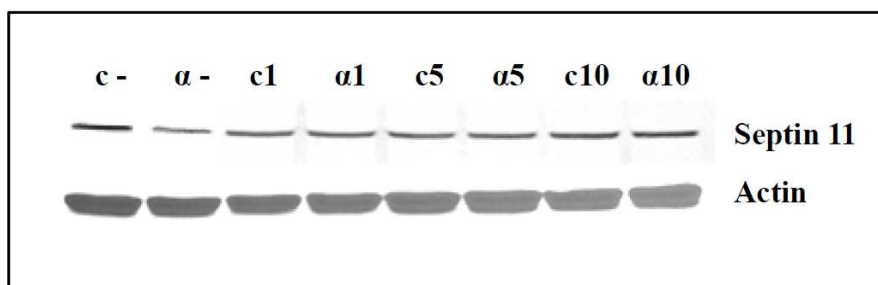


Figure 3.13: The effect of α -TOS on the protein level of septin 11 in Ist-Mes-2 cells treated with different concentrations of NAC.

The cells were exposed to 25 μ M α -TOS in EtOH for 24 h (α) and control cells (c) were treated with an equal volume of EtOH only. Further, cells were treated in the same way and were additionally incubated with different concentrations of NAC (1, 5 and 10 mM) for 24 h. Whole cell lysates were isolated and the protein levels estimated by WB analysis. Anti-actin IgG was used as the loading control. The images shown are representative of three independent experiments. (c = control cells without α -TOS and NAC; α = α -TOS treated cells without NAC; c1 = control cells without α -TOS and with 1 mM NAC; α 1 = α -TOS treated cells with 1 mM NAC; c5 = control cells without α -TOS and with 5 mM NAC; α 5 = α -TOS treated cells with 5 mM NAC; c10 = control cells without α -TOS and with 10 mM NAC; α 10 = α -TOS treated cells with 10 mM NAC).

3.4.2.6 Additional direction of the exploration of the effect of α -TOS on septin 11 and its role in MM cells

The above results document that α -TOS appears to regulate in the MM cell line Ist-Mes-2 the expression of the protein septin 11 on both the transcriptional and translational level. This follows from experiments using several approaches: the 2-D GE coupled with MS analysis, RT-PCR, qPCR and WB. Moreover, it appears that the effect of α -TOS on septin 11 is mediated by ROS that are produced as a result of the effect of the agent on Ist-Mes-2 cells. This is indicated by the finding that addition of NAC overrules the decrease of septin 11 in the cells exposed to the VE analogue. It was therefore of interest to further evaluate the role of septin 11 in MM cells and how its suppression by α -TOS may contribute to the anti-cancer efficacy of the agent. We therefore decided to test the effect of suppression of septin 11 in MM cells on their growth and capacity to form tumours.

Firstly, to achieve this goal, we decided to prepare septin 11-depleted cells by silencing this protein using shRNA. Using four different shRNAs, we failed to achieve this goal. At the stage when we were discussing how to overcome this hurdle so that an Ist-Mes-2 cell line with silenced septin 11 can be achieved, we had to change the direction of the research to focus more on the mechanism of apoptosis induction in MM cells by a novel and highly

promising mitochondrially targeted VE analogue, MitoVES, so that this part of the project is unfinished at this stage. The reason for re-directing our research was the planned clinical trial with MM patients using MitoVES (see Aim I). MM is a fatal disease and since MitoVES is a very promising selective and highly efficient anti-cancer agent, it should be tested since it can give hope to the mesothelioma patients. Therefore, Aim II is yet to be finalised, which is the plan for our work beyond this doctoral thesis. Indeed, we realise that results about the role of septin 11 in tumour suppression, thus far not well understood (in fact, septin 11 can also act as an oncogene, depending on the circumstances) can provide novel knowledge in the context of pathobiology of neoplastic disease with MM being a prime example of the pathologies that present an ultimate challenge in cancer management.

4 Discussion

MM is a rare, highly aggressive solid form of tumour that grows into separate plaques and nodules on the visceral and parietal pleural surfaces in over 75% of the cases, while a relatively low percentage of patients feature peritoneal MM. The neighbouring tissues are involved at an early stage, with the invasion of the chest wall, diaphragm, pericardium and interlobar apertures. The tumour commonly metastasises to the lungs, liver, bone and the adrenal gland (Hillerdal, 1982; Tomasetti *et al.*, 2009). The main cause for MM is exposure to asbestos fibers. MM is characterised by long latency periods from the exposure to clinical diagnosis. At present, there is no efficient therapy for patients with the disease. The median life expectancy following diagnosis of MM is <12 months. Because of the very aggressive nature of the treatment-resistant disease, MM represents a suitable model of cancer for testing novel potential anti-cancer drugs. MM is generally characterised by resistance to apoptosis: surmounting this difficulty would present one of the most promising strategies in MM treatment.

4.1 Discussion to Aim I

In the recent years, mitochondria have attracted the attention and have become a central subject of research because of their function as sensors and executioners of apoptosis (Green and Reed, 1998; Ferri and Kroemer, 2001; Wang, 2001). Apoptosis is an energy-dependent and genetically regulated form of cell death that is vital for normal development of an organism (Jacobson *et al.*, 1997). Alterations in the regulation of apoptosis are involved in the progression of various human pathologies, including neurodegenerative diseases and cancer (Thompson, 1995; Reed, 1999). It is now widely accepted that mitochondria represent effective targets that may provide the selectivity required for potent anti-cancer therapy (Neuzil *et al.*, 2001a; Don and Hogg, 2004). We have termed the group of small compounds with anti-cancer activity acting via mitochondrial destabilisation and apoptosis induction, often in a cancer-specific manner, as mitocans (Neuzil *et al.*, 2006, 2013). The agents were classified into several classes based on their molecular targets and mechanism of action (Neuzil *et al.*, 2013). Studies concerning mitocans have pointed to their unprecedented potential for cancer treatment due to the high specificity of many of these agents for cancer cells and due to their limited side effects towards normal cells

(Neuzil *et al.*, 2007). We have focused on class 5 mitocans, which comprises compounds targeting the ETC (Kluckova *et al.*, 2013), epitomised by VE analogues (Neuzil *et al.* 1999; 2001a,b; Zhang *et al.*, 2002; Yu *et al.*, 2003).

α -TOS, a redox-silent compound, represents a prime example of VE analogues. It has been shown to induce apoptosis in various cancer cells and to suppress the growth of many types of carcinomas in animal models (Neuzil *et al.*, 2001a; Tomasetti *et al.*, 2004a; Stapelberg *et al.*, 2005). At the molecular level, the agent behaves as a BH3 mimetic and sensitizes cancers cells to other drugs but, more importantly, it induces apoptosis by interacting with the UbQ-binding sites of the mitochondrial CII of the electron redox chain (Dong *et al.*, 2008; Dong *et al.*, 2009). Thereby it interferes with UbQ, the natural acceptor for electrons generated by the SDH activity of CII catalysing the conversion of succinate to fumarate. The electron flow is disrupted and the electrons recombine with molecular oxygen, resulting in the generation of superoxide anion radicals. These in turn activate the Hippo/Mst1 kinase in the cytosol, which directly phosphorylates the forkhead box protein FoxO1 that belongs to the conserved group of transcription factors with the forkhead DNA-binding domain (Lehtinen *et al.*, 2006). Phosphorylated FoxO1 is subsequently released from the inactive complex with the 14-3-3 protein and translocates to the nucleus, where it binds to the FoxO-binding sequence in the promoters of pro-apoptotic genes (Fu and Tindall, 2008) including *FASL* (Suhara *et al.*, 2002), *TRAIL* (Modur *et al.*, 2002), *NOXA* and *BIM* (Obexer *et al.*, 2007) inducing their transcription. The up-regulated expression of proteins TRAIL and FASL then results in the activation of caspase-8 and of the extrinsic apoptotic pathway, whereas the up-regulated BH3-only protein Noxa interacts with the anti-apoptotic Bcl-2 family protein Mcl-1, which leads to the dissociation of Mcl-1 from the pro-apoptotic Bcl-2 family protein Bak. Consequently, Bak forms pores in the MOM leading to the leakage of cytochrome *c* and further pro-apoptotic molecules and to the activation of the down-stream mitochondrial apoptotic pathway (Prochazka *et al.*, 2010; Valis *et al.*, 2011).

The finding that the molecular target for α -TOS are the UbQ-binding sites of the mitochondrial CII of the ERC inspired us to modify the VE analogue by tagging it with the mitochondria-targeting delocalised cationic TPP⁺ group. This stems from other studies where a variety of redox-active compounds was modified in this way, whereby it was

mitochondrially targeted; these agents include UbQ, VE as well as polyphenolic compounds (Smith *et al.*, 2003; Murphy and Smith, 2007; Biassutto *et al.*, 2010). The targeting of the anti-oxidants with the TPP⁺ group caused a 5-10-fold uptake of the compounds across the cellular plasma membrane as a consequence of the negative potential on the cytosolic face of the membrane (~ -50 mV) and an up to 500-fold uptake at the interface of the mitochondrial matrix and the MIM due to the high negative potential across the MIM (~ -180-200 mV) (Biassutto *et al.*, 2010). The resulting product of tagging VE succinate with the TPP⁺ group was mitochondrially targeted VE succinate (MitoVES) which proved to be about 20-50-fold more efficient in killing cancer cells compared to the parental compound while keeping the selectivity for cancer cells (Dong *et al.*, 2011 a, b). Intriguingly, too, MitoVES very efficiently killed proliferating endothelial cells while their quiescent counterparts were highly resistant, endowing the agent with yet another unexpected feature (Rohlena *et al.*, 2011).

Due to the poor prognosis for patients with MM and the finding that α -TOS is efficient against experimental MM (Tomasetti *et al.*, 2004a; Yin *et al.*, 2007), we decided to test the effect of MitoVES on various MM cell lines as well as on experimental MM. The data obtained from this work document that MitoVES is considerably more efficient against MM than its untargeted parental compound α -TOS, providing the agent a clinical importance, as detailed below. In this work we first tested and compared the efficiency of the two mitocans from the group of VE analogues, MitoVES and its parental compound α -TOS, to kill various MM cell lines. Even more so taking into consideration the fact that several established anti-cancer therapies are more toxic to non-malignant mesothelial cells than their malignant counterparts (Narasimhan *et al.*, 1998).

We started the work by studying the killing efficacy of MitoVES with the idea that, if there is a considerable increase, more in depth investigation of the molecular mechanism of this activity would be warranted. To get a more general understanding of the effect of MitoVES, we used several MM cell lines of different types: the human cell lines Ist-Mes-2 cells (epitheloid phenotype), MM-BI cells (mixed phenotype), Meso-2 and H28 cells (sarcomatoid phenotype) (Pass *et al.*, 1995), and the murine AE17 cells (Jackaman *et al.*, 2003). Thus, using crystal violet staining, we prepared individual killing curves and calculated from them the IC₅₀ values. The experiment documents that MitoVES is much

more efficient in killing the MM cells than its parental counterpart, with the IC₅₀ values for the mitochondrially targeted analogue of VE being in all tested MM cell lines by about two order of magnitude lower than for α -TOS. This result documents the highly superior efficacy of MitoVES in killing the very recalcitrant cancer cells and warrants further investigations (Narasimhan *et al.*, 1998). We have recently observed that MitoVES has very low toxicity to the non-malignant mesothelial cells Met-5A, further pointing to this agent as a selective compound (Dong *et al.*, 2011b).

In the next experiment, we attempted to find out whether the apoptotic cell death pathway is operational in the various MM cell lines exposed to both mitocans. From previous studies, we knew that both, α -TOS and MitoVES triggered apoptosis in various types of cancer cells. Thus, using the annexin V-FITC/PI staining and flow cytometry, we compared the rate of apoptosis and post-apoptotic necrosis induced by the VE analogues in the individual MM cell lines. As anticipated based on the killing curves experiments, MitoVES was much more efficient in apoptosis induction than its parental compound α -TOS. It eradicated all tested cells within 24 h at a concentration of $>2.5 \mu\text{M}$, while a similar efficacy in killing was reached with approximately $100 \mu\text{M}$ α -TOS. The obtained outcomes document that both agents are able to overcome the resistance of MM cells to apoptosis induction, and extend our original observation of high efficacy of apoptosis induced in an MM cell line (Dong *et al.*, 2011b).

As discussed above, both tested mitocans exert their apoptotic effect on cancer cells primarily by affecting mitochondria resulting in generation of ROS and dissipation of $\Delta\Psi_{\text{m,i}}$ (Neuzil *et al.*, 2001 a, b; Yu W *et al.*, 2003; Gogvadze *et al.*, 2010; Dong *et al.*, 2011 a, b). Thus, we tested whether the compounds cause production of ROS and a drop of $\Delta\Psi_{\text{m,i}}$ in all studied MM cell lines. For assessment of generation of ROS, we used three different probes. DHE was used for the estimation of the superoxide levels, DCF for the evaluation of the generation of hydrogen peroxide and MitoSOX for the determination of superoxide levels formed specifically within the mitochondrial compartment. Consistent with the notion that mitocans cause ROS generation and with our previous findings concerning MitoVES, the mitochondrially targeted VE analogue generated considerable levels of ROS at concentration as low as $5 \mu\text{M}$ at times less than 10 min using all three

probes. The fast manner of ROS generation is similar as observed before for MitoVES and neuroblastoma cells transfected with a redox-sensitive plasmid (Dong *et al.*, 2011b). However, α -TOS caused only a marginal generation of ROS in MM cells at a 20-fold higher concentration (100 μ M) and at 60 min. This finding indicates that α -TOS is much less efficient than its mitochondrially targeted analogue, and, also that it may exert its killing activity in MM cells via a different mode of action. This result also makes the MM cells different from other cells in which ROS generation by α -TOS was tested. These are, for example, Jurkat cells, which produce ROS when exposed to 50 μ M α -TOS within 30 min (Weber *et al.*, 2003).

As tested further, MitoVES was responsible for a rapid dissipation of $\Delta\Psi_{m,i}$ in most MM cell lines with a notable exception. Thus, in the human MM cell line MM-BI, only a moderate dissipation of $\Delta\Psi_{m,i}$ was observed after the exposure of the cells to MitoVES, and in the human Meso-2 cells, MitoVES caused even an increase in $\Delta\Psi_{m,i}$. Using the uncoupler CCCP we were able to determinate the maximum decrease of $\Delta\Psi_{m,i}$, which was in agreement with the dissipation of $\Delta\Psi_{m,i}$, induced by MitoVES. No dissipation of $\Delta\Psi_{m,i}$ was observed in Meso-2 cells exposed to CCCP. This suggests that there are considerable differences in the level of $\Delta\Psi_{m,i}$ in the various MM cell lines. Since the killing of the different cell lines with MitoVES does not follow the efficacy of the agent to dissipate $\Delta\Psi_{m,i}$, it is possible that dissipation is indicative to some extent of the effect of MitoVES but does not correlate directly with the killing efficacy of the agent. In stark contrast to MitoVES, α -TOS failed to dissipate $\Delta\Psi_{m,i}$ in all MM cell lines.

The distinctions in the generation of ROS levels and the dissipation of $\Delta\Psi_{m,i}$ are indicative of a potentially different mode of action between the two mitocans, MitoVES and α -TOS. MitoVES is directly targeted to mitochondria, whereas α -TOS can associate with different lipophilic structures (Weber *et al.*, 2003; Neuzil *et al.*, 2002; Dong *et al.*, 2011 a, b). Thus, α -TOS may induce apoptosis in two ways - by a direct effect of mitochondria or by affecting other organelles, in particular lysosomes (Neuzil *et al.*, 1999; 2002). Furthermore, α -TOS can affect signalling pathways, such as the c-Jun pathway in leukemia and breast cancer cells (Qian *et al.*, 1997; Yu W *et al.*, 2001), the protein kinase C pathway (Neuzil *et al.*, 2001) and the FGF2 autocrine loop in MM cells (Stapelberg *et al.*, 2005), which may also

influence its mode of action. Importantly, the data found in this work document, that MitoVES can generate ROS even at a low initial $\Delta\Psi_{m,i}$, suggesting that it will be efficient against different cancer cell types and at differently energised mitochondria.

Further, we were interested, whether MitoVES is directed to mitochondria of MM cells and whether it accumulates in these organelles. For this purpose, we synthesised fluorescently labelled analogues of MitoVES and α -TOS, MitoVES-F and α -TOS-F, respectively (*cf* Figure D.1). Using the mitochondrial marker TMRM and confocal microscopy, we were able to visualise mitochondria in MM cells. Almost exclusive localisation in mitochondria was shown for MitoVES-F while α -TOS-F was localised mostly to other compartments and, to some extent, to mitochondria. Pre-treatment of Ist-Mes-2 cells with CCCP prior to their exposure to MitoVES-F resulted in the dissipation of $\Delta\Psi_{m,i}$ and caused, subsequently, a loss of the exclusive localisation of the compound in mitochondria. This finding documents that MitoVES in contrast to its parental compound α -TOS is targeted to mitochondria and that its mitochondrial association is dependent on a high $\Delta\Psi_{m,i}$. In a follow-up experiment we were able to show the importance of $\Delta\Psi_{m,i}$ for the induction of apoptosis in Ist-Mes-2 cells exposed to MitoVES, as the pre-treated MM cells with CCCP failed to undergo efficient apoptosis when compared to cells which were not exposed to the uncoupler. Altogether, the data obtained here point to the importance of $\Delta\Psi_{m,i}$ for efficient killing of MM cells. These findings are consistent with recent data, in which dissipation of $\Delta\Psi_{m,i}$ suppressed the high killing efficacy of MitoVES towards proliferating endothelial cells (Rohlena *et al.*, 2011).

Previous studies revealed, that the VE analogue α -TOS and its mitochondrially targeted derivate MitoVES interact with the UbQ-binding sites of CII of the mitochondrial respiratory chain of cancer cells (Dong *et al.*, 2008; 2009; 2011a). Mammalian CII contains two UbQ-binding sites, the proximal (Q_p) and the distal (Q_d) site (Sun *et al.*, 2005). We suggested that while α -TOS can interact with both UbQ-binding sites (Dong *et al.*, 2008), MitoVES is able to reach only the Q_p site, due to the length of its aliphatic chain and due to its localisation across the mitochondrial matrix and the MIM, with the charged TPP^+ group in the matrix and the tocopheryl succinyl group in the MIM (Dong *et al.*, 2011a). We synthesised a range of homologues of MitoVES by shortening its aliphatic chain by two carbons at a time (*cf* Figure D.1) to show the importance of the length of the

aliphatic chain for efficient activity of MitoVES towards MM cells. The resulting homologues were tested for their effect on SQR activity, as well as their efficacy to induce apoptosis and to evoke ROS production and $\Delta\Psi_{m,i}$ dissipation. Using all mentioned methodologies, we can state that all tested shorter-chain homologues of MitoVES were much less efficient than the full length (11-C) homologue, with the C-9 homologue almost as active as the full length agent, while the shorter chain homologues C-7 and C-5 were comparatively inefficient. This finding is in compliance with our earlier studies (Dong *et al.*, 2011a, b).

Furthermore, we wanted to find out, how relevant is the composition and structure of MitoVES for its activity in MM cells. It was proposed earlier (Dong *et al.*, 2008; 2011a) that the free succinate group of MitoVES is required for its interaction with the UbQ-binding serin 68 in the Q_p site (Sun *et al.*, 2005). Therefore we tested a further homologue of MitoVES, MitoVES4TPP, in which the succinyl moiety of α -TOS was esterified with the TPP⁺ group attached to the α -TOS structure via a 4-C linker (*cf* Figure D.1). Testing this homologue in MM cells by measuring the SQR activity, apoptosis rate, generation of ROS and dissipation of $\Delta\Psi_{m,i}$, we were able to validate the proposal that the free succinate group of MitoVES is needed for its activity and interaction with the Q_p site. Thus, we can conclude, that both, the structure of MitoVES, as well as the length of its aliphatic chain are fundamental for exerting its killing activity in MM cells.

The above data on the structure-function relationship of MitoVES homologues invoke further questions and possibilities. The clear conclusion is that the length of the linker between the TPP⁺ group and the chromanol ring is 11 carbons and that a free carboxylate is essential for interaction of MitoVES with serin 68 of the Q_p site. Various approaches to modifications of VE analogues to understand the structure-function relationship have been taken. Perhaps the most extensive study is one by Birringer and colleagues (Birringer *et al.*, 2003). Here the authors modified all three domains of the basic structure of VE analogues, the functional, signalling and hydrophobic domains. It is clear that an ideal VE analogue in terms of its efficacy to kill cancer cells is one with a free carboxylate in the functional group, the presence of the chromanol ring and a C-11 aliphatic chain. For example, methylation of the succinyl group of α -TOS completely wiped out the activity. Removal of the chromanol structure (that is, generating a compound with a succinylated

phytyl ring) suppressed the apoptotic activity almost to zero. And finally, elimination of the phytyl ring such as the replacement by a carboxyl group resulted in Trolox succinate with no apoptogenic activity (Birringer *et al.*, 2003).

The length of the MitoVES phytyl chain is very important, since it allows the succinyl group that is esterified to the C-6 position of the chromanol ring to reach the Qp site. Attempts to synthesise homologues with longer aliphatic chains were not attempted. One reason is that the longer the chain, the harder the synthesis: we found that in this case, the intermediates are very hard to dissolve, which makes the synthesis a considerable challenge (Neuzil *et al.*, unpublished results). Further, such compounds would be difficult to get into cells. This can be reconciled with studies by Murphy and Smith (Murphy and Smith, 2007), in which they prepared mitochondrially targeted forms of UbQ with the TPP⁺ group at the end of the 40-carbon side chain (10 isoprenoid units), which is the length of the aliphatic chain of natural UbQ. After many attempts they arrived at the ideal structure, in which mitochondrially targeted UbQ had 10 carbon-long aliphatic chain. Perhaps the most important reason for keeping the maximum number of carbons in the aliphatic chain of MitoVES at 11 is, that this is the length of the phytyl chain of natural VE, which has been ‘designed’ via evolution to have ideal biological properties. We further tested for the effect on cancer cells an analogue, in which an acetyl group with free carboxylate is attached to the chromanol ring with an 11-C aliphatic chain and the TPP⁺ group via an ether link. We found the compound rather ineffective, probably due to the short length of the functional group (2 carbons) (Neuzil *et al.*, unpublished results), therefore, we did not include this agent in our studies on MM. For reasons mentioned above, MitoVE₁₁S was used in all subsequent studies.

It has been documented that there are small anti-cancer agents that can affect mitochondria by suppressing their respiration, in particular agents from Class 5 (Ralph *et al.*, 2010b; Neuzil *et al.*, 2013). To learn more about the mechanism how α -TOS and MitoVES affect mitochondria and induce cell death in MM cells, we tested the two VE analogues for their suppression of mitochondrial respiration. First, we assessed the effect of the two compounds on routine respiration in five different MM cell lines by evaluating the total oxygen consumption by whole cells in the standard cultivation medium without any uncoupler. We observed significant differences in routine respiration amongst the

individual MM cell lines, which were in the range of 15-30 pmol O₂ per s per 10⁶ cells. MitoVES was much more efficient in suppressing mitochondrial respiration than α -TOS, which proved to be almost inefficient, suppressing respiration by some 10-20% at 50-100 μ M. Very interestingly, we observed initial increase in respiration at low levels (1-2 μ M) of MitoVES, resulting most likely from mitochondrial uncoupling triggered by the agent. This corresponds to recent studies showing an uncoupling effect of MitoVES at low levels in intact cells and in isolated mitochondria, with suppression of respiration at its higher concentrations (Rodríguez-Enríquez *et al.*, 2012). It is possible that at low concentrations, the agent accumulates at the interphase of the mitochondrial matrix and the MIM at levels that are not sufficient to suppress the SQR activity, which is needed for ROS generation. While it is beyond this study to further investigate the molecular mechanism of uncoupling by MitoVES, it will be a subject of subsequent experiments.

The inhibition of respiration is connected with generation of ROS evoked by MitoVES and is in agreement with CII as an origin of radicals (Dong *et al.*, 2011a,b; Quinlan *et al.*, 2012; Moreno-Sánchez *et al.*, 2013). Using appropriate substrates and inhibitors for CI or CII, we assessed the contribution of the two complexes to mitochondrial respiration in permeabilised whole MM cells exposed to α -TOS and MitoVES. All tested MM cell lines were shown to respire primarily through CII. With the exception of MMBI and Ist-Mes-2 cells, CII contributed 2-3-fold more to the total respiration than CI. MitoVES uncoupled CII-dependent respiration at low levels in several of the tested MM cell lines and caused its sharp suppression at higher levels. The mitocan influenced the CI-dependent respiration as well, although here the inhibition was less noticeable and the initial uncoupling effect was not observed. No inhibitory effect of α -TOS on respiration via CI or CII was observed in all MM cell lines tested. The effect of MitoVES on CI is reminiscent of a recent study where α -TOS suppressed CI activity in leukemia cells (dos Santos *et al.*, 2012). The results need to be taken with the assumption that cell cultures are sed. This is particularly due to our recent finding, in which we assessed the effect of MitoVES on tumours. In this experiments, mice with breast tumours were treated with MitoVES for over 3 weeks. The animals were then sacrificed and tumour tissue shredded and evaluated for respiration via CI and CII in the Oxygraph. Interestingly, we observed about 50-60% inhibition of respiration via CII while respiration via CI was increased (Neuzil *et al.*, unpublished results). This is a very important finding that shows the molecular mechanism of MitoVES

in vivo. While it causes suppression of CII respiration, whereby (via ROS generation) killing cancer cells and suppressing the tumours, the malignant cells try to overcome their suppressed respiration by increasing CI respiration. We will further study this premise in the future, since it is of high importance and, most importantly, teaches us about the molecular mechanism of the agents in the context of a real tumour.

We found that MitoVES has a considerable effect on respiration via mitochondrial complexes, in particular CII. It is therefore possible, that the agent affects the assembly of mitochondrial complexes as well as their supercomplexes, referred to as respirasomes (Acin-Perez *et al.*, 2008). We therefore evaluated the effect of α -TOS and MitoVES on mitochondrial (super)complexes using NBE in two MM cell lines, Ist-Mes-2 and H28. We observed that mitochondrial complexes are assembled and form supercomplexes in both cell lines, these include the supercomplexes of CIII₂, CIII₂+CIV, CIII₂+CI, and CIII₂+CI+CIV_n. While MitoVES did not have practically any effect on CII, it destabilised the CI+CIII₂+CIV_n supercomplex, which is the most active form of the mitochondrial supercomplexes (presumably due to direct transfer of electrons from CI to CIII to CIV) (Acin-Perez *et al.*, 2008). α -TOS showed a mild effect on the CI+CIII₂+CIV_n supercomplex. We next tested the effect of MitoVES and α -TOS on the CI and CII activity using the in-gel assay following separation of the proteins in the cell lysates by clear native electrophoresis. MitoVES was found to relatively efficiently the CII activity, and to a lesser extent the CI activity, and some effect of α -TOS on the CII activity was also observed. Thus, we were able to demonstrate, that MitoVES much more efficiently influences the formation of respirasomes in MM cells, when compared to α -TOS. The mechanism by which MitoVES affects the mitochondrial complexes is unclear. In other experiments, we found that MitoVES but not α -TOS affected the mtDNA (coding for 13 subunits of mitochondrial complexes), in particular the level of the so called D-loop transcript, which is essential for the replication and transcription of the mitochondrial genome in a range of cancer cells, including MM cells (Neuzil *et al.*, unpublished data). Whether the effect of MitoVES on the mitochondrial supercomplexes is due to lower level of expression of some of their subunits or whether it destabilises them due to its chemico-physical properties is yet to be resolved and is beyond the scope of this work.

To definitively prove that the mitochondrial CII is the molecular target of MitoVES, we prepared MM cells with suppressed CII activity. For this purpose, Ist-Mes-2 cells were stably transfected with SDHC shRNA, causing silencing of the SDHC subunit of CII, which resulted in very low level of the SDHC protein and highly reduced SQR activity. At the same time, the SDH activity in the cells transfected with SDHC shRNA was similar to that of control cells, transfected with non-silencing shRNA, as the catalytic center of the CII SDH activity, causing conversion of succinate to fumarate, comprises of the SDHA activity. It has been shown that SDHA (probably together with SDHB) can function independently, albeit with lower activity, in the absence of SDHC and/or SDHD (Lemarie *et al.*, 2011; Grimm 2013). We then assessed respiration of the MM cells via CI and CII using the Oxygraph. CII-compromised cells respired to a lesser extend via CII than the corresponding control cells which were transfected with non-silencing shRNA. We further used flow cytometry and the annexin V/PI method to investigate the importance of CII for the induction of apoptosis by MitoVES. Low level of apoptosis was assessed in Ist-Mes-2 cells with silenced SDHC upon the exposure to MitoVES when compared to the control cells, which proved a significantly higher apoptosis level. This outcome clearly shows that CII is the molecular target of MitoVES. This finding is compatible with the effect of MitoVES on other cancer cell types (Dong *et al.*, 2011a,b). The novel issue here is in the context of MM being non-curable, which makes agents like MitoVES of high importance for potential translational studied. Understanding the molecular mechanism of the anti-mesothelioma activity of MitoVES is therefore essential for its further potential clinical utilisation.

In the next experiment, we wanted to verify the ultimate, whether MitoVES is efficient in suppressing experimental MM. Previous studies reported on MM inhibitory activity *in vivo* caused by treatment with α -TOS (Tomasetti *et al.*, 2004a; Stapelberg *et al.*, 2005). To support the data pointing to the superior effect of MitoVES to α -TOS, we tested the effect of the two mitocans on experimental MM that was prepared by grafting Ist-Mes-2 cells in immuno-deficient, athymic Balb/c *nu/nu* mice injected subcutaneously with the cells. When tumours formed, we treated the animals intraperitoneally with α -TOS or with MitoVES twice per week. Control mice were treated with the excipient only (the solvent for MitoVES or α -TOS). Ultrasound imaging enabled the quantification of the tumours. This method is superior to majority of established methodologies that are used for tumour

quantification, since it allows for non-invasive visualisation of complete tumours, including their parts embedded in the experimental animal, followed by very precise quantification. As expected and in line with the *in vitro* experiments, MitoVES was considerably more efficient in suppressing MM in the immunocompromised mice than α -TOS. Under the conditions described in section 3.4.1.6, MitoVES inhibited tumour growth by >90% while α -TOS was at a 15-fold higher concentration less efficient, with approximately 80% tumour growth inhibition. It is also important to add, that we did not observe any toxicity towards the drugs during the experiment. The intriguing finding that MitoVES suppresses MM is accentuated by the fact that the treatment with MitoVES resulted in complete tumour disappearance. This result showing superior efficacy of MitoVES endows this agent with a great promise to be developed into a clinically relevant anti-cancer agent.

All data obtained from this work consistently show that mitochondrial targeting of VES endows the agent with considerably higher efficacy to kill MM cells, both *in vitro* and *in vivo*, than found for the parental compound α -TOS, since it brings it to the immediate proximity of its molecular target of action. Very importantly, MitoVES is selective for cancer cells and for tumour tissue. This condition is a prerequisite for any further consideration of a compound for translational studies. There are several pieces of evidence for this. First, treatment of immunocompromised mice with Ist-Mes-2 xenografts with MitoVES did not result in any deleterious toxicity to the animals within the almost four weeks of the therapy, as judged from the behavioural pattern of the mice and no loss of their weight. Second, we found recently that the non-malignant immortalised mesothelial Met5A cells are resistant to MitoVES even at doses of 20-30 μ M (Dong *et al.*, 2011b), at which concentration the agent kills MM cells within hours. Third and perhaps most intriguingly, we have recently performed an experiment in which the FVB/N *c-neu* mice with spontaneous HER^{high} breast carcinomas (Guy *et al.*, 1992) were injected with MitoVES-F. Confocal microscopy of sections of the tumour, liver, heart and kidney revealed green fluorescence only in the malignant tissue, not in the normal organs, clearly documenting that MitoVES is selectively taken up by cancer cells (Truksa *et al.*, submitted for publication).

The reasons for selective uptake of MitoVES by cancer cells are not clear at the moment. We can speculate that the main factor for the translocation from the intrastitium to the MIM is due to the chemico-physical properties and depends on the differences between malignant and normal cells. Clearly, the TPP⁺ group at the end of the aliphatic chain endows MitoVES with high level of accumulation in the organelles with high potential. As stated earlier, the first barrier for the agent is the plasmatic membrane, which has a certain membrane potential ($\Delta\Psi_p$). Since cancer cells feature high levels of glycolysis (Vander Heiden *et al.*, 2009) and they need to maintain their cytosolic pH neutral even at elevated levels of lactate, they efficiently pump outside the cells protons, acidifying the interstitium (Kozin *et al.*, 2001). It can thus be expected that the $\Delta\Psi_p$ levels are greater for cancer than normal cells. This then causes a selective uptake of agents like MitoVES into cancer cells within the various tissues (Biassutto *et al.*, 2010). Of course, it is well known that the $\Delta\Psi_p$ is much greater in malignant cells than in their normal counterparts, therefore the killing efficacy of the former is greater (considering that perhaps some MitoVES is taken up by normal cells at all).

Another point to realise is the importance of the structure of MitoVES for the maximum anti-cancer efficacy. Thus, we observed some 90% suppression of experimental Ist-Mes-2 tumours in mice treated with MitoVES. In our recent work, MitoVES suppressed to a similar or greater extent spontaneous HER2^{high} breast carcinomas in the FVB/N *c-neu* transgenic mice and by some 70-80% experimental colorectal carcinomas (Dong *et al.*, 2011a,b). This is contrasted by a very recent study by Cheng and colleagues (Cheng *et al.*, 2013). In this work, the chromanol structure of α -TOS was modified by its tagging with the TPP⁺ group via an 11-carbon aliphatic chain attached to the chromanol ring moiety via a carboxylic bond. This compound suppressed the growth of cancer cells in a selective manner, however its IC₅₀ values for a series of breast cancer cell lines were inferior to those found by us for MitoVES. Perhaps most importantly, it suppressed tumours by some 20-30%, documenting that the complete structure of MitoVES is essential for maximising the anti-tumour efficacy of the agent.

The above results and considerations make MitoVES an ideally placed agent to be tested in cancer patients, and studies are being planned. The importance of MitoVES as a potential

future anti-cancer agent is fuelled by our additional recent work. Several years ago, we were treating a single mesothelioma patient with a-TOS given transdermally in Lipoderm[®]. The patient, a 68-year female, was predicted to live for some 4-5 months when she started to apply α -TOS. The therapy was very pleasant and caused a reduction in the size of the tumour, with no side effects. However, after about 4 years, the tumour suddenly started to increase and the patient succumbed to the disease. This can be reconciled with our study on the potential resistance of cancer cells to α -TOS. We found, that escalating the dose of α -TOS, non-small cell lung carcinoma cells developed resistance to the drug (including tumours derived from the resistant cells) (Prochazka *et al.*, 2012). The reason was a gradual adaptation of the cells to the agent, increasing the ATPase pump, the ABCA1 protein, lowering the intracellular level of α -TOS below the IC₅₀ value. However, MitoVES was able to kill the α -TOS-resistant cells, even more efficiently than the parental cells (Prochazka *et al.*, 2013). We can speculate that the reason for the susceptibility of these cells is the chemico-physical nature of MitoVES, which is taken up on the bases of the $\Delta\Psi_p$, bypassing the activity of the ATPase pump. We also found that the α -TOS-resistant cells show higher level of respiration via the mitochondrial CII, which may explain their higher susceptibility to MitoVES (Neuzil *et al.*, unpublished results). Given these findings and the notion that the patient apparently developed resistance to a-TOS, the idea of the use of MitoVES as an anti-cancer agent, in a single-drug or combinatorial therapy, is very tempting and warranted to be trialed clinically.

4.2 Discussion to Aim II

A further aim that was followed but not completed in this study was to find the signalling pathways of apoptosis mediated by α -TOS in Ist-Mes-2 cells. It has been reported in a number of papers, that α -TOS, besides causing generation of ROS (or maybe as a consequence of this), modulates a variety of signalling pathways (Zhao *et al.*, 2009). To name, these include the protein kinase C pathway (Neuzil *et al.*, 2001a), the MAPK-c-jun pathway (You *et al.*, 2002; Zhao *et al.*, 2007), the TGF β pathway (Yu *et al.*, 1997), the NF κ B pathway (Dalen *et al.*, 2003) or the HER2-PI3K-Akt pathway (Wang *et al.*, 2005). Since a-TOS acts on mitochondria (Neuzil *et al.*, 2007a,b) as its major molecular target, we

had in mind the potential ‘mitochondrial connection’ when focusing on the various pathways.

To delineate the possible candidates as members of the complex signalling pathways, we performed 2-D GE of the treated and untreated MM cells. Proteins that were resolved using lysates from the two types of conditions were compared to identify those with significantly different levels. MS analysis was then used to identify a variety of proteins with different expression levels after exposure of Ist-Mes-2 cells to α -TOS. The complete list of the identified proteins is included in Table 3.2, showing 12 proteins up-regulated more than 1.5-fold and 12 proteins down-regulated more than 1.5-fold. Subsequent proteomic and genomic molecular studies were performed to validate the altered expression levels of the individual proteins. Very briefly, of the proteins identified by MS, RT-PCR confirmed the down-regulation of septin 11 and the up-regulation of subunit beta of the H^+ transporting F1 complex of mitochondrial ATP synthase in Ist-Mes-2 cells exposed to α -TOS. Further studies showed that the protein and mRNA levels of septin 11 after the treatment of Ist-Mes-2 cells with α -TOS were decreased, as further documented by qPCR and western blot analysis. Pre-treatment of Ist-Mes-2 cells with the free radical scavenger NAC protected cells exposed to α -TOS from down-regulation of septin 11, suggesting that the down-regulation of the protein might be regulated by ROS induced by the VE analogue. In the next part of the Discussion, we look at the various proteins differentially regulated by α -TOS, focusing on their potential role in the signalling pathways modulated by the agent, in particular in the context of mitochondria.

From all the identified proteins (see Table 3.2) septin 11 represents a very interesting target regulated by α -TOS, as it has been reported by various studies to play an important role in carcinogenesis. Septin 11 belongs to a conserved family of GTP-binding proteins that assemble into filamentous structures and interact with actin and tubulin. In humans, the protein is encoded by the *SEPT11* gene (Kim *et al.*, 2004). From yeast to human, septins maintain a conserved role in cytokinesis and are implicated in a various other cellular processes including apoptosis, vesicle trafficking, migration, synaptogenesis, neurodegeneration, chromosome segregation and DNA repair (Kinoshita, 2003; Connolly *et al.*, 2011). To date, 14 septin orthologous genes have been identified in mammals (Connolly *et al.*, 2011). The septin family is characterised by complex genomics and

extensive (but not universal) splicing, resulting in a number of septin isoforms. Since septins have been shown to participate in a variety of fundamental biological processes, it is not surprising that their deregulation is involved in different types of cancer. Numerous studies have reported that septins play important roles in tumourigenesis and metastasis.

The knockdown of different genes encoding the septin proteins in dividing cells leads to the formation of multinucleated cells, incorrect attachment of chromosomes to the spindle apparatus, and improper completion of cytokinesis (Kinoshita *et al.*, 1997; Nagata *et al.*, 2003; Spiliotis *et al.*, 2005; Estey *et al.*, 2010; Connolly *et al.*, 2011). Based on these findings, it is enticing to assume that deletion or down-regulation of septins, leading to improper cell division and subsequently to aneuploidy or polyploidy, is an important way how altered gene expression may lead to the malignant transformation. Surprisingly, only few studies describe the loss of septin function or expression, with *SEPT11* in hepatocellular carcinomas (Huang *et al.*, 2010) and *SEPT9* in colon and breast carcinomas (Kalikin *et al.*, 1997; Russell *et al.*, 2000; Grützman *et al.*, 2008).

Most of studies have reported septins to be up-regulated and overexpressed in numerous cancer types, with *SEPT11* over-expression in addition to *SEPT2* over-expression and the associated alteration of isoform expression in the Von Hippel-Lindau (VHL) protein, giving rise to defective renal carcinoma cell lines and renal cancer (Craven *et al.*, 2006a,b), *SEPT9* over-expression in breast cancer (Montagna *et al.*, 2003; Gonzales *et al.*, 2007; 2009), head and neck cancer (Stanbery *et al.*, 2010) and ovarian carcinoma (Scott *et al.*, 2006), *SEPT8* over-expression in gastrointestinal tumours with KIT mutations (Kang *et al.*, 2006), *SEPT6* up-regulation in invasive melanoma cell lines and metastasis (Kim *et al.*, 2004; Jaeger *et al.*, 2007), *SEPT1* over-expression in oral squamous cell carcinomas (Kato *et al.*, 2007), and *Sept9* in rat hepatocellular carcinomas (Kakehashi *et al.*, 2010). So far, it is not understood, why septins are mainly up-regulated in solid tumours with the evidence that the knock-down of septins may result in aneuploidy.

Septins have been suggested by different studies to function either as oncogenes or as tumour suppressor genes. Interestingly, various septins have been proposed to have a dual function of tumour suppression and oncogenic activity (Connolly *et al.*, 2011). However, this field of investigation is in its preliminary stages of development and requires to be

supplemented and supported by further studies. The research on mammalian septins is emerging and will most likely provide new therapeutic targets and biomarkers for early detection of cancer in future (Connolly *et al.*, 2011).

In context to this work, we have considered for the first time a possible role of septin 11 in apoptosis mediated by the the mitocan α -TOS in MM cells. This has not been studied thus far. In our system, septin 11 was down-regulated upon treatment of the Ist-Mes-2 cells with the anti-cancer agent α -TOS, both on the protein and mRNA levels. Since α -TOS has been reported to affect cancer cells by way of ROS generation (Weber *et al.*, 2003; Stapelberg *et al.*, 2005) (although it appears that the level of ROS generated in MM cells as a response to α -TOS when compared to MitoVES is rather low), we tested their potential role in the decrease of its expression in response to the VE analogue. The antioxidant NAC caused suppression of the down-regulation, which was observed when Ist-Mes-2 cells were exposed to α -TOS in the absence of the antioxidant. This indicates that there is a mechanism by which ROS may regulate the level of septin 11, most likely affecting its transcriptional regulation.

We further wanted to study the role of septin 11 in the effect of α -TOS on MM cells. Therefore, we used shRNA to suppress the protein and see whether cells with low level of septin 11 have a different morphology, proliferation rate, or susceptibility to α -TOS. However, we did not achieve this goal due to the fact that in the first series of experiments, we did not obtain a single clone with down-regulated septin 11. At this stage, there was a need to change the strategy of the research due to a planned trial of MitoVES for MM patients. Therefore, the effect of the mitochondrially targeted VE succinate on MM cell lines and experimental mesotheliomas was tested, and this is the thrust of Aim I of the thesis. Notwithstanding this complicating point, septin 11 is a very interesting protein, whose role in driving tumourigenesis and as a target for anti-cancer agents has only recently started to be studied. In a recent report, it has been shown that several septins (septin 2, 8, 9 and 11) were overexpressed in paclitaxel-resistant breast cancer cells, while the motor protein dynein-1 was down-regulated. The septins deregulated the microtubule organisation, in particular its dynamics, with potential consequences for the organisation of signalling pathways (Froidevaux-Klipfel *et al.*, 2011). Given the fact that α -TOS

suppresses septin 11, it may not only directly kill cancer cells but also sensitise them to established therapies. In particular taking into consideration that MM cells are rather resistant to clinically used therapeutics (Narasimhan *et al.*, 1998).

Therefore, more in- depth studies have to be performed to understand the role of septin 11 in MM cells exposed to α -TOS and to identify the mechanism by which the mitocan influences the down-regulation of septin 11. The necessary studies are rather complex and include methodology such as the hromati immunoprecipitation and site-directed mutagenesis. These studies are in the stage of planning and will be executed in our laboratory in the future.

Mitochondria play a central role in providing eukaryotic cells with energy. The molecular apparatus involved in the synthesis of cellular ATP is the H^+ -ATP synthase, a molecular ‘rotatory-engine’ complex embedded in the MIM (Boyer, 1997; Yoshida *et al.*, 2001). Mitochondrial ATP synthase catalyzes ATP synthesis, using an electrochemical gradient of protons across the MIM within oxidative phosphorylation. The enzyme consists of two associated multi-subunit complexes - the soluble catalytic core (F1) and the membrane-bound component (Fo), forming the proton channel. The catalytic core comprises five different subunits, α , β , γ , δ and ϵ , assembled with a stoichiometry of 3 α , 3 β and a single representative of the other 3 subunits. The F_o proton pore consists of three main subunits A, B, and C, and (in humans) six additional subunits, d, e, f, g, F6, and 8 (or A6L).

Our results from 2D-GE and MS analysis revealed up-regulated β subunit of the H^+ transporting F1 complex of mitochondrial ATP synthase after the treatment of Ist-Mes-2 cells with 25 μ M α -TOS for 24 h. Furthermore, we were able to prove that that the up-regulation of ATP5B occurs at the mRNA level. The mRNA that encodes the catalytic subunit β of the mitochondrial H^+ -ATP synthase (ATP5B, β -F1-ATPase) is localized in a large ribonucleoprotein (RNP) complex (β -F1–RNP), which is subject to strict translational control within the development and the cell cycle as well as in tumourigenesis (Ortega *et al.*, 2010). Remarkably, it has been reported by various studies that the relative cellular expression level of β -F1-ATPase is considerably down-regulated in most prevalent human carcinomas in comparison to normal human tissues (Cuezva *et al.*, 2002, 2004; Isidoro *et al.*, 2005; Lopez-Rios *et al.*, 2007). This leads to a ‘bioenergetic signature’ of the biopsy

with potential clinical applicability as an indicator of cancer progression and answer to treatment (Cuezva *et al.*, 2009; Ortega *et al.*, 2010). Two alternative pathways have been proposed by which cancer cells may downregulate the β -F1-ATPase protein: a general down-regulation of mitochondrial components that is consistent with a suppression of the programme of mitochondrial proliferation as was shown for liver cancer, and a specific down-regulation of the expression of the β -F1-ATPase that is consistent with a selective suppression of the expression of the components involved in mitochondrial bioenergetic function as was shown in kidney and colon carcinomas (Cuezva *et al.*, 2002).

The conserved down-regulation of the β -F1-ATPase described in various human carcinomas is in agreement with the hypothesis, proposed in 1930 by Otto Warburg, that cancer cells may have impaired mitochondrial function and that this alteration would lead to an increased rate of glycolysis that is a common feature of most tumors (Warburg, 1930; Vander Heiden *et al.*, 2009). Although the glycolytic phenotype has been demonstrated at biochemical and molecular levels for a wide range cancer cells and tumours (Dang and Semenza, 1999; Semenza *et al.*, 2001; Ziegler *et al.*, 2001), the proposed impairment of mitochondrial function has not been directly observed in cancer biology (Pedersen, 1978). Up to date, the mitochondrial function in neoplastic transformation and in maintaining or promoting the transformed state remains still underexplored, although there is a clear link (Ralph *et al.*, 2010a, b).

At present, it is not clear by which mechanism α -TOS, which has been classified as a Class V mitocan (an electron redox chain targeting drug), stimulates the expression of the β -F1-ATPase in MM cells and why the cells possibly require to rise their mitochondrial ATP production upon the exposure to the VE analogue. Our findings might be linked to the observation that efficient execution of apoptosis requires the molecular components of the H^+ -ATP synthase (Matsuyama *et al.*, 1998; 2000), in addition to adequate supplies of ATP (Dey and Moraes, 2000; Harris *et al.*, 2000). It is also possible that since α -TOS affects the mitochondrial CII, other components of oxidative phosphorylation are stimulated as an adaptive response to treatment of the cells with the mitocan. This phenomenon is very interesting and is to be examined in a follow-up study.

Some of the other proteins that were identified by 2-D GE and MS analysis as differentially expressed in cells treated with α -TOS, that were not confirmed by subsequent techniques and which might be involved in carcinogenesis and in apoptotic signalling pathways induced by α -TOS are briefly described below.

One of the proteins, which were found to be up-regulated after treatment of Ist-Mes-2 cells with 25 μ M α -TOS for 24 h is the 14-3-3 γ protein. This protein is in humans encoded by the *YWHAG* gene (Autieri and Carbone, 1999; Horie *et al.*, 1999) and belongs to the highly conserved 14-3-3 family of proteins that exert diverse effects on the signal transduction pathways of cells by binding to the phosphoserine-containing proteins in the context of RSXpSXP or RXY/FXpSXP domain (Yaffe *et al.*, 1997). This binding generally leads to modulation of the activity of the binding partner (Jin *et al.*, 2006). Nine different isoforms, designated α , β , γ , δ , ϵ , η , σ , τ and ζ of mammalian 14-3-3 proteins have been described. These 14-3-3 isoforms are encoded by diverse genes, except for α and δ , which are the phosphorylated forms of β and ζ , respectively (Aitken *et al.*, 1995). High levels of the 14-3-3 proteins have been detected in the brain, while low levels have been shown in most mammalian tissues. The *YWHAG* gene has been reported to be highly expressed in brain, skeletal muscle, and heart (Horie *et al.*, 1999). The 14-3-3 proteins can dimerise, associate with the Raf protein and control its kinase activity (Tzivion *et al.*, 1998). The 14-3-3 γ protein can also interact with Protein kinase c. Furthermore, Rad24, a member of the 14-3-3 family of proteins, is involved in the replication checkpoint by controlling the localization of Cdc25 in the fission yeast *Schizosaccharomyces pombe* (Lopez-Girona *et al.*, 1999). These findings indicate that the 14-3-3 proteins may be involved in the survival, differentiation, proliferation, and transformation of cells (Horie *et al.*, 1999). Interestingly, the 14-3-3 proteins also interact with the BH3-only protein Bad and are responsible for the release of Bcl- x_L , which inhibits apoptosis (Zha *et al.*, 1996b).

In our recent paper, we have defined a novel Hippo/Mst1-FoxO1-Noxa signalling pathway, which involves its regulation by 14-3-3 proteins and which controls apoptosis in cancer cells exposed to anti-cancer agents as α -TOS (Valis *et al.*, 2011). Here the 14-3-3 protein forms an inactive complex with the transcription factor FoxO1. Upon exposure to α -TOS, the phosphorylated FoxO1 protein is released from the complex and activates the expression of pro-apoptotic genes. The detailed pathway is described earlier. However, it is

not clear at present, which isoform of 14-3-3 protein is in complex with the FoxO1 protein in untreated cells and why the 14-3-3 protein γ is up-regulated in Ist-Mes-2 cells upon exposure to α -TOS. Regardless, the regulation of 14-3-3 by α -TOS is very interesting and is worth further work.

Vimentin represent another protein, which has been identified as up-regulated after exposure of MM cells to α -TOS. Vimentin belongs to the intermediate filament protein family and is pervasively expressed in normal mesenchymal cells. The protein preserves the integrity of a cell and ensures resistance against stress. Vimentin has been found to be over-expressed in various epithelial cancers, including breast cancer, prostate cancer, lung cancer, tumours of the central nervous system and MM (Satelli and Li, 2011). Vimentin's over-expression in cancer is associated with faster tumour growth, invasion, metastasis and poor prognosis. Thus, the protein represents an attractive potential target for cancer therapy. However, the role of the protein in cancer progression remains relatively unknown and more research is required to determine its specific role. At this stage, we are not able to explain, why higher expression level of vimentin was found in Ist-Mes-2 cells treated with α -TOS. Again, it could be an adaptive response of the cells to the exposure. More research is needed to estimate the role of vimentin in MM cells exposed to the VE analogue and the way how the protein is regulated.

Another interesting protein that has been identified as up-regulated in MM cells exposed to α -TOS by 2-D GE and MS analysis but not by subsequent methodologies is protein disulfid isomerase-related (PDIR) protein 5 (also known as PDIA5). It is a specialized member of the group of protein disulfide isomerases, which comprise a large family of enzymes responsible for catalysing the proper oxidation and folding of newly synthesised membrane and secreted proteins in the luminal space of the endoplasmic reticulum (ER). The PDI proteins are localized in the ER and comprise one or more thioredoxin-like domains, which commonly possess an oxidoreductase activity mediated by a conserved catalytic CxxC motif. Within the catalytic motif, the N-terminal cysteine builds a mixed disulfide with the substrate, whereas the C-terminal cysteine ensures its release. Glutathione is involved in PDI-mediated disulfide bond formation of a protein in the cell (Hayano *et al.*, 1993). Most members of the PDI family comprise additional non-catalytic thioredoxin-like domains lacking the CxxC motif. These domains participate in substrate

or co-chaperone recognition, or function as spacers for positioning the catalytic elements. PDIA5 was found to be involved in the folding of α_1 -antitrypsin and the N-linked glycoproteins (Vinaik *et al.*, 2013). Interestingly, PDI functions also as a β -subunit of prolyl-4-hydroxylase (Pihlajaniemi *et al.*, 1987; Hayano and Kikuchi, 1995), a further protein that has been identified as up-regulated after the treatment of Ist-Mes-2 cells with α -TOS.

One of the proteins, which has been found to be down-regulated after treatment of Ist-Mes-2 cells with α -TOS is glutathione synthetase. It is the second enzyme involved in the glutathione biosynthetic pathway, which catalyses the condensation of γ -glutamylcysteine and glycine to form glutathione (Njålsson and Norgren, 2005). Glutathione is a tripeptide, which comprises glutamate, cysteine and glycine. It is present in most mammalian cells and takes part in fundamental biological functions, including free radical scavenging and maintaining the redox tone in cells (Ristoff and Larsson, 2007). This is interesting from that point of view of α -TOS being an inducer of ROS generation (as described above) which subsequently leads to the activation of the apoptotic pathway. α -TOS mediated down-regulation of glutathione synthetase will result in decreased glutathione levels in the cells and consequently to lower free radical scavenging capacity of the cells, which will be prone to succumb to apoptosis.

Some of the identified proteins, including septin 11 and β -F1-ATPase, which showed an altered expression level in Ist-Mes-2 cells exposed to α -TOS have been reported to be involved in various important cellular processes including apoptosis, cell division and proliferation, carcinogenesis and metastasis. However, the mechanism by which these proteins are regulated and which functions they exert in MM cells upon exposure to the VE analogue is not understood yet and has to be clarified in a follow-up study. The PhD candidate will focus on these investigations in the future research projects with the idea to unravel the role of these proteins, identified via 2-D GE and MS analysis, in tumour progression and how these proteins are regulated by α -TOS and, also MitoVES.

The context of this work is within the potential cure of MM, a thus far fatal disease. Scientifically, it is believed that the acquired outcomes from this work will contribute to the emerging notion that mitocans hold a substantial promise to be developed into efficient

anti-cancer drugs that will be utilised to help curb the increasing incidence of neoplastic diseases, as epitomised here by the extremely hard-to-treat mesothelioma. It needs to be emphasised, too that this work is likely to generate novel intriguing results of fundamental scientific importance.

5 Conclusions

The main aim of this work was the study of the effect of mitocans from the group of VE analogues epitomised by α -tocopheryl succinate (α -TOS) and its mitochondrially targeted derivate MitoVES on MM cells. The term mitocans was recently defined by our group and refers to a group of anti-cancer drugs that selectively induce apoptosis in cancer cells by destabilisation of their mitochondria (Neuzil *et al.*, 2006; Neuzil *et al.*, 2013). Both tested agents were known from previous studies to eradicate selectively various types of cancer cells and to inhibit the growth of many models of cancer *in vivo*, while causing only a marginal toxicity to non-malignant cells. Thus, we decided to test the effect of both mentioned anti-cancer agents on the treatment-resistant, fatal MM. Several human and murine MM cell lines have been used as well as a MM experimental mouse-model. The obtained data for α -TOS and MitoVES were compared and are briefly summarized below:

- It was shown that MitoVES was considerably more efficient in killing MM cells than its parental analogue α -TOS with IC_{50} lower by two orders of magnitude.
- Directed accumulation of MitoVES in mitochondria was documented using its fluorescently labelled analogue and confocal microscopy.
- MitoVES triggered apoptosis in MM cells via destabilisation of mitochondria. This was followed by dissipation of mitochondrial membrane potential and generation of reactive oxygen species.
- The efficacy of MitoVES was reduced when its aliphatic chain spanning the chromanol and TPP^+ groups of the compound was shortened, which led to the idea that its potential target might be complex II of the mitochondrial electron transfer chain.
- The role of complex II in the effect of MitoVES on cancer cells was corroborated by the finding that MM cells with suppressed succinate quinonereductase showed resistance to MitoVES.
- Finally, MitoVES provided a stronger anti-MM activity in an experimental mouse model when compared to α -TOS.

Collectively, the here listed results indicate that mitochondrial targeting of vitamin E succinate endows the compound with significantly higher ability to kill MM cells, bringing it to the proximity of its molecular target. This gives the drug considerable clinical significance. All aims of the first part of the study have been achieved.

The second part of the study focused on the identification of proteins that are up-regulated or down-regulated in MM cells exposed to α -TOS, with the idea of finding potentially important signalling pathways that play a role in apoptosis included by the vitamin E analogue in MM cells. Two-dimensional gel electrophoresis followed by mass spectrometry resulted in the identification of 12 proteins that were up-regulated more than 1.5-fold and 12 proteins down-regulated more than 1.5-fold. Of these, most proteins were not confirmed by subsequent approaches (RT-PCR, qPCR, western blotting). Of those, that were confirmed, septin 11 was the focus of additional studies due to its role in tumourigenesis and a potential role in resistance of tumours to treatment. Since we did not manage to prepare cells with silenced *SEPT11* (due to lack of time and the need to focus on the molecular mechanism of the action of mitocans on MM cells, see above), the functional aspects of down-regulation of septin 11 in response to α -TOS were not ascertained. This work needs to be concluded, and this will be the subject of a future project of our group.

All data obtained in this study contribute to the understanding, how the tested mitocans exert their selective killing effect on MM cells and other cancer cells. In particular, this work characterises the molecular mechanism by which a very efficient and selective mitochondrially targeted vitamin E succinate kills MM cells. This knowledge will be, in particular, utilised in the preparation of clinical tests with MitoVES and mesothelioma patients. Since this is a fatal cancer that is on the rise, this work can be seen as beneficial from the clinical point of view. Also, and very importantly, given the fact that mesothelioma is one of the hardest-to-manage neoplastic diseases, successful use of MitoVES for MM patients will set the ground for its testing in other types of tumour. Therefore, the potential impact of this work goes beyond malignant mesothelioma and has a wider implication.

6 References

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Appendix A – Material

Material	Supplier
<i>Cell culture</i>	
Antibiotic/Antimycotic Solution (100x)	Sigma-Aldrich®
Cell culture flasks (T75, T150) and cell culture plates (12-well, 96-well)	Techno Plastic Products AG (TPP)
DMEM	Gibco® or Lonza
Foetal Bovine Serum (FBS)	Gibco® or Bovagen
Phosphate buffered saline (PBS)	Institute of Molecular Genetics (IMG) AS media store
RPMI 1640	Gibco® or Lonza
Trypsin/EDTA	IMG media store
<i>VE analogues</i>	
α -Tocopheryl succinate (α -TOS)	Sigma-Aldrich®
<i>Cell viability assay</i>	
Crystal violet	Sigma-Aldrich®
Paraformaldehyde	Fluka
<i>Apoptosis and necrosis assay</i>	
Annexin V-binding buffer 10x	BD Biosciences
Annexin V-FITC	BD Biosciences
Propidium Iodide (PI)	Sigma-Aldrich®
<i>ROS generation</i>	
2',7'-Dichlorodihydrofluorescein diacetate (DCF-DA)	Sigma-Aldrich®
Dihydroethidium (DHE)	Sigma-Aldrich®
MitoSOX™ Red reagent	Sigma-Aldrich®
<i>Assessment of mitochondrial membrane potential</i>	
Carbonyl cyanide 4-(trifluoromethoxy)phenyl-hydrazone (CCCP)	Sigma-Aldrich®
TMRM	Sigma-Aldrich®
<i>Confocal microscopy</i>	
CCCP	Sigma-Aldrich®
Hoechst 33342	Fluka
TMRM	Life Technologies

SQR activity	
ATP	Sigma-Aldrich®
DCPIP	Sigma-Aldrich®
Decylubiquinone	Sigma-Aldrich®
Rotenone	Sigma-Aldrich®
Succinate	Sigma-Aldrich®
Isolation and Solubilisation of mitochondria	
Aminocaproic acid	Sigma-Aldrich®
Bis-Tris	Sigma-Aldrich®
Digitonin	Sigma-Aldrich®
Protease Inhibitor Cocktail	Sigma-Aldrich®
Sucrose	Sigma-Aldrich®
NBE and Hr-CNE	
Deoxycholate	Sigma-Aldrich®
Dodecyl maltosid	Sigma-Aldrich®
NADH	Sigma-Aldrich®
NativePAGE™ Novex® 3-12% Bis-Tris Gels	Life Technologies
NativePAGE™ Novex® 4-16% Bis-Tris Gels	Life Technologies
NTB	Sigma-Aldrich®
Low fat milk	Sigma-Aldrich®
Phenazine methosulfate	Sigma-Aldrich®
PVDF (polyvinylidene difluoride) membranes	GE Healthcare
Serva Blue G-250	Serva
Sodium succinate	Sigma-Aldrich®
Tricine	Sigma-Aldrich®
Preparation of SDHC-deficient cells	
Fugene HD transfection reagent	Active Motif
Puromycin	Sigma-Aldrich®
SureSilencing human SDHC shRNA or NS plasmid	SuperArray Biosciences
Mitochondrial Respiration and CII activity	
ADP	Sigma-Aldrich®
Antimycin A	Sigma-Aldrich®
BSA	Sigma-Aldrich®
Cytochrome c	Sigma-Aldrich®

Digitonin	Sigma-Aldrich [®]
Ethylene glycol tetraacetic acid (EGTA)	Sigma-Aldrich [®]
FCCP	Sigma-Aldrich [®]
Glutamate	Sigma-Aldrich [®]
HEPES	Sigma-Aldrich [®]
KH ₂ PO ₄	Merck
K-lactobionate	Sigma-Aldrich [®]
Malate	Sigma-Aldrich [®]
Malonic acid	Sigma-Aldrich [®]
MgCl ₂ x 6H ₂ O	Scharlau
Oligomycin	Sigma-Aldrich [®]
Rotenone	Sigma-Aldrich [®]
Succinate	Sigma-Aldrich [®]
Succrose	Roth
Taurine	Sigma-Aldrich [®]
<i>Determination of protein content</i>	
BCA Protein Assay kit	Thermo Scientific
EZQ Protein Quantitation Kit	Molecular Probes
Pierce Coomassie Plus Protein Assay Reagent	Thermo Scientific
<i>Animal experiments</i>	
Balb-c <i>nu/nu</i> mice	Jackson Laboratories
Corn oil	Sigma-Aldrich [®]
<i>2-D Gelelectrophoresis (2-D GE) and MS analysis</i>	
Acetonitrile (= MeCN)	Sigma-Aldrich [®]
α -Cyano-4-hydroxycinnamic acid (CHCA)	ProteoChem
Agarose Type I	Sigma-Aldrich [®]
Benzonase [®] Nuclease (25 U/ μ l)	Novagen
Bromphenol Blue	Sigma-Aldrich [®]
CHAPS	GE Healthcare Life Sciences
DL-dithiothreitol (DTT)	Sigma-Aldrich [®]
4-ethylmorpholine acetate	Sigma-Aldrich [®]
Immobiline dry strip cover fluid	GE Healthcare Life Sciences
Iodoacetamide (IAA)	Sigma-Aldrich [®]
18 cm IPG strips (pH 4-7, non-linear)	GE Healthcare Life Sciences

Novex™ Colloidal Blue Stain	Invitrogen
Pharmalyte™, broad range pH 3-10	GE Healthcare Life Sciences
Plus One SDS	GE Healthcare Life Sciences
Plus One urea	GE Healthcare Life Sciences
Protease inhibitor mix (100x solution)	GE Healthcare Life Sciences
Sequencing grade modified trypsin	Promega
Thiourea	GE Healthcare Life Sciences
Trifluoroacetic acid	Thermo Scientific
Omix C18 Zip Tips (10 µl)	Varian
Zeba spin desalt columns (0.5 ml)	Thermo Scientific
RNA isolation	
Aurum Total RNA mini kit	BioRad
Chloroform	Penta
RNeasy mini kit	Quiagen
TRIzol reagent	Invitrogen
Reverse trascription	
ProtoScript first strand synthesis kit	New England Biolabs
RevertAid cDNA first strand synthesis kit	Fermentas
RT-PCR	
PCR master mix	Promega
qPCR	
SYBRGreen Jump Start Taq Ready Mix	Sigma
Agarose-gelelectrophoresis	
Agarose type I	Sigma-Aldrich®
6x Gel loading dye, blue	New England Biolabs
Gel red dye	Biotinum
Western Blot (WB)	
Nonidet P 40 (NP-40)	Fluka
Phosphatase inhibitors (20x)	Active Motif
Protease inhibitor mix (100x solution)	GE Healthcare Life Sciences
Precision Plus Protein™ standards – All Blue	BioRad
Sodium deoxycholate	Fluka
SuperSignal® West Femto Maximum Sensitivity Substrate	Thermo Scientific
SuperSignal® West Pico Chemiluminescent Substrate	Thermo Scientific

<i>SDS PAGE gels (for 2-D GE and WB)</i>	
Acrylamid/bisacrylamid, 29:1 (3.3% C)	BioRad
Ammonium persulfate (APS)	GE Healthcare Life Sciences
N,N,N',N'-tetramethylethylenediamine (TEMED)	Fluka
Non-fat dry milk bovine	Sigma-Aldrich®
<i>generally used chemicals</i>	
Acetic acid	Penta
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich®
EDTA	Sigma-Aldrich®
Ethyl alcohol (EtOH)	LachNer
Ethyl alcohol for UV spectroscopy	Penta
Glycerol	Sigma-Aldrich®
Glycine	Serva
Hydrochloric acid	Fluka
2-mercaptoethanol	Fluka
Methanol (MeOH)	Penta
Potassium hydroxide	Chemapol, LACHEMA
Sodium chloride	Serva
Sodium dodecyl sulphate (SDS)	Sigma-Aldrich®
Tris(hydroxymethyl)aminoethan (Tris)	Sigma-Aldrich®

Appendix B – Buffers, solutions and media

SQR activity assay:

SQR assay buffer:

10 mM KH_2PO_4 , pH 7.8, 2 mM EDTA, 1 mg/ml BSA

Mitochondrial respiration:

Mir05 medium:

0.5 mM EGTA, 3 mM $\text{MgCl}_2 \times 6\text{H}_2\text{O}$, 60 mM K-lactobionate, 20 mM taurine, 10 mM KH_2PO_4 , 20 mM HEPES, 110 mM sucrose, 1 g/l BSA. Adjust the pH to 7.1 (5 N KOH) at 30°C, aliquote and store at -20°C.

Buffers for isolation of mitochondria:

Sucrose-Tris Buffer (STE):

250 mM sucrose, 10 mM Tris, 1 mM EDTA. Adjust the pH at 7.0. Add 1% protease inhibitor cocktail (100 μl PIC/10 ml STE). Store at - 20 °C.

Buffers for solubilisation of mitochondria:

Extraction Buffer (EB):

1.5 M aminocaproic acid (131.15 g/mol), 50 mM Bis-Tris (209.24 g/mol), 0.5 M EDTA. Adjust pH to 7.0, store at 4°C.

Buffers for BNE:

BNE Sample buffer:

0.75 M aminocaproic acid (131.18 g/mol), 50 mM Bis-Tris (209.24 g/mol), 0.5 M EDTA .

Adjust the pH at 7. 5% w/v. Serva Blue G-250 - add glycerol at 12% final concentration.

Cathode Buffer:

15 mM Bis-Tris (209.24 g/mol), 50 mM Tricine (179.17 g/mol). Adjust the pH at 7 and store at 4°C.

Blue Cathode buffer:

15 mM Bis-Tris (209.24 g/mol), 50 mM Tricine (179.17 g/mol), 1 l – 0.02% Serva Blue G-250 0.2 g. Filter and adjust the pH at 7. Store at 4°C.

Anode Buffer:

50 mM Bis-Tris (209.24 g/mol). Adjust the pH at 7. Store at 4°C.

Buffers for Hr-CNE:

Hr-CNE Sample loading buffer: (same as for BNE)

Hr-CNE-3 Cathode Buffer:

Cathode buffer (BN page protocol) + 0.05% deoxycholate + 0.01 % dodecyl maltosid.

Anode buffer: (same as for BNE)

Buffers for semi-wet transfer immunoblotting:

25 mM Tris (121.4 g/mol), 25 mM glycine (75.1 g/mol), MeOH, pH 9.2. Store at 4°C.

Buffers for in-gel catalytic activity assay for CI and CII:

Fixation solution:

50% MeOH, 10% acetic acid.

Activity assay buffer:

5 mM Tris/HCl, pH 7.4.

NADH:NTB reductase assay buffer:

25 mg NTB and 100 μ l of NADH (10 mg/ml) add to 10 ml of the *Activity assay buffer*.

Succinate:NTB reductase assay buffer:

25 mg NTB, 200 μ l sodium succinate (1 M), 8 μ l phenazine methosulfate (250 mM dissolved in DMSO) add to 10 ml of the *Activity assay buffer*.

Buffers used for 2-D GE:

Lysis Buffer:

7 M urea, 2 M thiourea, 4% CHAPS.

Rehydration buffer:

Lysis buffer, 60.6 mM DTT, 1% ampholytes pH 3-10, 0.002% bromphenol blue

Solutions used for isoelectric focusing (1st dimension):

15 mM DTT

Solutions used for IPG strip Equilibration prior to SDS-PAGE:

- *Stock equilibration solution:*

50 mM Tris (pH 6.8), 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, H₂O. Filter and store at room temperature.

- *Equilibration solution 1 (+DTT):*

2% DTT, stock equilibration solution

- *Equilibration solution 2 (+IAA):*

2.5 % IAA (0.5 g), Stock equilibration solution (17.4 ml), H₂O (2.6 ml), few grains of bromphenol blue, total volume of 20 ml.

Solutions used for SDS-PAGE (2nd dimension):

- *Acrylamid/bisacrylamid (30% T/2.67% C):*

29.2% acrylamid, 0.8% bisacrylamid, H₂O. Filter, aliquote and store at -80°C.

- *5x running buffer for SDS-PAGE:*

125 mM Tris, 960 mM glycine, 5% SDS, H₂O.

- *Separating gel for SDS-PAGE (10%):*

375 mM Tris-HCl, pH 8.8, 0.1% SDS, 10% acrylamid/bisacrylamid, H₂O. Mix and allow to degas for 30 min, then add 0.05% TEMED and 0.05% ammonium persulfate.

The solution for separating gel is filled between two glass plates cca 2 cm under the top of the glass. Allow to polymerise, then add the solution for the stacking gel (cca 1.5 ml/gel). Overlay with 70% EtOH and allow to polymerise for cca 45 min. Wash with H₂O before use.

- *Stacking gel for SDS-PAGE (4%):*

125 mM Tris-HCl, pH 6.8, 0.1% SDS, 4% acrylamid/bisacrylamid, H₂O. Mix and allow to degas for 15 min, then add 0.1% TEMED and 0.05% ammonium persulfate.

Solutions used to visualise separated proteins:

- *Fixation solution:*

10% acetic acid, 50% MeOH, H₂O.

- *NovexTM Colloidal Blue Staining Kit (Invitrogen):*

Use according to the manufacturer's instructions.

Solutions used for protein identification:

- *Cleavage buffer:*

50 mM 4-ethylmorpholine acetate, 10% acetonitrile, and sequencing grade trypsin (50 ng/μl, Promega).

Buffers used for Western Blotting:

RIPA (Radio-immunoprecipitation assay) lysis buffer:

50 mM Tris (pH 8), 150 mM NaCl, 1% NP-40, 0.1% SDS, 1 mM EDTA, 0.5% (w/v) sodium deoxycholate. Fill up with deionised water. Adjust pH to 7.5, aliquote and store at -20°C.

Sample loading buffer:

8% SDS (w/v); 40% glycerol (v/v); 20% 2-mercaptoethanol (v/v); 0.04% bromphenol blue (w/v); 0.25 M Tris-HCl, pH 6.8. Store at -20°C.

Running buffer (10 x conc.): (for 1 l)

30 g Tris base, 144 g glycine, 100 ml 20% SDS. Bring to 1 l with water. Store at 4°C.

Transfer Buffer: (for 1 l)

3.03 g Tris base, 14.4 g glycine, add to 800 ml with water, mix and add 200 ml MeOH. Store at 4°C.

Separating gel for SDS-PAGE (10%):

375 mM Tris-HCl, pH 8.8, 0.1% SDS, 10% acrylamid/bisacrylamid, 29:1 (3.3% C), water. Mix and allow to degas for 30 min, then add 0.05% TEMED and 0.05% ammoniumper-sulphate.

Stacking gel for SDS-PAGE (4%):

125 mM Tris-HCl, pH 6.8, 0.1% SDS, 4% acrylamid/bisacrylamid, 29:1 (3.3% C), H₂O. Mix and allow to degas for 15 min, then add 0.1% TEMED and 0.05% ammoniumper-sulphate. The solution for the separating gel is filled between two glass plates and overlayed with 70% EtOH. Allow to polymerise, wash with water, then add solution for the stacking gel, insert the comb and allow to polymerise. Wash with water before use.

Buffers used for agarose-gel electrophoresis:

Tris/Acetate/EDTA (TAE) buffer (50x concentrated):

242 g Tris base, 57.1 ml glacial acetic acid, 37.2 g Na₂EDTA·2H₂O, add water to 1 l.

2% Agarose gel:

Dissolve 2 g agarose in 100 ml TAE buffer. Boil for a while using a microwave, add 1:10,000 Gel Red (10.000x concentrated) (Biotinum). Fill into the gel chamber and insert the sample comb. Allow to polymerise and remove the comb before pipetting the DNA samples in the gel sockets.

Appendix C – Antibodies, Primers

Table C.1: List of Antibodies

Antibody	Supplier
Anti-NDUFS1 (79 kDa)	Abcam
Anti-SDHA (70 kDa)	Abcam
Anti-UQCRC2 (48 kDa)	Abcam
Anti-MTCO2 (26 kDa)	Abcam
Anti-ATP5A (60 kDa)	Abcam
Anti-VDAC IgG	Abcam
Septin 11 (N-12) (49.4 kDa)	Santa Cruz Biotechnology
Actin (I-19) (43 kDa)	Santa Cruz Biotechnology
Anti-ATP5B (57 kDa)	Sigma-Aldrich®
Anti-goat IgG secondary antibody	Sigma-Aldrich®
Anti-rabbit IgG secondary antibody	Sigma-Aldrich®
Anti-SDHC IgG	Santa Cruz Biotechnology
β-Actin (13E5) Rabbit mAb (HRP Conjugate)	Cell Signalling

Table C.2: List of Primers.

The primers were ordered from Metabion International.

All primer sequences are shown in the 5' → 3' direction.

Gene	Forward	Reverse
Primers used for RT-PCR		
AldDeh	ATGAGTTTGTGGAGCGGAGC	CTCTCCCAACAACCTCCTCT
ARP3	TAGCAGCACGGAGCAGACGG	CCATCTCCACTGTCTATTACCG
ATP5B	GAGGTGTCTGCATTATTGGG	CGTAATGCTCACTGCCAACA
GlutSynth	GGCTGAGGGAGTATTGCTGA	AGCTGGCAGAGATGGTGTTG
HSPA8	GCCTACACCCCAGCAACCAT	AGACCAGCAATAGTTCCAGC
Mortalin	GCCGTCTGGAGCGCTTGTTTG	ATCGCCGGCCAATGAGACGC
OAS	TACCGTGGCCACGTTCCAGG	GCTGTGATGTTGAACTCATCA
P4H	GCTGGTGGAGTTCTATGCCCCCT	GTCAGGCAGGGTGGTGGCAG
PDIR	CTGGCAATCTGGGTGGTCCTG	TTCGGGCTCAGGTCAACTTTCA
P0	TCGACAATGGCAGCATCTAC	ATCCGTCTCCACAGACAAGG
RuvB-like1	GAAGAGCACTACGAAGACGC	GTCTCCTTTATTTCGCAGCCC
RuvB-like 2	CATCAAGGAGGAGACGGAGA	TGGGTGCGAGAGTTGATGAC
Septin 11	GTTGGTGAGACAGGCATTGG	GGACCCCATGCTGACCAGT
TBP-7	CAGGACGAGGTGAAGCGAAT	ATCTGGCTTCTGGTCTGAGG
Vimentin	AAGAACACCCGCACCAACGA	TCAAGGTCAAGACGTGCCAG
14-3-3 γ	CTTTGCCGCTTTTGTGCCTC	AAGTCTCCCATTCCCTCTCC
Primers used for qPCR		
P0	TCGACAATGGCAGCATCTAC	ATCCGTCTCCACAGACAAGG
Septin 11	GAGCCGGAGCCCGAGCACTA	CCAGCTGGTCAGGGAGGCTG

Appendix D - Structures of VE analogues

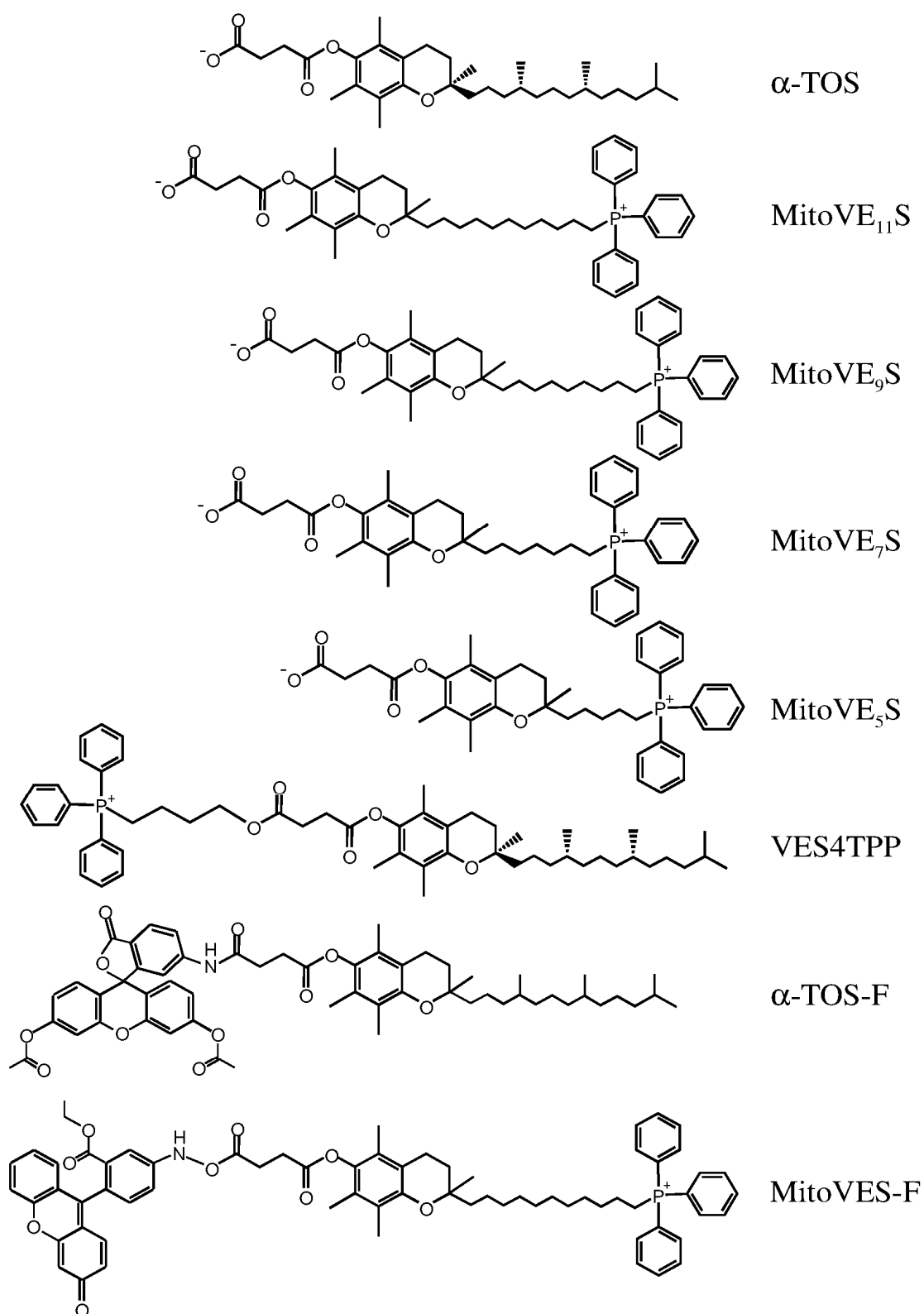


Figure D.1: Structures of compounds used in this study.