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Mitochondria - a target for anticancer therapy

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Stockholm 2014

Published by Karolinska Institutet. Printed by US-AB universitetsservice, Karolinska Institutet.

ISBN 978-91-7549-766-2

Für meine Familie

Abstract

Mitochondria possess a central role in several cellular metabolic pathways, maintenance of calcium homeostasis, production of reactive oxygen species (ROS) and in the regulation of various cell death modalities. A majority of cancers demonstrate aberrations in mitochondrial functions, which were shown to contribute to tumourigenesis. In addition, many mechanisms of chemotherapy-resistance are located upstream of the mitochondria in cell death pathways. Thus, destabilization of mitochondria and permeabilization of the outer mitochondrial membrane (OMM), a point of no return in apoptosis induction, represent promising strategies for anticancer therapy.

One major aim of this thesis was to identify therapeutic approaches to overcome resistance of cancer cells to conventional chemotherapeutic drugs. We could show in **Paper I** that chemotherapy resistance mechanisms in cancers, mediated by various oncogenic signalling/mutations, could be overcome by targeting Complex II of the mitochondrial respiratory chain. Treatment of Neuroblastoma (NB) cells with α-tocopheryl succinate (α-TOS), a redox-silent analogue of vitamin-E, which was shown to target Complex II, mediate ROS-production and an increase of cytosolic calcium levels, could induce apoptosis in cancers cells irrespective of their MycN or p53 status. We propose that this is based on the ability of α-TOS to induce both mechanisms of OMM permeabilization, in a Bax/Bak-dependent manner, as well as calcium-dependent induction of mitochondrial permeability transition (MPT).

In **Paper II and III** we investigated the possibility of sensitizing cancer cells to conventional anticancer drugs in a co-treatment setting with compounds targeting Complex II. In case of α-TOS **(Paper II)**, the obtained results revealed contrasting effects for the chemotherapeutic drugs etoposide and cisplatin. In case of etoposide, α-TOS was able to sensitize cancer cells in a dose-dependent manner. Whereas strikingly, in case of cisplatin, low concentration of α-TOS protected cells from cisplatin-induced toxicity. We demonstrated that the succinate moiety of α-TOS is mediating this protective effect via stimulation of Complex II activity. However, when Complex II was inhibited using thenoyltrifluoroacetone (TTFA) **(Paper III)**, a specific inhibitor of the ubiquinone binding site of Complex II, cells could be sensitized to both, etoposide- and cisplatin-induced cytotoxicity. This chemosensitizing effect was shown to rely on Complex II-mediated ROS-production.

For the study that was concluded in **Paper IV**, a different approach was utilized. Citrate, a substrate of the tricarboxylic acid cycle, was shown to induce cytotoxicity in cells. The underlying mechanism was speculated to be based on citrate's inhibitory effect on several crucial glycolytic enzymes and its ability to chelate calcium. We could demonstrate that although these features contribute, the main cause of cell death induced by citrate is the activation of initiator caspases. The underlying mechanism was proposed to be the kosmotropic property of citrate.

In summary, the findings of this PhD thesis clearly underline the potency of exploiting mitochondria for anticancer therapy. Particularly Complex II plays an intriguing role in the sensitivity towards chemotherapy and represents an attractive target that should be further explored in future projects. In addition, new roles of well-known mitochondrial substrates were revealed.

List of publications

I. Kruspig B, Nilchian A, Bejarano I, Orrenius S, Zhivotovsky B, Gogvadze V. **Targeting mitochondria by α-tocopheryl succinate kills neuroblastoma cells irrespective of MycN oncogene expression.** *Cell Mol Life Sci. 2012 Jun;69(12):2091-9.*

II. Kruspig B, Zhivotovsky B, Gogvadze V. **Contrasting effects of α-tocopheryl succinate on cisplatin- and etoposide- induced apoptosis.** *Mitochondrion. 2013 Sep;13(5):533-8.*

III. Kruspig B, Skender B, Zhivotovsky B, Gogvadze V. **Inhibition of Mitochondrial Complex II by Thenoyltrifluoroacetone Potentiates the Efficacy of Anticancer Therapies.** *Manuscript*

IV. Kruspig B, Nilchian A, Orrenius S, Zhivotovsky B, Gogvadze V. **Citrate kills tumor cells through activation of apical caspases.** *Cell Mol Life Sci. 2012 Dec;69(24):4229-37.*

Additional publications (not included in the thesis)

I. Kruspig B, Zhivotovsky B, Gogvadze V. **Mitochondrial substrates in cancer: Drivers or passengers?** *Mitochondrion. 2014 Nov;19(A):8-19.*

II. Rasmuson A, Kock A, Fuskevåg OM, Kruspig B, Simón-Santamaría J, Gogvadze V, Johnsen JI, Kogner P, Sveinbjörnsson B., **Autocrine prostaglandin E2 signaling promotes tumor cell survival and proliferation in childhood neuroblastoma.** *PLoS One. 2012;7(1):e29331.*

Table of contents

List of abbreviations

3-NPA	3-nitropropionic acid
2DG	2-deoxy-D-glucose
ACLY	ATP-citrate lyase
AIF	Apoptosis-inducing factor
ANT	Adenine nucleotide translocase
Apaf-1	Apoptotic protease activating factor-1
ATP	Adenosine tri-phosphate
Bad	Bcl-2 associated death promoter
Bak	Bcl-2 homologous antagonist/killer
Bax	Bcl-2 associated X protein
Bcl-2	B-cell lymphoma 2
Bcl-X_L	Bcl-2 related gene, long isoform
BH	Bcl-2 homology
Bid	BH3- interacting-domain death agonist
Bim	Bcl-2 interacting mediator of cell death
BITC	Benzyl isothiocyanate
Caspase	Cysteine-dependent aspartate-specific protease
CIC	Citrate carrier
CL	Cardiolipin
dATP	Deoxy-adenosine tri-phosphate
DCA	Dichloroacetate
DIM	3,30-diindolylmethane
DISC	Death inducing signalling complex
DR	Death receptor
EndoG	Endonuclease G
ER	Endoplasmic reticulum
ETC	Electron transport chain
FADD	Fas-associated death domain
GABA	γ-aminobutyric acid
HDAC	Histone deacetylase
Hif	Hypoxia-inducible factor
HK	Hexokinase
HSP	Heat shock protein
IAP	Inhibitor of apoptosis
IMM	Inner mitochondrial membrane
LDH	Lactate dehydrogenase
Mcl-1	Myeloid cell leukaemia sequence 1
MOMP	Mitochondrial outer membrane permeabilization
MPT	Mitochondrial permeability transition
mTOR	Mammalian target of rapamycin
NB	Neuroblastoma
OMM	Outer mitochondrial membrane
OXPHOS	Oxidative phosphorylation
PARP	Poly(ADP-ribose) polymerase
PBR	Peripheral benzodiazepine receptor

PDH	Pyruvate dehydrogenase
PET	Positron emission tomography
PFK	Phosphofructokinase
PHD	Prolyl hydroxylase domain
PI3K	Phosphoinositide 3-kinase
Rb	Retinoblastoma
ROS	Reactive oxygen species
SDH	Succinate dehydrogenase
SQR	Succinate:ubiquinone oxidoreductase
TCA	Tricarboxylic acid cycle
TNF	Tumour necrosis factor
TNFR	Tumour necrosis factor receptor
TTFA	Thenoyltrifluoroacetone
UbQ	Ubiquinone
VDAC	Voltage-dependent anion channel
αKG	α-ketoglutarate
α-TOS	α-tocopheryl succinate

1. General introduction

Cancer

According to GLOBOCAN, a project of the International Agency for Research on Cancer and the World Health Organization, in 2012 there were worldwide 14.1 Million new cases of cancer and 8.2 Million cancer-related deaths. These numbers strikingly demonstrate that cancer is one of the leading causes of death in the world, with an increasing prevalence. Cancer is a generic term for a big variety of diseases, which generally are named after the tissue of origin; alternative names include tumour malignancy or neoplasm. Tumours may arise from almost any cell type of the body and display very heterogeneous phenotypes.

The development of cancer, also called tumourigenesis or carcinogenesis, is classically viewed as a multistep process, developing over a longer period of time and based on the development of several different genetic aberrations in the pre-malignant cell[1]. The process involves activation of oncogenes, such as *Myc* or *Ras*, and inactivation of tumour-suppressor genes like *p53* or the Retinoblastoma protein (pRb).

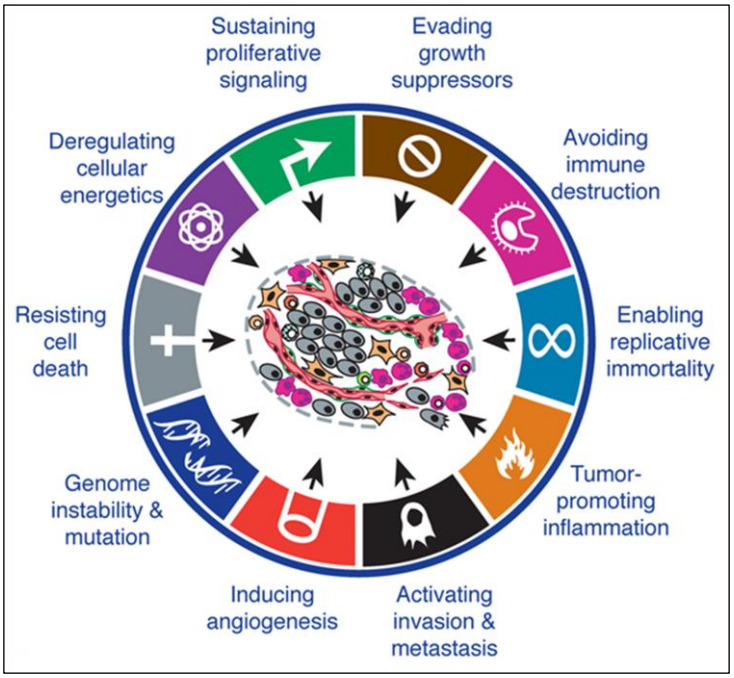

Fig. 1: Hallmarks of Cancer[2] (reprinted and adapted with permission from Elsevier BV).

Despite the big variety of cancers, common features exist (see Fig. 1) and comprise uncontrolled cell proliferation, increased vascularization

(angiogenesis), spreading over the adjacent tissue and formation of metastasis in other parts of the body[2]. Two of the so called “Hallmarks of Cancer”, resisting cell death and deregulation of cellular energetics, will be discussed in depth in separate sections of this thesis. The main cause for cancer-related deaths is the development of metastasis, due to interfering with the physiological functions of the tumour bearing tissue or organ.

In contrast to adults, where cancer can be viewed as an age-related disease, which is caused spontaneously or due to exposure to different type of risk factors, the development of cancer in children is greatly different. In adults cancers develop mainly from mature tissues, whereas childhood tumours arise from defective development and have a much shorter latency than adult malignancies[3, 4]. According to the current view of the process of embryonic tumourigenesis, each embryonic malignancy shares at least three different common features: an excess of prenatal proliferation, evasion of cell death under the hostile early postnatal development and an increased rate of genomic instability[5].

Neuroblastoma

Neuroblastoma (NB) is an embryonic malignancy of the sympathetic nervous system. It is the most common extracranial solid tumour in children and the overall most common cancer in infants younger than one year of age. NB accounts for approximately 7% of all cancer-related deaths in children. In Sweden NB has a mean annual incidence rate of 0.7 cases/100000 children younger than 15 years of age. Interestingly, more boys are affected than girls with a male/female ratio of 1.22. The highest incidence rate have children in their first year of life and it is decreasing thereafter with age. The age of the child at diagnosis is also the most significant prognostic marker, as infants with less than one year of age have a survival rate of 80.8% whereas patients with the age of 5-9 years of age have a probability of survival of 50.0%. Survival rates have been increasing significantly for children diagnosed in the recent past, most pronounced after 2001, with a probability to survive of 77% for children diagnosed between 2006-2010 *vs*. 59% in the time period of 1996-2000[6]. This positive development is mainly due to improved diagnosis and therapeutic approaches, as well as a better understanding of the underlying mechanisms for NB development. Approximately 1-2% of NBs have a

hereditary background, mainly caused by germline mutations in the paired-like homeobox 2B gene (PHOX2B)[7] or the anaplastic lymphoma kinase (ALK) gene[8, 9].

NB arises from neural crest cells during embryonic development and is a result of aberrant sympathetic nervous system development. Neural crest cells are pluripotent sympathetic neural pre-cursors, originating from the ectoderm and forming the top of the neural tube, which will develop into the spinal cord. The neural crest itself is a transitory structure, whereas the neural crest cells persist. They lose their epithelial attachment, migrate throughout the whole body, and give rise to most of the cells of the peripheral nervous system, Schwann cells, the enteric nervous system and adrenal medullary cells[10, 11].

Primary tumours can be found anywhere along the sympathetic nervous system, with a majority in the adrenal medulla. Tumours close to the spinal column may expand and cause compression of the spinal cord, with a potential risk of paralysis. Lower-stage NBs often regress spontaneously or are getting encapsulated and can be surgically removed without further complications. Aggressive higher-stage tumours often infiltrate local organ structures, thereby surrounding vesicles and nerves, making it very difficult for the surgeon to remove the malignant tissue. Via the hematopoietic system, NB typically metastasises to the surrounding lymph nodes or the bone marrow. But metastasis can also be found in the liver[12].

According to the International Neuroblastoma Risk Group staging system, localized tumours are divided into two groups, L1 or L2, depending on the presence or absence of image-defined surgical risk factors. Further criteria are the existence of metastasis (group M) and if the patient is younger than 18 months of age at diagnosis (group MS)[13, 14].

Four broad risk groups were defined in terms of the 5-year event-free survival rates: very low risk (>85%), low risk (>75 - ≤85%,), intermediate risk (≥50 - ≤75%), and high risk (<50). These groups were based on age at diagnosis, the INRG tumour stage, histologic category, grade of tumour differentiation, DNA ploidy, and copy-number status of the *MycN* oncogene as well as of chromosome 11q.

Amplification of the *MycN* oncogene characterizes a subset of 25-30% of NBs and correlates with a highly aggressive phenotype and poor prognosis in patients[15-17]. MycN belongs to the Myc family of transcription factors that play a

key role in the regulation of a variety of cellular processes, such as cell proliferation, differentiation and apoptosis. The role of MycN in the treatment of NB is not fully clear as reports utilizing cellular models were showing that an overexpression of *MycN* causes a marked sensitization of NB cells towards anticancer therapy[18, 19]. These findings are in contrast with the clinical features of *MycN* amplified tumours, which very frequently relapse after chemotherapy. Another chromosomal aberration associated with bad prognosis is the deletion of chromosome 11q locus[20]. In contrast, the second group of DNA-copy aberrations, whole-chromosome gains that result in hyperdiploidy, are associated with a favourable outcome for the patient[21].

The big heterogeneity of NB also results in different therapeutic strategies, mainly depending on the risk category of the tumour. Some tumours regress or differentiate without any treatment, whereas others do not respond to intensive multimodal therapeutic approaches. There has been a trend to reduce the intensity for low-risk tumours with a favourable prognosis, in order to lower the risk of severe side-effects. Therefore, low-risk localized tumours may be surgically removed without any other adjuvant treatment. Intermediate-risk malignancies with extension in to local lymph nodes and/or metastases into the bone marrow additionally receive a moderate intense chemotherapy. In contrast, high-risk NBs with metastases to the bone-marrow/bone and segmental chromosomal aberrations are treated with a combination of intensive chemotherapy, surgery, radiotherapy and stem cell rescue, proceeded by maintenance therapies such as retinoic acid and immunotherapy with antibodies against disialganglioside 2 (GD2). The majority of high-risk tumours firstly respond to treatment but relapse in many cases and develop resistance mechanisms against therapy[22, 23].

Cell Death

Historical context

Cell death was reported and described as early as 1842 by Carl Vogt and has been studied by several other scientists during the second half of the 19th century. Although these histologists described already typical morphological features of several modes of cell death, at that time the process was regarded as an accident and scientists did not realize its importance for biology or the underlying mechanisms[24]. Only with time researchers, in particular

developmental biologists, started to understand that cell death is not a passive but might be also a well-controlled process with crucial importance for cellular homeostasis in the organism. The term programmed cell death was first introduced by the work of Lockshin and Williams, who discovered that the death of muscle cells in insects is dependent on hormonal regulation and preceded by activation of lysosomes, thus represents a biologically controlled process[25]. In 1972, John Kerr, Alastair Currie and Andrew Wyllie published a seminal paper, in which the terminology and phenotype of the before poorly described process of programmed cell deletion was further specified and named apoptosis. As indicated in a footnote in the original paper, the term apoptosis is ancient Greek, describing the falling of petals from flowers or leaves from trees, and was suggested by Professor James Cormack from the University of Aberdeen[26]. The authors divided the morphological changes accompanied with apoptosis into two main stages, cytoplasmic shrinkage and nuclear fragmentation, with the formation of so-called apoptotic bodies, which then undergo the uptake and engulfment by macrophages. Furthermore, it was proposed that both, physiological elimination of normal cells, as well as pathological removal of potentially harmful cells, such as cancer cells, are mediated by this mechanism. Necrotic cell death, in contrast to apoptosis, represented a more passive mode of cell death, characterized by swelling and rupture of the dying cells, with leakage of intracellular content into the extracellular space, ultimately leading to an immune response.

The underlying mechanisms of apoptosis were since then intensively studied, in particular after the realization that evasion of apoptosis is one of the hallmarks of cancer. *Caenorhabditis elegans*, a transparent 1mm long nematode was the preferred model system, in which the majority of apoptosis research was conducted. In 2002 the work of Sydney Brenner, Robert Horvitz and John Sulston on the genetic regulation of organ development and programmed cell death was awarded the Nobel Prize in Physiology or Medicine[27]. Based on their work on the development of this nematode, today it is clear that also in humans the cell population is highly regulated by interplay of cell division, differentiation and cell death. In particular the finding by the group of Robert Horvitz, that one of the identified apoptosis-related genes, *ced-3*, is coding for a cysteine protease (later on termed caspase) and was conserved from *C. elegans* to mammals[28, 29]. These findings kindled big

interest for this field and resulted in numerous following studies, exploring various aspects of apoptosis. Over time several other modes of cell death were discovered and are in depth described by the recommendation of the Nomenclature Committee on Cell Death[30, 31].

Mechanisms of apoptosis

As mentioned earlier, apoptosis is a genetically controlled and evolutionary conserved mode of cell death, which can be triggered by various intra- or extra cellular stimuli. Apoptotic cell death is involved in various physiological and pathological processes, such as embryonic development, maturation of the immune system and tissue homeostasis, as well as part of a defence mechanism to remove potentially dangerous cells. The dying cell undergoes characteristic structural and biochemical changes. Morphological changes include cell shrinkage, nuclear condensation and cell membrane blebbing. Characteristically, apoptosis requires RNA- and protein synthesis[32], as well as the presence of deoxyadenosine-5-triphosphate (dATP)[33]. Cell death by apoptosis can be triggered by an extrinsic, receptor-mediated, or intrinsic, mitochondria-mediated pathway. In both distinct pathways a defined signalling cascade leads to the activation of proteolytic activity and ultimately apoptotic cell death. Depending on the type of stimulus, a distinct pathway is activated that additionally depends on the genetic background and cellular context. The associated pathway includes activation of a series of proteolytic proteins, cysteine-aspartic proteases cysteine, or also known as caspases, and cleavage of several hundreds of proteins by this enzyme family. Other crucial biochemical features include permeabilization of the outer mitochondrial membrane (OMM) in a big majority of apoptotic signalling pathways and the exposure of phosphatidylserine, a so-called eat me signal, on their cell surface, attracting macrophages and leading to endocytosis of apoptotic cells.

Apoptotic signalling pathways

As mentioned above, apoptosis can be triggered by a variety of stimuli. In the extrinsic, or receptor mediated, pathway, the ligation of surface receptors (death receptors) by their specific ligands, leads to their activation and transduction of an apoptotic signal into the cell. The death receptor family comprises 8 members and belongs to the tumour necrosis factor (TNF)

receptor superfamily. The most important members are the tumour necrosis factor receptor 1 (TNFR1; or DR1) with the complementary ligand TNFα, CD95 (also DR2, APO-1 or Fas) and its ligand CD95L, as well as the two TNF-related apoptosis-inducing ligand receptors 1 and 2 (TRAILR1/TRAILR2; also DR4 and DR5, respectively) with the TNF-related apoptosis-inducing ligand (TRAIL)[34-37]. Upon binding of their specific ligands, CD95 and TNF receptors undergo homo oligomerization and formation of trimeric receptors. These receptors contain a so called death domain, which is crucial for formation of a so called death-inducible signalling complex (DISC). Depending on the particular type of receptor the DISC complex contains either the adapter molecule Fas-Associated Death Domain (FADD) or TNFR-associated death domain protein (TRADD) and pro-caspase-8/-10. The DISC complex serves as activation platform of these initiator caspases, which undergo autoproteolytic cleavage and cause triggering of downstream effector caspases and ultimately apoptotic cell death. The intensity of caspase-8 activation at the level of the DISC is regulated by the FADD-like interleukin-1β–converting enzyme (FLICE)-like inhibitory protein (FLIP)[38]. FLIP shares great structural similarity with caspase-8 but lacks its enzymatic activity. Replacement of caspase-8 by FLIP in the DISC leads to a decreased activation of this initiator caspase and thereby also an inhibition of apoptosis.

Depending on the cellular background, caspase-8 may induce downstream activation of effector caspases via two distinct pathways. In type I cells, which are characterized by high levels of DISC formation and as a results a strong activation of caspase-8/-10, caspase-3/-7 can be directly activated by caspase-8/-10. Whereas in type II cells, there is a lower formation of DISC and active caspase-8, which in turn requires a further amplification of the apoptotic signal via the mitochondrial pathway[39]. These organelles, initially thought to be solely involved in regulation of metabolic pathways, in particular generation of ATP, were found to play a crucial role in apoptotic signalling and execution[40]. The mitochondrial apoptotic pathway includes cleavage of the BH3-only protein BH3-interacting domain death agonist (BID) by caspase-8, which leads in turn to an activation the proapoptotic B cell lymphoma 2 (Bcl-2)-associated X protein (BAX) and Bcl-2 antagonist or killer (BAK). Oligomerization and incorporation of these proteins into the OMM leads to formation of pores in the OMM and mitochondrial outer membrane permeabilization (MOMP). As a

result, various proteins of the mitochondrial intermembrane space are released into the cytosol, including cytochrome *c,* which forms the apoptosome complex in the cytosol, together with the apoptosis activating factor-1 (Apaf-1), pro-caspase-9 and dATP. The apoptosome acts like an activation platform for pro-caspase-9, which thereafter triggers the activation of the effector caspase-3.

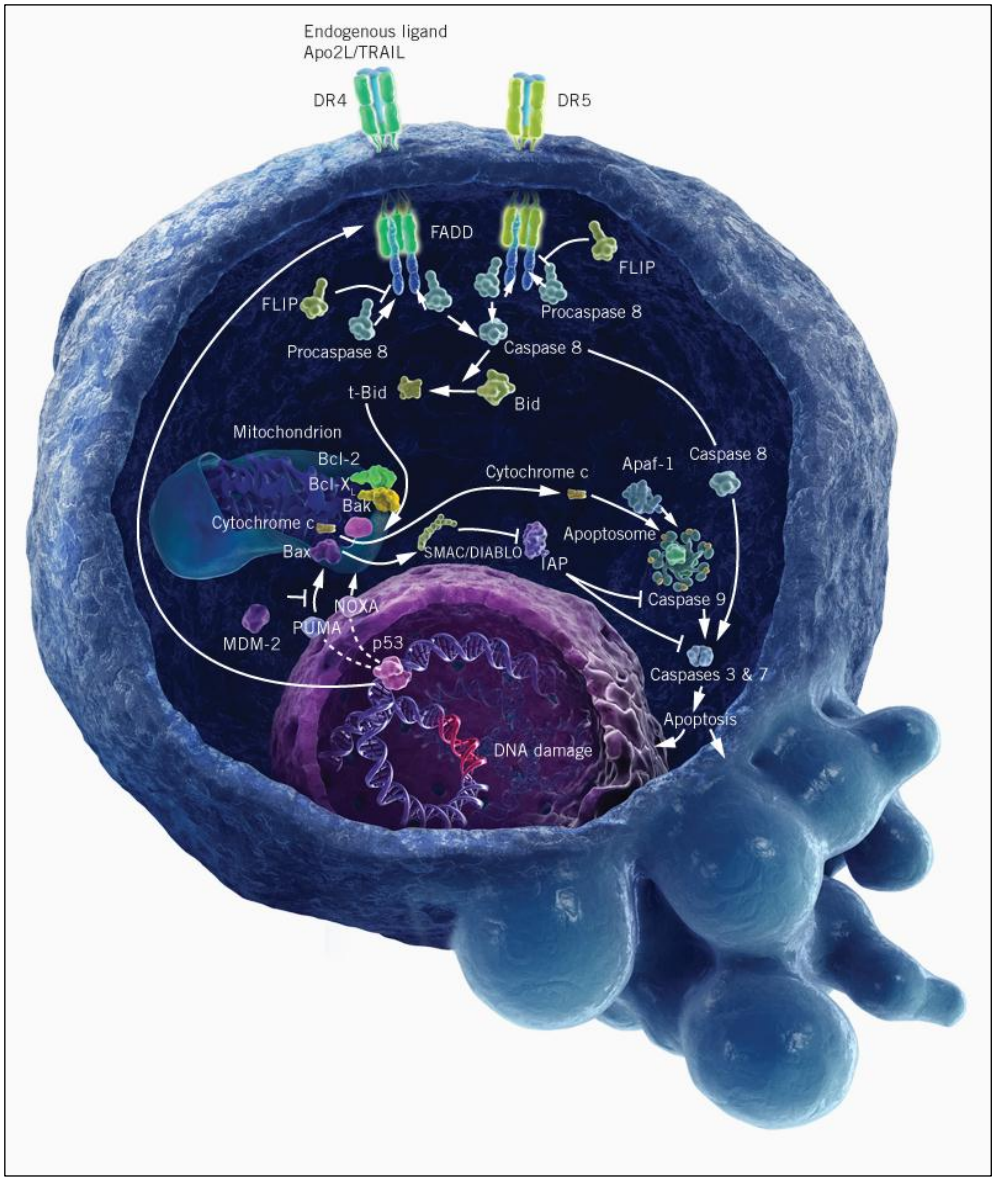

Fig. 2: Extrinsic- and intrinsic apoptotic pathway. © 2014 Genentech USA, Inc.

The intrinsic apoptotic pathway, is activated as the name suggests, by stimuli originating from inside of the cell, such as DNA damage induced by UV- or γ-irradiation, cytotoxic drugs or reactive oxygen species (ROS). These death signals can either directly or indirectly cause MOMP and the release of pro-apoptotic proteins from the mitochondria. This process is tightly regulated by the members of the Bcl-2 family, which will be discussed in detail in another section of this thesis. The proapoptotic proteins that are released from the

intermembrane space of the mitochondrial comprises the aforementioned cytochrome *c*[33], apoptosis-inducing factor (AIF)[41, 42], second mitochondrial activator of caspases (Smac)/direct IAP binding protein with low pI (Diabolo)[43, 44], mammalian homolog of the bacterial high temperature requirement protein (HTRA2/Omi) [45, 46] and Endonuclease G (EndoG) [47, 48]. Particularly, the release of cytochrome *c* is very well studied and is regarded as a point of no return in apoptosis induction. The translocation of this otherwise essential protein of the respiratory chain, results as mentioned earlier, in activation of pro-caspase 9, and subsequent induction of the caspase-cascade by activation of the effector caspase-3. The intrinsic pathway may also be executed in a caspase-independent manner, mainly mediated by translocation of EndoG and/or AIF from mitochondria into the nucleus, where their mode of action includes chromatin condensation, as well as formation of high-molecular weight DNA fragments.

Mechanisms of OMM permeabilization

As aforementioned mentioned, the main regulatory mechanism for MOMP in apoptosis is controlled by the Bcl-2 family of proteins. Bcl-2 was originally discovered as an previously unknown gene in studies related to chromosomal translocations in human follicular lymphoma[49] and was later on found to be associated with tumourigenesis and regulation of cell death[50]. Overexpression of Bcl-2 was shown to inhibit cell death induced by various stimuli. The family of Bcl-2 and associated proteins constitutes of more than 30 members and can be divided into three groups, depending on the number of Bcl-2 homology (BH) domains and their pro- or antiapoptotic function. Bcl-2 itself, together with the other antiapoptotic members (e.g. Bcl-X_L and Mcl-1), as well as the proapoptotic effector proteins Bax and Bak, share different combinations of four short regions of homology (BH1-4). In contrast, the very heterogeneous subgroup, which consists of a variety of other Bcl-2 proteins such as Bid, Bim, Bad, Noxa and Puma, contain only the conserved BH3 domain and are therefore called BH3-only proteins[51, 52]. Members of this subgroup serve as sensors for intracellular apoptotic stimuli, such as DNA damage or endoplasmic reticulum (ER) stress and therefore can be categorized as proapoptotic. Their mode of activation can be divers, including transcriptional upregulation, such as in case of Puma or Noxa, posttranslational modifications (Bad) or proteolytic

cleavage (Bid). After their activation, the BH-3 only proteins translocate to the OMM, where they either bind antiapoptotic Bcl-2 proteins or directly activate Bax or Bak. Permeabilization of the OMM requires an oligomerization of activated Bax or Bak, and it was shown that the absence of both of these effector proteins render cells resistant to several apoptotic stimuli[53]. Under normal conditions Bax resides mainly in the cytosol, whereas Bak and other Bcl-2 proteins are associated with the OMM via their carboxy-terminal anchor[54]. Several different modes for Bax/Bak activation have been proposed. The so-called derepressor model, assumes a constant active form of Bax and Bak, only being repressed by antiapoptotic Bcl-2 proteins[55-57]. According to this model, BH3-only proteins displace, upon apoptotic stimuli, Bax/Bak from the binding site at Bcl-X_L or Bcl-2 and thereby lead to their release and induction of OMM permeabilization. The second, direct activator, model, postulates in contrast a need for direct activation of Bax/Bak by the BH3-only proteins. Furthermore, it divides them into two classes, “direct activators” and “sensitizers”. According to this model, the truncated form of Bid (tBid) and Bim are able to directly activate Bax/Bax via N-terminal conformational change, under the premise of their prior deliberation by sensitizer proteins such as Bad or Noxa[58, 59].

A more refined version of the direct activator model is the so-called “embedded together” model, which focuses on the interaction of the pro- and antiapoptotic Bcl-2 family proteins with the mitochondrial lipid bilayer[60, 61]. According to this model, which emphasizes the importance of the interaction of the Bcl-2 proteins with the OMM, the MOMP induced by tBid-activated Bax is a multistep process, initiated by the insertion of tBid into the membrane. This in turn recruits Bax, which undergoes conformational changes and allowing it to insert into membrane as well. Membrane-bound Bax recruits other cytosolic Bax and leads to their oligomerization and ultimately MOMP[62]. Currently, the role of the mitochondria-specific phospholipid cardiolipin (CL) in this process is (controversially) discussed. The current view is that in resting cells CL is mainly located in the inner mitochondrial membrane (IMM) and translocates to the OMM under apoptotic conditions. These CL-enriched OMM microdomains are the platform for full caspase-8 activation in type II cells, which leads to cleavage of Bid and causing the insertion of its truncated form tBid to the CL-enriched microdomains[63, 64]. As discussed above, this leads to activation and

oligomerization of Bax, causing MOMP. Cytochrome *c* is believed to be anchored to the IMM via CL. Thus, for its full release, cytochrome *c* catalyses the peroxidation of CL, thereby liberating itself from the IMM. Despite these findings, the exact role of CL for this process is still not fully clear and requires further exploration[63].

In conclusion, the control of MOMP by the Bcl-2 family is based on the balance between the pro- and antiapoptotic members of this family in the OMM, and is critical for cell fate decisions.

Another mechanism of MOMP is the activation of the mitochondrial permeability transition (MPT) pore. This mechanism is long-known and was first described 35 years ago when Haworth and Hunter were showing that a mitochondrial calcium-overload causes drastic changes in the mitochondrial morphology and potentially leads to opening of a non-specific pore in the IMM[65]. This phenomenon is based on the ability of mitochondria to accumulate and retain Ca^{2+}. Mitochondria take up calcium electrophoretically via the mitochondrial calcium uniporter, driven by their membrane potential. In comparison with the ER, the mitochondrial calcium pool is low under physiological conditions, but these organelles were shown to accumulate large amounts under pathological conditions when the level of this cation increases drastically. Opening of the MTP pore leads to mitochondrial osmotic swelling, dissipation of their membrane potential and rupture of the OMM. This causes a release of mitochondrial content, such as proapoptotic proteins like cytochrome *c* or AIF, into the cytosol, ultimately leading to stimulation of apoptotic cell death. MTP pore opening was originally associated with necrotic cell death but was also shown to trigger apoptosis in response to different stimuli, such as drugs inducing a pathological increase of calcium concentration in the cytosol. Other activating factors include inorganic phosphate (Pi)[65] and ROS[66], whereas ATP[67] and cyclosporine A[68, 69] were shown to have an inhibitory effect. The exact composition of the MPT pore is still not fully clear and a matter of discussion among the scientific community. According to the traditional view, this multimeric protein complex consists of the voltage-dependent anion channel (VDAC), adenine nucleotide translocase (ANT), and the matrix protein cyclophilin D. In addition, the c subunit of the ATP synthase F1[70] and phosphate carrier (PiC)[71] have been discussed as participating components. The specific role of MPT in apoptosis is so far not fully understood, as it has

been speculated that not the whole mitochondrial population might undergo MPT, and that mechanisms exists to reseal the MPT pore, thereby enabling mitochondria to recover from the insult caused by the apoptotic stimuli[72]. Thus, particularly mitochondria in close proximity to so-called calcium hot-spots, such as contact sites between mitochondria and ER, are vulnerable for MPT induction, as calcium concentrations at theses spots might by far exceed average cytosolic concentration[73]. In mitochondria with opened MPT pore, subsequent uncoupling and loss of membrane potential will occur, leading to an active hydrolysis of ATP, in order to sustain the membrane potential.

Apoptosis and cancer

Already in 1972 Kerr, Wyllie, and Currie speculated about a role of apoptosis in cancer development and postulated that the expansion of tumour cells is not merely determined by the rate of cell proliferation but also by the number of dying cells[26]. Apoptosis is involved in spontaneous regression of tumours, while, as mentioned earlier, defects in apoptosis contribute to tumour progression and resistance to treatment. Therefore, evasion of apoptosis was highlighted by Hanahan and Weinberg as one of the hallmarks of cancer in their seminal review[74]. First experimental indication for a role of apoptosis in cancer development was the finding that the Bcl-2 protein, which was found to be overexpressed in follicular lymphoma, possesses antiapoptotic function[75]. In a transgenic mouse model, Bcl-2 enhanced the Myc-induced formation of B cell lymphomas, by increasing the survival of the malignant cells[76]. This cooperation of Myc and Bcl-2 in tumourigenesis was intensively studied and resulted in a consensus view that overexpression of oncogenes leads to induction of apoptosis, which represents one of the major safety mechanisms of the body to remove potentially dangerous cancerous cells. Other examples for the interplay between tumourigenesis and defects in apoptotic signalling include findings regarding the mandatory inactivation of the p53 tumour suppressor in pRb-mediated tumours in the choroid plexus[77]. p53 is a key proapoptotic regulator that acts as an intracellular damage sensor and can transcriptionally upregulate proapoptotic members of the Bcl-2 family and thereby induce apoptosis. In fact, p53 mutations are the most common feature of tumours that evade apoptosis, and its inactivation can be found in more than 50% of all cancers. In addition, tumours utilize a big variety of strategies to

circumvent induction of apoptosis, including the aforementioned overexpression of antiapoptotic Bcl-2 members (e.g. Bcl-2, Bcl-X_L), downregulation of death receptors[78], overexpression of IAPs[79] or c-FLIP[80], as well as silencing of crucial apoptotic proteins, such as caspase-8[81, 82].

Evasion of apoptosis is not only relevant for tumourigenesis but also for anticancer treatment. The presence of defects in the apoptotic signalling pathway often leads to failure of chemo- or radiotherapy, as the main aim of these approaches is induction of apoptotic death. Chemotherapy represents a selective pressure for the tumour to develop further defects in the apoptotic machinery. It was shown that NB tumours relapsed after chemotherapy with additional mutation in p53[22], rendering these cancers more resistance to drug-induced apoptosis.

Role of mitochondria in cancer

The role of mitochondria in cancer has been greatly underestimated by the research community for a long period of time, as researchers focused primarily on cancer genetics and ignored the seminal findings by the German biochemist and Nobel Laureate Otto Warburg, who predicted as early as 1927 the importance of these organelles for tumourigenesis[83]. In recent decades his early findings were confirmed and further developed. Besides, it has been shown that disturbances of mitochondrial functions are not only key features of cancer, but also of other, mainly neurodegenerative, disorders, e.g., Parkinson's, Alzheimer's, and many other diseases. The role of mitochondria in various pathologies is based on their central role in many vital physiological processes in the cell, including ATP production, calcium homeostasis regulation, and their ability to produce ROS, as well as discussed earlier, in the execution and regulation of apoptosis.

Metabolic dysregulation in cancer has long been regarded as a mere by-product of tumourigenesis to support tumour growth and survival. Based on the findings by Otto Warburg, and after a rediscovery in recent years, it became more and more apparent that metabolic changes in cancer are not only a consequence of malignant transformation, but seem to be essential for this process and are therefore regarded as a crucial hallmark of cancer[2].

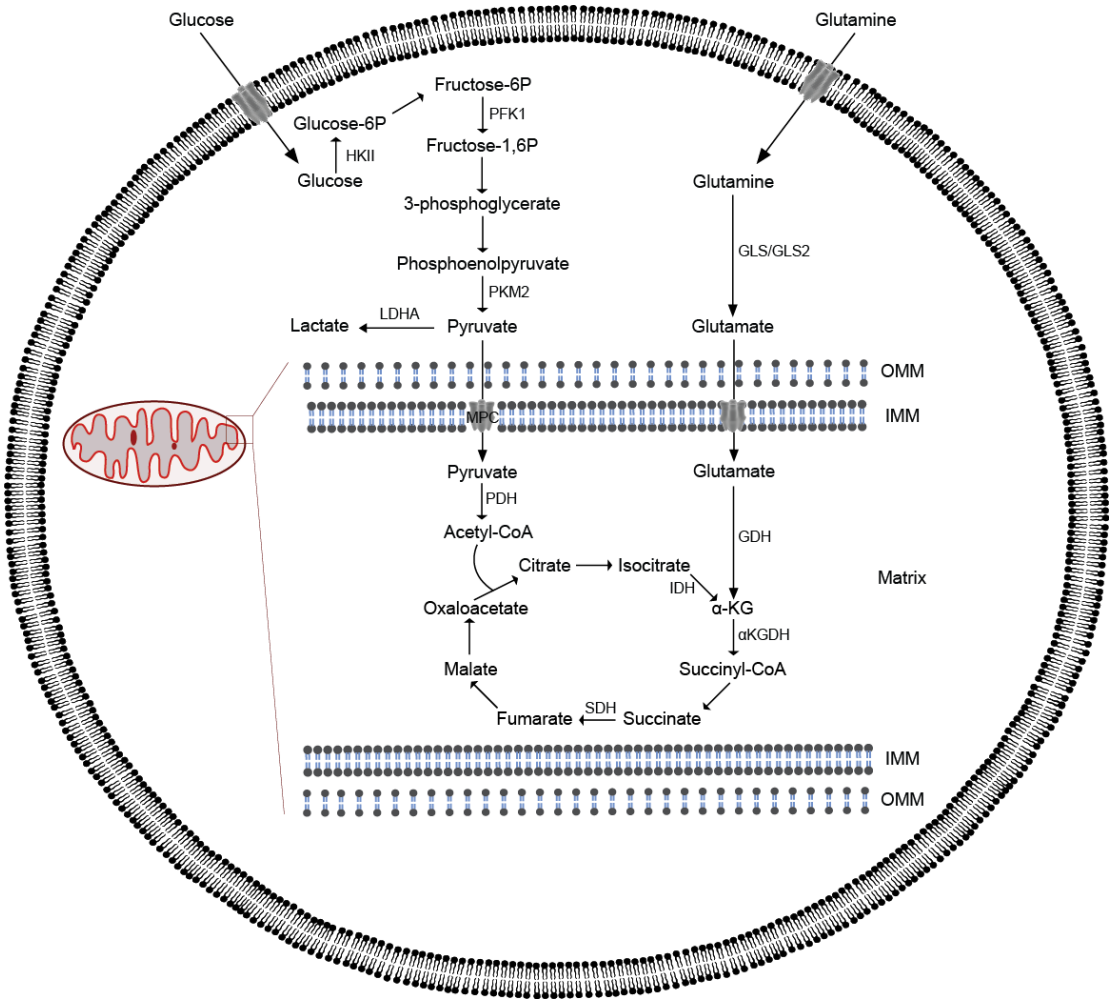

Fig. 3: Cellular glucose and glutamine metabolism. Overview of cellular glucose and glutamine metabolism, as well as the mitochondrial tricarboxylic acid cycle. Figure adapted from BioCarta and republished with permission[84].

Most cells utilize glucose as their main energy source, which is metabolized after its uptake in a set of glycolytic enzymatic reactions to form pyruvate (Fig. 3). In normal cells, under normoxic conditions, most of the ATP is produced via oxidative phosphorylation (OXPHOS) in the mitochondria, whereas in a majority of cancer cells there is a shift towards glycolysis. This metabolic shift leads to changes in the fate of pyruvate, the end product of glycolysis. Normally, pyruvate is directed to the mitochondria where it is metabolized by pyruvate dehydrogenase (PDH) to enter the tricarboxylic acid cycle (TCA) cycle and fuel OXPHOS. In contrast, in most tumour cells, the activity of PDH is suppressed, causing a reduced flow of pyruvate to the mitochondria, and a decrease in OXPHOS and pyruvate to be mainly converted to lactate by lactate dehydrogenase (LDH). Named after Otto Warburg, the “Warburg Effect” can be found in the majority of tumours. The reliance of tumour cells on glucose is

widely used for visualisation of tumours using fluorodeoxyglucose (18F-FDG) positron emission tomography (PET) and therapy by utilizing the non-metabolizable glucose analogue 2-deoxyglucose to block glycolysis and cancer growth. Glycolysis, despite being less efficient in terms of ATP yield per glucose molecule when compared to OXPHOS-driven energy production (2 vs. 36), renders tumour cells more resistant to oxygen-deprivation as a result of excessive growth or high metabolic activity and poor oxygen supply. Furthermore, it provides the cell with resources to sustain proliferation[84]. For a long time, it was assumed that these metabolic changes are caused by defects in mitochondria but later on explained by alterations in metabolic regulatory signalling. Thus, one of the most commonly activated signal transduction pathways in cancer, the phosphoinositide 3-kinase (PI3K)[85] and mammalian target of rapamycin (mTOR)[86] pathways were shown to induce a pseudo-hypoxic state, independently of oxygen, by stabilization of the hypoxia-inducible factor 1α (Hif1α), which causes a strong stimulation of glycolysis via upregulation of key enzymes in glucose transport and utilization.

2. Introduction to the study

Targeting mitochondria for anticancer therapy

Due to the importance of mitochondria in the development of various pathologies, they are also viewed as promising targets for therapeutic interventions in these diseases. For anticancer therapy, their importance in cancer metabolism, production of ROS and their central role in regulation of cell death are exploited. The rationale underlying the majority of strategies of targeting these organelles is to circumvent mechanisms of tumours to evade apoptosis and thereby facilitate anticancer therapy. These obstructions in cell death pathways are mainly mediated upstream of the mitochondria within the apoptotic pathway, such as mutations or downregulation of apoptotic effectors or regulators (e.g. initiator caspases, p53,) or due to overexpression of antiapoptotic members of the Bcl-2 family. Bearing in mind that OMM permeabilization and release of proapoptotic proteins e.g. cytochrome *c* from mitochondria are regarded as a point of no return in apoptosis induction, approaches to destabilize mitochondria represent a very intriguing tactic to combat cancer and overcome chemoresistance mechanisms. In the following paragraph major approaches and drugs for targeting mitochondria in anticancer therapy will be discussed.

BH3 mimetics

BH3 mimetics are small molecules that have close structural or functional similarity to BH3-only proteins and were developed using rational structure-based drug design. Most BH3 mimetics that are currently under preclinical and clinical development aim to disrupt interaction of proapoptotic proteins with antiapoptotic members of the Bcl-2 protein family, such as Bcl-2 or Bcl-X_L, which are frequently overexpressed in tumours. One of the first small-molecule inhibitors was ABT-737, which targets Bcl-2, Bcl-X_L and Bcl-W[87]. Further studies revealed that tumour sensitivity to ABT-737 was dependent on their expression level of Mcl-1, another antiapoptotic member of the Bcl-2 family that is not a target of ABT-737. Cancers with high levels of Mcl-1 were highly resistant against ABT-737, which led to the development of a combined treatment approach of ABT-737 with agents reducing Mcl-1 levels, such as

sorafinib. Studies that were conducted using this treatment regime showed promising results *in vitro* and *in vivo*[88], but the therapeutic potential of ABT-737 was greatly hampered by its poor physiochemical properties, making this compound not orally bioavailable. This limitation was overcome by the next generation of Bcl-2 inhibitors, ABT-263 or navitoclax[89]. Despite its improved characteristics in terms of bioavailability and promising results from clinical trials[90, 91], the clinical efficacy of ABT-263 was significantly reduced due to its deleterious effect on circulating platelets. This hematologic toxicity is caused by the inhibition of Bcl-X_L, a critical survival factor of platelets. These findings lead to a further improvement and development of the third generation of Bcl-2 inhibitors, ABT-199, which is characterized by a high Bcl-2–selectivity[92]. Due to its specificity for Bcl-2, sparing Bcl-X_L, hematologic toxicity could be greatly reduced, and allowed higher doses of the drug, resulting in a vastly increased anticancer efficacy. ABT-199 was further shown to enhance the antitumour effects of tamoxifen in estrogen receptor-positive breast carcinoma[93], as well as to have therapeutic potential in multiple myeloma [94] and acute myeloid leukaemia[95].

Targeting MPT

Targeting MPT, the second mode of OMM permeabilization, can be facilitated by a large number of compounds, either indirectly by depleting cells of endogenous MPT-inhibitors such as glucose, ATP, glutathione and creatine phosphate, or directly by agents inducing ROS production or an increase in cytosolic calcium concentrations. Other approaches are drugs which directly interact with the components of the MTP core complex. To this end, several ligands of ANT, a core component of the MPT pore, were shown to induce cancer cell death via the mitochondrial apoptotic pathway. Including the ANT-cross linker 4-(N-(S-glutathionylacetyl) amino) phenylarsenoxide (GSAO), which was shown to inhibit the ATP/ADP antiporter activity of ANT, causing subsequent ROS production and inhibition of tumour angiogenesis by targeting mitochondria in proliferating endothelial cells[96]. This compound particularly targets the endothelial component of tumours, reflecting their higher amounts of mitochondria as compared to tumour cells. Another class of compounds that were shown to induce MPT and cell death are retinoic acid derivatives, such as all-*trans*-retinoic acid, which are commonly known for their ability to induce

differentiation and dormancy in NB or certain forms of leukaemia. Interestingly, their ability to activate ANT-dependent MPT, was shown to be independent from their function in gene-expression regulation[97]. The underlying mechanism was shown to be mainly dependent on an increase in cytosolic calcium levels, one of the hallmarks of MPT-induction, as firstly described for the synthetic retinoid ST1926[98]. Whether or not ST1926 directly targets ANT is unknown, but its clinical potential is currently under investigation in a clinical trial using ST1926 as a monotherapy in ovarian cancer[99].

The peripheral benzodiazepine receptor (PBR), a potential MTP pore-interacting protein in the OMM, is overexpressed in a variety of tumours[100, 101]. Surprisingly, PBR was also implicated in regulation of Bcl-2-protein mediated apoptosis, as PBR was shown to block the three major antiapoptotic members of this family, Bcl-2, Bcl-X_L and Mcl-1. Thus, representing an interesting interplay between both forms of OMM permeabilization. Furthermore, PBR ligands such as PK11195 or RO5-4864 were shown to have antitumour effects *in vitro* and *in vivo*, and were able to sensitize cancer cells to conventional chemotherapeutic drugs, irrespectively of Bcl-2 overexpression[102, 103].

Metabolic inhibitors

As discussed earlier, tumours show a wide range of specific metabolic aberrations, which biochemically distinguish them from normal tissues. These alterations are exploited as targets for treatment of cancer. One of the major changes is the hyperglycolytic state in tumours, consequently, the inhibition of glycolysis is one of the main rationales for targeting tumour metabolism. The synthetic analogue of glucose, 2-deoxy-D-glucose (2DG), was extensively investigated and tested as a promising chemotherapeutic drug. This compound competitively inhibits glucose uptake by the GLUT proteins. After transport into the cell, 2DG is phosphorylated by hexokinase (HK) to form 2-deoxy-D-glucose-6-phosphate (2DG-6-P), which is not further metabolizable, rather accumulates in the cell and noncompetitively inhibits HK, thereby the entire glucose metabolism and partially also OXPHOS. Initial results showed a promising chemosensitizing effect in combination with cisplatin in human head and neck cancers as well as breast cancer, furthermore clinical trials are ongoing in pancreatic cancer[104-106]. Another interesting target is the interaction between HK and VDAC in the OMM, as HK was found to be frequently

overexpressed in tumours and bind to VDAC more tightly than in non-malignant cells[107]. 3-bromopyruvate is the best studied HK inhibitor and proved to exert anticancer effects on hepatic and pancreatic cancers[108]. Other HK inhibitors include the plant hormone methyl jasmonate and a short peptide derived from the HK2 amino terminus, which were shown to detach HK from mitochondria and induce MPT-dependent cell death in cancer cells[109, 110].

One of the most critical enzymes for the glycolytic shift in tumour cells is pyruvate dehydrogenase, which determines the fate of pyruvate, the end product of glycolysis. PDH is mainly regulated by pyruvate dehydrogenase kinase (PDK), which phosphorylates PDH and inhibits its enzymatic activity. Dichloroacetate (DCA), a PDK inhibitor, therefore indirectly can stimulate PDH activity and enhance conversion of pyruvate to acetyl-CoA, thereby stimulating OXPHOS. This reversal of the Warburg effect was shown to lead to a reduced mitochondrial membrane potential and inhibited cell growth, as well as an increased ROS production and ultimately cell death in a big variety of tumours[111-114]. These findings led to several clinical trials, including in malignant brain tumours, in which DCA was shown to improve the outcome for patients, without causing any major adverse effects[115, 116]. Another approach aiming to reverse the glycolytic shift is the inhibition of LDHA, the critical enzyme of the second possible metabolic route for pyruvate. Downregulation of LDHA using shRNA led to increased mitochondrial respiration, reduced mitochondrial membrane potential and decreased tumour cell proliferation[117].

Targeting the electron transport chain

The electron transport chain, consisting of in total 5 macromolecular protein complexes, is a very attractive target for chemotherapy, with the underlying rationale to inhibit individual complexes, induce increased production of ROS and ultimately facilitate tumour cell death. In particular Complex I and III have been shown to be the source of mitochondrial ROS production, although a contribution of Complex II was also shown recently[118, 119].

Complex I

The list of Complex I inhibitors comprises more than 60 classes of compounds, with rotenone being the most well studied and classical representative of this group[120]. Most of the compounds though, including rotenone, show severe

neurotoxicity and therefore are disregarded as potential anticancer drugs[121]. Deguelin, an analogue of rotenone, which has a much lower affinity to Complex I and also has an inhibitory effect on Akt and nuclear factor-κB, was shown to have antitumour activity[122, 123]. This was in part based on the ability of deguelin to interact with the heat shock protein 90 (HSP90) but also due to inhibition of Complex I, as cells with defects in the respiratory chain were insensitive to deguelin[124, 125].

Metformin, a widely used antidiabetic drug, was shown to have additional antineoplastic properties, as population based studies suggest that treatment with metformin is associated with a reduced cancer risk and improved prognosis. Nevertheless, the exact mechanism underlying these findings were unknown but was speculated to be based on inhibition of mitochondrial Complex I[126, 127]. Recent publications further confirm this view, showing a direct inhibitory effect on Complex I, reduced mitochondrial respiration and overall limiting the ability of cancer cells to cope with energetic stress. This leads to inhibited cell proliferation in the presence of glucose and tumour cell death under glucose deprivation[128-131]. Therefore, metformin, a drug prescribed to over 120 million patients worldwide, shows big potential to be utilized as a cancer therapeutic.

Another Complex I inhibitor is tamoxifen, a drug routinely used in the clinical setting to treat breast cancer. It is an antagonist of the estrogen-receptor but furthermore was also shown to inhibit Complex I at the Flavin site. Thus, the anticancer effect of tamoxifen might be at least in part also due to induction of ROS production at Complex I, in particular in cancer cells lacking the estrogen-receptor[132].

Complex II

Complex II, which will be discussed in detail in the following section, may also be blocked in its activity by several compounds. Thenoyltrifluoroacetone (TTFA) is the archetype representative of this group and was utilized to resolve the crystal structure of Complex II[133, 134]. TTFA binds to the ubiquinone (UbQ)-binding site of Complex II and thereby blocks electron transfer, which causes electron slippage and superoxide production. The clinical usage of TTFA needs to be further assessed as only limited information exists, which point out a potential hepatotoxicity by TTFA treatment[135].

The redox-silent vitamin E analogue α-tocopheryl succinate (α-TOS) was shown as early as 1982 to have antiproliferative effects in mouse melanoma cells[136] and, furthermore, proved to be effective in a big variety of experimental cancers such as colon, breast, and NB, in both *in vitro* and *in vivo* settings[137-139]. The underlying mechanism for apoptosis induction by α-TOS was investigated in several studies, providing different models for its mode of action, but without finding the specific molecular target of α-TOS. In a study by Dong and colleagues this target was identified to be the ubiquinone-binding site of Complex II, and the authors provided evidence for the involvement of superoxide production in this process[140]. Later on, this model was extended by findings that α-TOS additionally triggers an increase of cellular calcium levels, which in combination with ROS production leads to mitochondrial destabilization and cell death[141]. This compound is particularly attractive as a potential anticancer drug as it was shown to be selective for cancers, while being non-toxic to normal cells[142].

In contrast to α-TOS, malonate and 3-nitropropionic acid (3-NPA) target Complex II at the SDHA subunit and thereby block the SDH activity and metabolization of succinate. These compounds were also found to induce ROS production and cell death but showed severe neurotoxicity and even were associated with development of Huntington's disease[143-145]. These severe side-effects are likely to be caused by the simultaneous inhibition of transfer of electrons into the UbQ pool and in particular by arresting the TCA cycle. Thus, the selective inhibition of the UbQ-binding site by α-TOS or TTFA, without fully blocking the TCA cycle, represents a more attractive approach than targeting the SDH function of Complex II.

Complex III

Complex III, also known as coenzyme Q: cytochrome *c* – oxidoreductase, integrates electrons from both Complex I and II, and, therefore, is another major source of superoxide. It contains two UbQ-binding sites, Q_i and Q_O, and mediates the oxidation of two molecules of $UbQH_2$ at the Q_i site with the concomitant reduction of one UbQ at the Q_O site. This so called "Q cycle" was shown to be inhibited by the secondary bacterial metabolite antimycin A, which binds to the Q_i site and induces superoxide formation at the Q_O site[146]. Antimycin A is able to induce apoptosis in cancer cells via ROS production[147],

but interestingly was also shown to act as BH3 mimetic and inhibit antiapoptotic proteins of the Bcl-2 family[148].

Resveratrol, a compound that can be found in red wine and in the skin of different kind of berries, is usually associated with cytoprotective properties, although its beneficial effects are still highly debated and several different modes of action have been suggested. This plant-derived polyphenol is at the same time a very efficient inhibitor of the respiratory chain, in particular of Complex III, which in turn can induce mitochondria-dependent apoptosis[149]. The contrasting effects of resveratrol might be concentration-dependent and stem from the differential interaction with other cellular processes. The clinical application is currently investigated in several clinical trials, including ongoing studies in colorectal cancer.

Another class of natural compounds, isothiocyanates, exemplified by benzyl isothiocyanate (BITC), shows intriguing potential for their usage as anticancer drugs. These compounds can be found in vegetables like broccoli or cabbage and possess an inhibitory effect on Complex III. Hence, BITC induces ROS production from Complex III and apoptosis in cancer cells[150, 151]. Strikingly, these effects were specific for tumour cells, while sparing non-malignant cells. In addition, BITC suppressed angiogenesis and tumour cell growth in xenograft models, making BITC a very attractive compound to be further studied for its potential usage in chemotherapy[152, 153].

Complex IV

Cytochrome *c* oxidase, or Complex IV, receives electrons from cytochrome *c* and relays them to molecular oxygen to produce water. Being the last element of the electron transport chain, regulation of Complex IV activity modulates functioning of the whole respiratory chain. Regulation of Complex IV activity allows maintaining the efficiency of the respiratory chain under pathological conditions. Thus, during hypoxia, the master glycolytic transcriptional regulator, Hif-1, was shown to cause an isoform switch of Complex IV, which leads to a higher activity[154].

Transcriptional regulation is the most common underlying mechanism of Complex IV inhibitors. For example N-(4-hydroxyphenyl) retinamide, also known as fenretinide, destabilizes the mRNA transcript of the subunit III of Complex IV, which leads to an inhibition of the whole complex and ROS-

mediated apoptosis in cancer cells[155]. Interestingly, the well-known and commonly used anticancer drugs doxorubicin and daunorubicin, are also inhibiting Complex IV by two distinct mechanisms, transcriptional downregulation of subunit II and V and direct interaction with Complex IV. This interference with Complex IV was associated with side effects of these drugs in form of cardiotoxicity[156, 157].

Complex V

The ATP-synthase, also known as Complex V of the respiratory chain, utilizes the proton gradient which is built up by the Complexes I-IV to produce the majority of cellular ATP in non-malignant cells.

Blocking the ATP-synthase by the specific inhibitor oligomycin causes apoptotic cell death, but analogues of this compound showed a poor specificity for cancer cells, which limits their possible utilization for chemotherapy[158, 159].

The metabolite 3,30-diindolylmethane (DIM) is also able to block the ATP-synthase. It causes ROS formation, apoptosis and furthermore inhibits the cell cycle by induction of p21 expression[160]. In various cancer cell lines and in *in vivo* models, DIM, in addition to its antiproliferative and proapoptotic activity, proved to significantly inhibit angiogenesis[161]. DIM has been successfully tested in humans as treatment against a benign noninvasive neoplasia (recurrent respiratory papillomatosis), which is caused by papilloma virus infections[162, 163]. Currently, clinical trials are ongoing in patients suffering from cervical dysplasia and prostate cancer.

Complex II of the respiratory chain

Succinate dehydrogenase (SDH) or succinate:ubiquinone oxidoreductase (SQR), commonly known as Complex II, is a unique enzyme as it participates in both the TCA cycle and the electron transport chain. Complex II consists of four subunits, which are divided according to their functional contribution, into the SDH, consisting of the subunits SDHA and SDHB; and the SQR-part, comprising SDHC and SDHD[133]. Unlike all other complexes of the mitochondrial respiratory chain, Complex II is entirely encoded in the nucleus, and therefore lacks any contribution of the mitochondrial genome for its expression. Localized in the inner mitochondrial membrane, facing the matrix, the SDH part of Complex II is catalysing the oxidation of succinate to fumarate

and the concurrent production of $FADH_2$. The subunits SDHC and SDHD form the intramembranous SQR part of Complex II and are mediating the transfer of electrons from the first catalytic step to reduce ubiquinone to ubiquinol and to be finally transferred to the respiratory Complex III. Other structural abnormalities further highlight the special role of Complex II within the respiratory chain. In contrast to Complexes I, III and IV, which were shown to form a so called respirasome, Complex II resides relatively separated from this supramolecular complex[164, 165]. It was established that complexes responsible for the leakage of electrons are Complex I and III. For many years, Complex II was not considered as a source of ROS. However, various recent publications focused attention on Complex II as an important site of ROS production in the form of superoxide[118, 119, 166, 167], contributing to ROS-mediated execution of apoptotic cell death[168]. First mechanistic insight provided the finding that overexpression of SDHC induced cell death and cells lacking this subunit of Complex II were much less sensitive towards several chemotherapeutic agents, such as cisplatin, doxorubicin and etoposide[169]. Later on, this feature was proposed to be mediated by a specific disintegration of Complex II, triggered by pH changes after treatment with apoptotic stimuli, causing a dissociation of the SDHA/SDHB subunits, which functionally represent the SDH fraction of the complex. This results in an impairment of the SQR activity of the membrane-bound SDHC/SDHD subunits, while leaving the SDH activity of the dissociated SDHA/SDHB part intact, which in turn was shown to excessively produce superoxide and ultimately facilitate cell death[170]. Based on these findings Complex II was suggested to act as a cell death sensor, responding to acidification upon toxic stimuli by its disassembly and thereby further facilitating ROS-mediated apoptosis[171].

3. Aims

The general aim of this PhD study was to further explore mitochondria as targets for anticancer therapy, with a particular focus on Complex II of the mitochondrial electron transport chain and the role of various mitochondrial substrates in modulation of apoptotic cell death. In the individual papers the following specific aspects were investigated:

Paper I:

- Can treatment with α-TOS overcome chemoresistance of NB cells?
- Role of MycN in NB sensitivity to chemotherapy.

Paper II:

- Potential of α-TOS to be used in a co-treatment setting with conventional chemotherapeutic drugs.

Paper III:

- Can specific inhibition of Complex II by TTFA sensitize chemoresistant tumour cells to anticancer drugs?

Paper IV:

- Mechanism of citrate-induced toxicity and exploration of its potential usage for chemotherapy.

4. Materials and methods

Detailed description of the material and methods that were utilized during this PhD project can be found in the constituting papers I-IV. A brief summary is given bellow.

Mammalian cell culture

In this thesis a panel of NB cell lines was employed for the majority of the conducted experiments. All NB cell lines and the non-small cell lung cancer cell line U1810 were grown in RPMI-1640 medium and supplemented with 10% (v/v) heat-inactivated foetal bovine serum (FBS), penicillin (100 U/ml) and streptomycin (100µg/ml). For the growth of Tet21N cells, 200 µg/ml geneticin and 100 µg/ml hygromycin were additionally added, as they are stably transfected with a Tet-Off system for *MycN* overexpression[172, 173]. For switching off *MycN* expression in Tet21N cells 0.1 µg/ml doxycycline was added to the cell culture medium. The human colon carcinoma cell line HCT116 was grown in DMEM medium with the same additions as above. During experiments cells were maintained in a logarithmic growth phase and kept at 37°C with 95% humidity and 5% CO_2 supply.

Western blot analyses

For immunodetection of proteins, Western Blotting was utilized in the different papers included into this thesis. Following treatment and harvesting, a sample was taken for measuring protein concentration via the bicinchoninic acid (BCA) colorimetric assay. Subsequently, Laemmli's loading buffer was added, samples boiled for 10min, and subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) at 100V, followed by transfer to nitrocellulose membranes for 2h at 120V. After membranes were blocked with 5% non-fat milk in phosphate-buffered saline (PBS) at room temperature for 1h, they were probed with the desired primary antibody overnight. Membranes were rinsed and incubated with a horseradish peroxidase-conjugated secondary antibody (1:10000) at room temperature for 1h. Blots were visualized using ECL TM (Amersham Biosciences, Buckinghamshire, UK) and X-ray film.

Evaluation of apoptosis

Apoptotic morphology and nuclear condensation

Characteristic morphological features of apoptotic cells, including cell membrane blebbing, cell shrinkage and nuclear condensation were assessed using a confocal microscope and staining of cells with the cell permeable DNA staining dye HOECHST. Results were expressed as percentage of apoptotic cells.

Caspase-activation

Caspase-processing and specific substrate cleavage

Caspases require processing in order to be activated. This cleavage can be assessed utilizing Western Blot and specific antibodies. To this end, antibodies against caspase-3, caspase-2 and caspase-8 were used in the experiments included in the thesis. Specific cleavage products were detected and used as readout for caspase-processing and activation. As a further parameter of cell death and the activity of caspase-3, the cleavage of one of its substrates, poly(ADP-ribose) polymerase (PARP), was detected using an antibody directed against the cleaved form of this protein. Under physiological conditions PARP has the function to detect and signal single-strand DNA breaks (SSB) in the nucleus.

Fluorometric caspase activity assay

Caspase activity assay is a relatively easy, fast, and convenient tool for the detection of caspase activation in cells undergoing apoptosis. This quantitative and sensitive method utilizes various substrates that are recognized and cleaved by appropriate caspases. At present, a wide variety of different synthetic low molecular weight caspase substrates containing appropriate peptide (tetrapeptide or pentapeptide) sequences with caspase cleavage sites are provided by a number of suppliers. The peptides can be conjugated with fluorogenic [7-amino-4-methylcoumarin (AMC)] or chromogenic (p-nitroaniline) groups, which are released when the substrate is cleaved by a particular caspase. The released AMC emits green fluorescence at 450nm when analysed with a fluorometer or a fluorescence microtiter plate reader (excitation, 360nm). The rise of the fluorescence signal is proportional to the amount of cleaved substrate and depends on the extent of caspase activity.

Caspase assays, as well as many other methods of detection of enzyme activity, are sensitive to different factors. Thus, optimal pH, salt concentration, a proper buffer composition as well as the presence of a stabilizing agent are important for the measurement of enzymatic activity. These requirements differ between the caspases. The pH optimum for caspases ranges between 6.5 and 8. In general, high salt concentration blocks caspase activity; however, the activity of some caspases (i.e., caspase-5 and -7) is enhanced when 0.2M NaCl and 5mM Ca^{2+}, respectively, are included in the reaction buffer. The activity of most caspases can be assayed in buffers based on HEPES (0.1M), but MES (0.1M) buffers are to be preferred when the activity of caspase-2 and -9 are investigated. In addition, stabilizing agents that keep the caspase subunits together (sucrose or PEG), detergents that lyse the cells, such as CHAPS and/or NP-40 and reducing agents, such as dithiothreitol (DTT), should be included in the caspase assay buffer. A reducing environment is necessary for the activity of caspases since they contain a cysteine residue in the active site.

Annexin V/PI staining of cells

One of the characteristic changes during apoptosis is the translocation of phosphatidylserine (PS), a phospholipid and component of the cell membrane, from the inner to the outer leaflet of the plasma membrane. This PS exposure can be detected with the human anticoagulant, annexin V, a Ca^{2+}-dependent phospholipid-binding protein, which features a high affinity for PS. A commercial available kit was utilized with a recombinant annexin V, conjugated to fluorescein (FITC annexin V), and mixed with the red fluorescent propidium iodide (PI). The cell-impermeable nucleic acid binding dye PI discriminates living and apoptotic cells from cells that have undergone necrosis with a ruptured cell membrane. After staining, samples were analysed using FACS and the following excitation/emission settings: 494/518nm for FITC annexin V and 535/617nm for DNA-bound PI.

OMM permeabilization and release of cytochrome *c*

Cells were digitonin-permeabilized (final concentration 0.01% w/v) for 15min at room temperature, spun down at 13000 x g and subsequently separated into

supernatant (cytosolic) and pellet (mitochondrial) fraction. Samples were prepared for Western Blot as described above and subjected to 15% SDS-PAGE at 100V followed by electroblotting to nitrocellulose for 2h at 120V. Membranes were blocked for 1h with 5% non-fat milk in PBS at room temperature and subsequently probed overnight with a mouse anti-cytochrome *c* antibody (BD Biosciences, San Jose, CA).

Assessment of oxidative stress

ROS-measurements

The amount of cytosolic hydrogen peroxide in cells was assessed in **Paper I** using the genetically encoded, cytosolic fluorescent indicator pHyPer-dCyto (Evrogen, Moscow, Russia)[174]. Cells were seeded on coverslips and after one day transiently transfected with the pHyPer-dCyto plasmid, utilizing the Lipofectamine LTX/Plus reagent (Invitrogen, Carlsbad, CA). After one additional day and the desired treatment, levels of hydrogen peroxide were analysed. In **Paper III** mitochondrial superoxide production was measured using the specific probe MitoSOX™ Red. Cells were stained with 5µM MitoSOX™ Red for 30min, washed three times and subsequently analysed. All experiments were conducted on a Zeiss LSM 510 META confocal laser scanning microscope (Zeiss Jena, Germany). Time lapse experiments were performed utilizing the POC-R cell cultivation system (Zeiss, Jena, Germany) at 37°C and humidified air/CO_2 (5%) atmosphere. The drugs were bath-applied and fluorescence recorded for the desired time span. Measurements were repeated three times and superoxide/ hydrogen peroxide production was expressed as x-fold increase in comparison with the starting point of the experiment. In **Paper III** mitochondrial superoxide production was additionally determined and expressed as percentage of MitoSOX-positive cells. Samples were seeded, treated as indicated, stained with MitoSOX and subsequently analysed using the aforementioned confocal microscope setup. Percentages of MitoSOX-positive cells were counted manually.

Content of protein sulfhydryl groups

In **Paper I**, the content of sulfhydryl (SH) groups, as an indicator for oxidative stress, was measured using Ellman's reagent (5,5'-dithiobis-(2-nitrobenzoic acid) or DTNB), a chemical used to quantify the number or concentration of

thiol groups in a sample[175]. In brief, following treatment and harvesting, 500000 cells were resuspended in 200µl of ATP releasing buffer. For the reaction mixture, 100µl of sample lysates, 400µl of water and 200µl of DTNB reagent were mixed. Extinction was read at 412nm after 5min, using a spectrophotometer and results were expressed in arbitrary units.

Measurement of mitochondrial oxygen consumption

Oxygen consumption was monitored with an oxygen electrode (Hansatech Instruments, Norfolk, UK) and analysed with the OxygraphPlus software (Hansatech Instruments, Norfolk, UK). The results were normalized to either number of cells or amount of protein. Activity of individual respiratory complexes and rate of maximum respiration were analysed with the help of different mitochondrial substrates and the following specific inhibitors: rotenone (Complex I), TTFA (Complex II), cyanide (Complex IV), oligomycin (ATP synthase) and the uncoupling agent carbonyl cyanide m-chloro phenyl hydrazine (CCCP).

Measurement of cellular calcium fluxes

Mitochondrial calcium capacity

In order to assess the susceptibility of cells to undergo calcium-dependent MPT, mitochondrial calcium capacity was measured utilizing a calcium-sensitive electrode (Thermo Scientific, Beverly, MA, USA). To this end, cells were harvested, resuspended in a physiological buffer (150mM KCl, 5mM KH_2PO_4, 1mM $MgSO_4$, 5mM succinate, 5mM Tris, pH 7.4) and then added to the chamber of the calcium electrode. After a short stabilization period (2min), cells were permeabilized with digitonin (5µg/10^6 cells) and individual pulses of calcium (20nmol) were added to cells until MPT was induced, accompanied with the release of accumulated Ca^{2+}. The calcium capacity was expressed as nmoles of Ca^{2+}/10^6 cells.

Measurement of cytosolic calcium levels

Cytosolic calcium levels were analysed using the cell-permeable calcium-sensitive fluorescent dye Fluo-4AM (Invitrogen-Molecular Probes, Eugene, OR) and a confocal microscope (Zeiss LSM 510 META). Cells were seeded on glass slides and incubated for 30min at 37°C and 5% CO_2 in a Krebs-Ringer

solution [119mM NaCl, 2.5mM KCl, 1.0mM NaH_2PO_4 (monobasic), 2.5mM $CaCl_2$ $2H_2O$, 1.3mM $MgCl_2$ $6H_2O$, 20mM HEPES, 11mM D-glucose (dextrose) $C_6H_{12}O_6$, pH 7.4] containing 5µM Fluo-4/AM. After a washing step cells were examined, images acquired for the desired amount of time/frequency and drugs of interest were bath-applied.

5. Short summary of the papers

Paper I:

Kruspig B, Nilchian A, Bejarano I, Orrenius S, Zhivotovsky B, Gogvadze V. **Targeting mitochondria by α-tocopheryl succinate kills neuroblastoma cells irrespective of MycN oncogene expression.**
Cell Mol Life Sci. 2012 Jun;69(12):2091-9.

The amplification of the *MycN* oncogene is a feature of a very aggressive and highly proliferative subset of NBs, which is characterized by a poor prognosis in patients. But the significance of MycN in tumourigenesis and cancer therapy is still not fully elucidated as on the other hand *MycN* overexpression was shown to sensitize cells to apoptosis induction[18]. To further investigate the role of MycN in sensitivity towards drug-induced cell death we took advantage of a NB cell line stably transfected with a Tet-Off system, which allows to specifically switch off the overexpression of the proto-oncogene *MycN* by addition of the antibiotic doxycycline[173]. As chemotherapeutic drugs two different compounds were utilized, the conventional anticancer drug cisplatin and the redox-silent vitamin E analogue α-TOS. α-TOS belongs to a relatively new class of compounds directly targeting mitochondria, so called Mitocans, and was previously shown to induce cell death via binding to Complex II and induction of calcium influx in to the cell. Several methods for detecting cell death were applied, such as Western Blot and specific antibodies against apoptotic markers, fluorometric measurement of caspase activity, confocal live cell imaging to analyse changes in intracellular calcium levels as well as ROS production.

The main outcome of this study was that MycN-dependent differences in the sensitivity towards cisplatin, could be overcome by targeting mitochondria with α-TOS. This cytotoxic effect was shown to be in part dependent on α-TOS mediated calcium influx into the cells, leading to induction of MPT-dependent apoptosis. Additionally, we could show that α-TOS is able to induce cell death in a panel of NB cell lines, independent of their p53 and MycN status.

Paper II:

Kruspig B, Zhivotovsky B, Gogvadze V.

Contrasting effects of α-tocopheryl succinate on cisplatin- and etoposide-induced apoptosis.

Mitochondrion. 2013 Sep;13(5):533-8.

Targeting mitochondria i.e. via α-TOS, has been put forward as a promising strategy for tumour cell elimination by many laboratories, including ours. In particular the combination of conventional, mainly DNA-damage inducing drugs, with substances targeting mitochondria, are currently being tested and discussed. In this paper we were comparing the effect of α-TOS in a co-treatment setting with the two conventionally used anticancer drugs, cisplatin and etoposide. To this end, we utilized a variety of cancer cell lines (NB, colon and lung), which were exposed to different concentrations of α-TOS in combination with etoposide and cisplatin. As readout for the induced toxicity several parameters of apoptosis were analysed using Western Blot, fluorometric quantification of caspase-activity, as well as apoptotic morphology using confocal microscopy.

Obtained results demonstrate the contrasting effects of low and higher concentrations of α-TOS in combination with etoposide and cisplatin. When combined with cisplatin, low concentration of α-TOS strongly attenuated toxicity, whereas in a co-treatment with etoposide a sensitization could be observed. Higher α-TOS concentrations caused sensitization towards both cisplatin and etoposide. We propose that α-TOS is being cleaved by cytosolic esterases into its two components, vitamin-E and succinate. In addition, we could demonstrate that the succinate moiety mediates the protective effect against apoptotic stimuli by stimulation of Complex II activity. These findings imply to use α-TOS with caution when combined with other chemotherapeutic drugs.

Paper III:

Kruspig B, Skender B, Zhivotovsky B, Gogvadze V

Inhibition of Mitochondrial Complex II by Thenoyltrifluoroacetone Potentiates the Efficacy of Anticancer Therapies.

Manuscript.

Cisplatin, an alkylating agent, is commonly used to treat a variety of cancers, though chemotherapy often fails due to development of cisplatin-resistance or from unwanted side effects associated with therapy. One strategy to avoid these shortcomings is to increase the cytotoxic efficacy of cisplatin by co-administering a chemopotentiating drug, thereby facilitating cisplatin dose reduction. In this study, the mitochondrial Complex II inhibitor TTFA was combined with cisplatin to determine whether mitochondrial destabilization can potentiate the therapeutic effectiveness of the drug. A panel of NB cell lines with varying sensitivity towards chemotherapy was used and several different apoptotic markers and parameters of mitochondrial function were analysed.

Our results demonstrate that the specific inhibition of the ubiquinone-binding site of Complex II by TTFA synergistically sensitized otherwise resistant NB cells towards cisplatin. The underlying mechanism was identified to be mediated by TTFA-induced ROS production in the mitochondria, leading to permeabilization of the OMM and activation of the mitochondrial apoptotic pathway. We could further show that TTFA's chemosensitizing effect requires Complex II activity, as inhibition of the SDHA subunit led to a partial abrogation of the effect, and, the only cell line that could not be sensitized to cisplatin, exhibits an impaired Complex II function. These findings demonstrate the potential of TTFA to be utilized as an adjuvant to increase the efficacy of cisplatin in treating certain types of NB.

Paper IV:

Kruspig B, Nilchian A, Orrenius S, Zhivotovsky B, Gogvadze V.

Citrate kills tumor cells through activation of apical caspases.

Cell Mol Life Sci. 2012 Dec;69(24):4229-37.

The metabolic peculiarities of tumour cells, such as the Warburg effect, referring to the increased glycolysis even under normoxic conditions, represent an interesting target for anticancer therapy. Numerous approaches have been employed in order to suppress various steps of glycolysis, such as treatment with 2-deoxyglucose or 3-bromopyruvate. Citrate, a substrate of the Krebs cycle was shown to possess an inhibitory effect on several critical enzymes of the glycolytic pathway, such as phosphofructokinase or pyruvate dehydrogenase. In addition, citrate also plays an important role in other metabolic processes like fatty acid synthesis. When applied exogenously to malignant cells, citrate was shown to either induce cell death directly or sensitize cells to drug-induced apoptosis. The aim of this study was to further investigate the mechanisms of citrate-induced cell death. A panel of NB cell lines was exposed to citrate and several apoptotic markers were analysed, using Western Blot, fluorometric quantification of caspase-activity, as well as confocal microscopy to assess apoptotic morphology. Furthermore, levels of cellular ATP were measured using a bioluminescent assay, glycolytic activity by quantification of lactate production in the cell and calcium levels were measured using a calcium-sensitive electrode.

We demonstrated that the apoptotic effect of citrate is mediated by the activation of the initiator caspases-2 and -8 and not via inhibition of the glycolytic pathway as it was proposed by other publications investigating the cytotoxic effect of citrate. Furthermore, as apoptosis induction was independent of the receptor-mediated pathway, we proposed a model of kosmotropic activation of the apical caspase 2- and -8 by citrate.

6. Discussion

Role of Complex II in cancer

SDH mutations promote tumourigenesis

Reflecting this crucial function in cell death, it is of no surprise that Complex II was additionally found to be a tumour suppressor in several tumour entities. The first direct connection to cancer was established in familial paraganglioma and pheochromocytoma cases that were found to carry germline mutations in SDHB, SDHC and SDHD[176-179]. Later, mutations were also reported in renal cell carcinoma and gastrointestinal stromal tumour (GIST)[180, 181]. Besides its role in cell death, another mechanism was discovered explaining how Complex II mutations can facilitate tumourigenesis. Early reports linking SDH mutations with familial cancer cases revealed a concomitant activation of hypoxia-inducible genes[182], which was shown to be due to cytosolic accumulation of succinate, a result of impaired SDH function, ultimately leading to stabilization and activation of Hif-1α[183]. Under normoxic conditions, this master regulator of hypoxic adaptation is targeted for proteasomal degradation by a process involving the von Hippel-Lindau (VHL) ubiquitin-ligase and Hif-prolyl hydroxylase domain (PHD) enzymes, which serve as intracellular oxygen-sensors since oxygen is a crucial cofactor for their enzymatic activity. Besides oxygen this enzyme requires several other cofactors, including α-ketoglutarate (αKG), which is metabolized to succinate during this reaction. Elevated cytosolic levels of succinate cause impairment of the 2-oxoglutarate (2-OG)-dependent Hif1-PHD activity by product inhibition, creating a so-called pseudo-hypoxic state due to Hif-1α stabilization, independent of cellular oxygen levels. Mutations in SDH subunits can lead to activation of an oncogenic signalling pathway, leading to Hif-1α stabilization and concomitant expression of genes involved in glycolysis, angiogenesis, metastasis, and thereby facilitate tumourigenesis. Interestingly, the cytosolic level of succinate was also shown to be elevated by inflammatory processes independently of SDH mutations. Under these conditions, succinate is being derived from glutamine-dependent anaplerosis, as well as the γ-aminobutyric acid (GABA) shunt and was shown to serve as an inflammatory signal, and also leading to Hif-1α activation[184].

Hence, the tumorigenic effect of inflammatory processes could be in part due to an increase in the cytosolic level of succinate.

Lately, similarly to isocitrate dehydrogenase (IDH) mutations, mutations in SDH and in fumarate hydratase (FH) were reported, leading to accumulation of succinate or fumarate, respectively, and concomitant inhibition of several α-KG-dependent dioxygenases by product inhibition[185]. As discussed earlier this class of enzymes, which includes histone demethylases and DNA hydroxylases, plays an important role in hydroxylation reactions of several different substrates in the cell, such as proteins and DNA. As a result of SDH mutations the accumulation of succinate was linked to a hypermethylated phenotype in GIST, as well as paraganglioma[186, 187]. The severity of these epigenetic modifications, leading to gene silencing, correlates with the aggressiveness of certain tumour subtypes, as the highly malignant SDHB-mutated tumours were also shown to have the most pronounced hypermethylated state[187]. These findings highlight a new interesting interplay between the TCA cycle and tumour-specific epigenetic changes, and further represent a potential therapeutic target in these malignancies. Clinically relevant are the findings that patients harbouring mutations in SDHB and SDHD also had a higher probability of increased succinate levels in plasma[188], which suggests exploring this method for diagnostic purposes, thereby providing a more cost-effective and easy screening for cancers harbouring SDH mutations.

Role of Complex II in chemotherapy

Complex II and succinate are not only associated with tumourigenesis but also utilized as targets for anticancer therapy. Therapeutic approaches vary depending on the type of tumours and their specific characteristics. Despite only representing a small fraction of tumours, as mentioned earlier, certain familial cancers carry SDH mutations that were shown to be important for tumour growth. As a promising new approach for treatment of these cancers, cell-permeable αKG derivatives were successfully tested in both cellular and xenograft models. The underlying rationale is that αKG, a cofactor for PHD enzymes, restores enzymatic activity of these hydroxylases, causing Hif-1α destabilization and thereby reverses the pseudo-hypoxic condition and pro-tumorigenic effect[189, 190].

For the vast majority of tumours that lack mutations in Complex II subunits, different chemotherapeutic drugs targeting Complex II have been tested. One of the most promising compounds was found to be the redox-silent vitamin E analogue α-TOS, which was described in detail earlier. In **Paper I** we could demonstrate that α-TOS is able to kill cancer cells irrespective of their p53- or MycN status and lack of the initiator caspase-8, which frequently is the underlying mechanisms of resistance to chemotherapy. We believe that this is based on the ability of α-TOS to induce both mechanism for OMM permeabilization, Bax/Bak-dependent and MPT-dependent. It was shown previously that α-TOS can upregulate Noxa, a proapoptotic member of the Bcl-2 family of proteins, which is able to displace antiapoptotic members from Bax/Bak and thereby facilitate OMM permeabilization and apoptotic cell death. In the absence of *MycN* overexpression, this upregulation of Noxa was strongly attenuated, while the apoptotic response was similar in both cell lines, with- or without *MycN* expression. These findings suggest that the MPT-dependent mechanism for OMM permeabilization is more important for the outcome of α-TOS. We could confirm this hypothesis, as α-TOS induced similar influx of calcium into cells, independent of *MycN* expression and apoptosis could be significantly abolished by chelating of intracellular calcium or inhibition of mitochondrial calcium uptake. Furthermore, ROS production was also found to be independent of *MycN* expression, and in combination with mitochondrial calcium uptake, facilitated OMM permeabilization. The potential of α-TOS to overcome chemoresistance was further demonstrated in cells carrying mutations in p53 and lacking caspase-8 expression, in which α-TOS could induce apoptosis in a similar manner.

The cancer specificity of α-TOS is thought to be based on a higher antioxidant capacity and elevated activity of intracellular esterases in non-malignant cells, which can cleave and thereby detoxify α-TOS into its two non-toxic components, the antioxidant α-tocopherol and succinate[191-193]. Big effort has been made to improve the biochemical properties of α-TOS by introducing structural modifications, resulting in a more specific accumulation in the mitochondria and higher efficacy[194, 195]. It should be taken into account that despite its well documented toxic effect, we could show that when used at low concentrations in a co-treatment setting with conventional anticancer drugs, α-TOS could either sensitize cells to cell death, in case of combination with

etoposide, or protect cells, when treated together with cisplatin **(Paper II)**. We could further demonstrate that the underlying mechanism for this protective effect was mediated by the succinate moiety of α-TOS, which is deliberated upon its esterase-dependent cleavage. Thus, α-TOS should be used with caution in a co-treatment setting with conventionally used anticancer drugs.

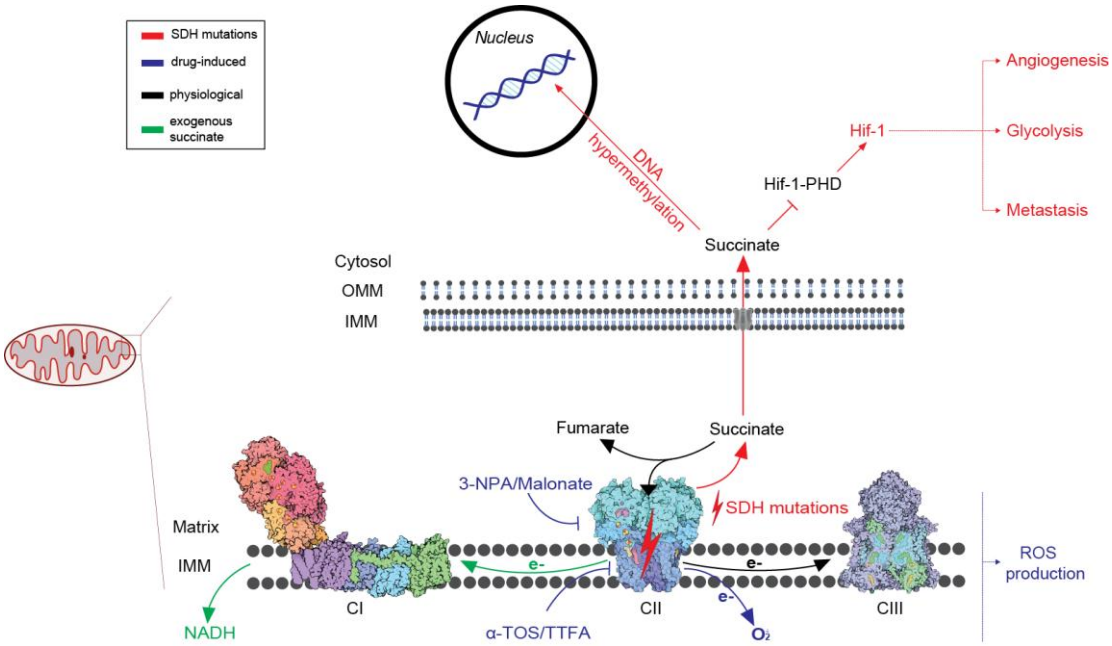

Fig. 4: Role of Complex II in cancer metabolism and tumourigenesis, and potential chemotherapeutic interventions. Under physiological conditions (black), succinate, a substrate of the TCA cycle, is oxidized to fumarate by the SDHA subunit of Complex II/SDH, and electrons are transferred further via subunit SDHB to the ubiquinone binding site formed by subunits SDHC and SDHD. Ubiquinone is reduced to ubiquinol and subsequently relays the electrons to Complex III of the respiratory chain. Specific drugs (blue) may be used to inhibit this process at different subunits of the SDH complex. 3-NPA and malonate inhibit the SDHA subunit and thereby prevent oxidation of succinate. α-TOS and TTFA bind and inhibit the ubiquinone binding site, which may lead to leakage of electrons and the formation of superoxide radicals. Mutations (red) in different subunits of SDH are found in several cancers and can cause ROS formation and accumulation of succinate. Increased cytosolic succinate levels may induce Hif-1α stabilization by product inhibition of HIF-1-PHD, resulting in concomitant pseudo-hypoxic conditions, and thus induction of angiogenesis, glycolysis, and metastasis. Further, accumulation of succinate in the cytosol may lead to DNA hypermethylation by inhibition of αKG-dependent dioxygenases. Exogenous (green) supply of succinate can induce a reversed electron flow, monopolization of the respiratory chain, and an increase in the pool of pyridine nucleotides, and thereby protect mitochondria from oxidative stress and OMM permeabilization. Figure adapted from BioCarta and David Goodsell & RCSB Protein Data Bank and republished with permission[84].

Based on our findings we concluded that targeting Complex II can modulate the cellular response to anticancer treatment. Stimulation of Complex II activity by supplying exogenous succinate protected cells from cisplatin-induced

apoptosis as assessed by caspase-3-like activity, release of cytochrome *c*, or PARP cleavage **(Paper II)**. Succinate is usually only poorly taken up by cells, but when applied at high concentrations it can slowly penetrate through the cell membrane. The protective effect of succinate is apparently based on the ability of this substrate to monopolize the respiratory chain of mitochondria[196]. This monopolization causes an even greater reduction of mitochondrial pyridine nucleotides than that achieved by substrates of Complex I[197]. Various processes were shown to be affected by the redox state of mitochondrial pyridine nucleotides, such as detoxification of ROS via the glutathione-dependent defence system[198], or MPT-induction[199]. Thus, when mitochondria are oxidized by succinate they can accumulate 3–4-times more calcium before opening of the MPT-pore, as in comparison with mitochondria using NAD-dependent substrates[200]. Moreover, oxidation of succinate makes mitochondria more resistant to oxidative stress. In the absence, as well as in the presence, of the oxidant t-butylhydroperoxide, mitochondria retained more calcium with succinate than with beta-hydroxybutyrate as the respiratory substrate[201].

In contrast, inhibition of Complex II by TTFA markedly stimulated cell death induced by sub-toxic doses of conventional chemotherapeutic drugs, such as cisplatin, etoposide and doxorubicin (**Paper III**) and was able to sensitize otherwise resistant cancer cells to treatment. This ROS-dependent chemo-sensitization mechanism exemplifies the potential role of Complex II in chemotherapy and suggests a combination therapy of conventional anticancer drugs with Complex II inhibitors.

Citrate as a potential anticancer drug

Citrate plays a central role in cellular metabolism as a substrate of the mitochondrial TCA cycle and also as a crucial switch between metabolic pathways, including OXPHOS, glycolysis, fatty acid synthesis and gluconeogenesis. Citrate is formed in the mitochondria either by the canonical reaction by citrate synthase out of acetyl-CoA and oxaloacetate, or via a reversed TCA cycle derived from αKG mediated by IDH. Within the mitochondria the primary function of citrate is to fuel the TCA cycle and thus, contribute to cellular ATP production via OXPHOS. In addition, citrate may act as an allosteric regulator of several metabolic enzymes. Citrate was shown to repress the TCA cycle by inhibition of PDH- and SDH-activities[202, 203], whereas

after its cytosolic translocation via the specific citrate carrier (CIC)[204, 205], it acts as a glycolytic inhibitor. Thus, in normal cells citrate, together with ATP, plays a pivotal role in the mediation of the Pasteur effect[206], via attenuation of the enzymatic activity of phosphofructokinase 1 (PFK1). A similar effect was demonstrated for 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFK2)[207]. In addition to its role in regulating catabolic processes, cytosolic citrate stimulates anabolic reactions such as lipid synthesis by enhancing acetyl-CoA carboxylase activity and by serving as a source for acetyl-CoA itself via cleavage by ATP-citrate lyase (ACLY), ultimately inducing fatty acid synthesis. ACLY was further shown to be important for metabolic modulation, mediated by oncogenic PI3K/AKT signalling[208], and its knockdown caused tumour suppression and cell differentiation[209, 210]. The level of citrate in the cytosol is determined by the mitochondrial export rate and can be modulated by differential expression of the CIC gene. Several regulatory transcription factors, such as Sp1[211], or NF-κB, can induce or inhibit CIC expression linking CIC and cytosolic citrate levels with pro-inflammatory pathways[212]. In tumour cells CIC was found to be transcriptionally upregulated and its inhibition has anti-tumour activity, potentially opening a therapeutic window for specific CIC inhibitors such as 1,2,3-benzene-tricarboxylate (BTA)[213]. Furthermore, citrate-mediated changes of fructose 2,6-bisphosphate (F2,6P) levels significantly influence tumour cell proliferation. Inhibition of PFK2 suppressed, whereas overexpression of PFK3 stimulated cell proliferation[214, 215]. Hence, changing cytosolic citrate levels is a powerful tool for modulation of cellular energy metabolism, as well as important anabolic processes.

Recently, another cancer related role of citrate was proposed, suggesting the potential usage of citrate for therapeutic approaches in cancer. Citrate was shown to either induce cell death directly in various tumour cell lines[216, 217] or facilitate toxic effects of conventional anticancer drugs such as cisplatin in malignant pleural mesothelioma cells[218]. These toxic effects were explained by the ability of citrate to chelate calcium, as well as to suppress glycolysis and thereby reducing cellular ATP levels, cellular growth and the capacity to repair cisplatin-induced DNA damage. A reduction of Mcl-1 levels has been reported to be a consequence of treating cells with citrate, causing loss of viability and ultimately cell death[219].

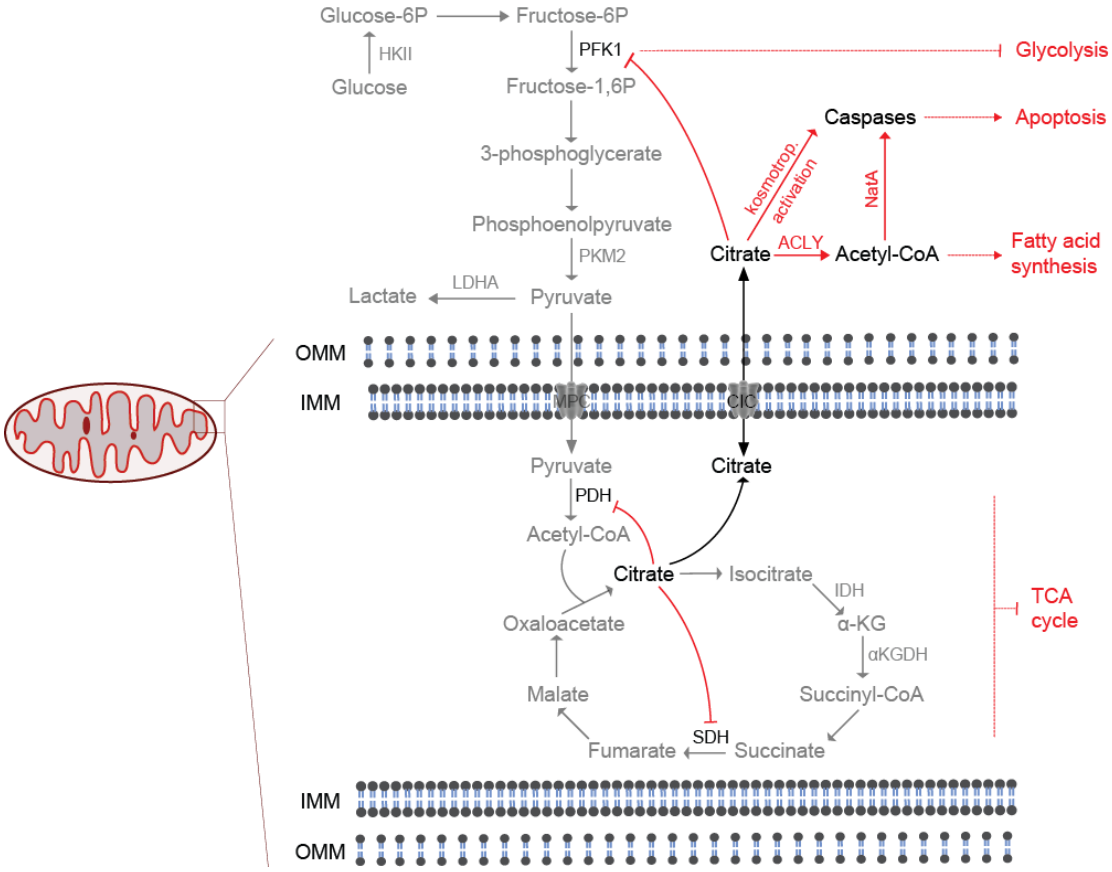

Fig. 5. Effects of citrate treatment on cellular metabolism and mechanisms of citrate-induced toxicity. Citrate treatment may cause suppression of the TCA cycle by inhibition of PDH and SDH activity, as well as glycolysis, by attenuating PFK1. Increased cytosolic citrate can enforce fatty acid synthesis after conversion to acetyl-CoA by ACLY. Dimerization and activation of apical caspase-2, -8, induced by the kosmotropic feature of citrate, as well as post-transcriptional modification by N-α-acetylation of caspase-2, -3, -9 can facilitate apoptotic cell death. Figure adapted from BioCarta and reprinted with permission[84].

Even in a clinical setting the anticancer effect of citrate was demonstrated. Two cancer patients suffering from medullary thyroid cancer or primary peritoneal mesothelioma were reported to have improved in their health condition after oral administration of citrate as a sole anticancer treatment[220, 221]. In **Paper IV** we are proposing an alternative mechanism of citrate-induced toxicity, showing activation of initiator caspases by citrate in a set of NB cell lines. Sensitivity of cells to citrate depended on the expression of caspase-8. This member of the initiator family of caspases can be activated by a so-called ‘‘induced proximity model” via adaptor-mediated clustering of zymogens[222]. Strikingly, in a study using a cell free system, citrate was able to induce both dimerization and activation of caspase-8. The underlying mechanism was proposed to be the kosmotropic feature of citrate[223, 224]. Kosmotropes are salts that promote and stabilize water–water interactions and thereby are able to stabilize

intermolecular interactions in macromolecules such as proteins. This kosmotropic property of citrate might also be involved in the activation of apical caspases *in vivo*. Similar to caspase-8, we could demonstrate that caspase-2 can also be stabilized and activated by citrate.

After conversion to acetyl CoA, citrate was shown to affect caspase activation also by another mechanism, in particular, modulation of protein N-alpha-acetylation. Acetyl-CoA is a key cofactor for N-alpha-acetylation and changes in its cytosolic abundance affect the activity of N-acetyltransferase protein complexes (NatA, NatB, NatC, NatD and NatE) and by these means also the posttranscriptional modification and activation of target proteins, including proapoptotic proteins such as caspase-2, -3 and -9[225]. This exemplifies the tight interplay between metabolism and apoptosis, which was shown for several metabolic pathways[226-229]. Strikingly, this interconnection between apoptotic pathways and cellular metabolism is not unidirectional, since the apoptosis-related protein Bcl-X_L was shown to influence regulation of mitochondrial membrane potential[230, 231]. Overexpression of this antiapoptotic Bcl-2 family member, a common feature in tumours in order to avoid apoptosis, was further shown to significantly decrease level of cytosolic citrate, leading to an inhibition of N-alpha-acetylation of caspases and thereby contributing to the antiapoptotic function of Bcl-X_L[232]. Citrate demonstrated a synergistic effect with ABT-737, a small molecule inhibitor of antiapoptotic members of the Bcl-2 family, which is currently being tested in clinical trials. In combination citrate and ABT-737 strongly inhibited the expression of Mcl-1 and thereby caused prominent apoptotic cell death[219]. The underlying mechanism of citrate-mediated downregulation of this antiapoptotic protein is unclear yet, but the authors speculated that citrate could cause a drop of the cellular ATP level by inhibition of glycolysis, leading to AMPK pathway activation, which in turn could lead to a GSK3 β-mediated phosphorylation and consequent proteasomal degradation of Mcl-1.

Summarizing the current knowledge of citrate's role in cancer, being an important metabolic switch involved in tumour-related adaptations and having potential to be used as an anticancer agent, citrate is a very attractive metabolite and it will be of great importance to further elucidate how the different molecular features of citrate are orchestrating its overall effect on cancer cells.

7. Conclusions and outlook

Recently, mitochondria emerged as promising targets for anticancer therapy, and various therapeutic approaches as well as drugs affecting mitochondrial functions have been investigated. These mitochondria targeting drugs, also called “Mitocans”[233], aim at different aspects of mitochondrial function. One of the prime targets is the electron transport chain, with the underlying rationale to inhibit individual complexes, induce increased production of ROS and ultimately tumour cell death. Complex II inhibition can be facilitated by several different drugs and was previously shown to induce tumour cell death. Despite of numerous reports documenting the potential of Complex II to serve as a chemotherapeutic target, many aspects of the underlying mechanism and the relevance of the different Complex II subunits have not been resolved yet. With our findings we could demonstrate that targeting Complex II can overcome chemoresistance caused by various mechanisms such as mutations of the crucial tumour suppressor p53, overexpression of the oncogene *MycN*, as well as silencing of the important apoptotic protein caspase-8. Furthermore, we could show that in particular the SQR part of Complex II, formed by the subunits SDHC and SDHD, is a promising target, as its inhibition leads to ROS production and facilitation of apoptosis. We are planning to further explore this intriguing approach and are currently testing small molecular inhibitors that were biochemically optimized to target these drugs specifically to the mitochondria. By doing so we hope to significantly increase the efficacy of Complex II inhibition and a decrease of the optimal drug concentration. In the future, these compounds, after *in vitro* validation, should be tested *in vivo* for their therapeutic potential and to rule out unwanted toxicity in non-malignant tissues.

Another aspect of this work was to understand the role of mitochondrial substrates in cancer and their importance in chemotherapy. Treatment of cells with exogenous succinate, the substrate of Complex II, could significantly reduce toxicity induced by a variety of chemotherapeutic drugs. Elevated succinate levels can be a result of mutations in the subunits SDHA or SDHC, leading to an accumulation of succinate. A second possibility is treatment of cells with low concentrations of α-TOS, which can be cleaved by intracellular

esterases into vitamin-E and succinate. The role of succinate in sensitivity towards apoptosis should be further elucidated as recent findings suggest a proapoptotic function of succinate in toxicity induced by ischemic reperfusion[234].

In contrast to succinate, citrate was shown in multiple studies to induce cell death and we could reveal a new mechanism underlying this cell toxicity. A proposed model of activation of initiator caspases *in vitro*, based on kosmotropic properties of citrate put forward a potential usage of this substrate in a co-treatment setting with conventional anticancer drugs. A sensitization of cells to chemotherapy by citrate should be first tested *in vitro*, and in case of success, be confirmed in *in vivo* cancer models.

8. Acknowledgements

This PhD project was carried out at the Institute of Environmental Medicine, Division of Toxicology, Karolinska Institutet and was supported by grants from: Swedish and the Stockholm Cancer Societies, Swedish Childhood Cancer Foundation and Swedish Research Council.

During my time at the Karolinska Institutet I worked together and collaborated with many people, and I want to thank all of you for your input, support and creating a very enjoyable working atmosphere. Nevertheless, there are some people to whom I want to express my gratitude in particular:

First and foremost I want to sincerely thank my two supervisors, **Associate Professor Vladimir Gogvadze** and **Professor Boris Zhivotovsky** for giving me the best support and doctoral education I could imagine. I am still extremely grateful that you welcomed me so warmly in the lab, first as a trial period and later on as a PhD student. Your constant encouragement, support, trust and the many invaluable advices you shared with me over all these years, had a very big influence on me, as a scientist and person.

Vladimir… where to start and what to write?! I think only people who know you personally will truly understand when I say what a great pleasure it has been to work together with you over all of these (6) years!! I was extremely lucky to have been under your supervision and I want to cordially thank you for all the things you have taught me about mitochondria and all the discussions about many, many other scientific and non-science related topics. I truly appreciate all the things we shared in- and outside of the lab and all the valuable things in life you introduced me to.

Boris, for being such a scientific role model and your truly inspiring, passionate, kind, determined and respectful leadership. It has been a great pleasure working together with you and learning so many things about how science truly works. I want to thank you for always having time for me to discuss science and your advices concerning my future plans.

Sten, I want to sincerely thank you for all the practical scientific input in our meetings and assistance with preparation of the manuscripts. Your devotion and passion for science is truly inspiring.

Magnus, it was a big pleasure working together with you, taking care of the plants you left behind in your old office and discuss science and football together! **Vitaliy, "**porco zio"… "This is clearly the end" my friend! ☺ At least of our time together in the lab. Thank you for your help, scientific- and football discussions and of course for the wonderful (though too few) times on the tennis court. **Birce**, "tschiki tschicki tschik .. lai lai lom"… Without you, especially evenings in the lab would have been very boring and I want to sincerely thank you for all the nice time we spent together!

I also want to thank former members of the group with whom I worked and spend a lot of time together. In particular to my friend **Olga** for being so patient with me over all these years and her legendary special birthday surprises; **Gabi**, for always having a lot of fun together; **Azizazi,** my most favourite Master student ever, it was a big pleasure working together with you!

Emiii…. for teaching us so many wonderful very useful phrases, which we still use and will never forget. And of course your amazing opera performances in the cell lab, elevator and all other places you thought no one would hear you! ☺ **Nadeem**, thank you for all the interesting cultural and political discussions. My friend **Dorien**, not only have you brought the best cakes and cookies to the lab in all this years, but you also managed to come back and visit us more than anybody else! Thank you for that!

Special thanks also to two visitors and friends from Brno, **Iva,** for all the fun we had together in Stockholm, Brno and Mikulov, and for introducing me to my most favourite Czech beer! **Belma,** for your patience and great time together in the lab with a lot of interesting & light intellectual discussions! I also want to say a big thanks to **Flor,** for all the extremely "spontaneous" lunches (at exactly 11:43!) and for being just a great friend.

Anna, Jeremy, Lian, Kadri, Giulia, Belen and of course **Katarina** for all the fun time in- and outside of the lab together!! I also want to say thanks to **Erik**, **Helin** and the members of their groups, in particular to **Helin** as I would not be in this lab without you!

To all my friends, in particular my "Bruder" **Jens, Kareem, Hermi, Ines** and **Christian**… I want to thank you for your great friendship and support.

My dear **Olga**, without you I would not be in Stockholm in the first place and would not have been able to manage all these years here without your

unconditional support and care. I can’t thank you (and **Punyatinha)** enough for this!

Last but definitely not least: möchte ich mich von ganzem Herzen bei meiner Familie für Ihre Liebe und Unterstützung jeglicher Art bedanken! Ihr seid die beste Motivation und Ansporn den man sich vorstellen kann.

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