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MITOCHONDRIA TARGETING AND ITS CONSEQUENCES FOR CELL DEATH IN NEUROBLASTOMA

Kadri Valter

Karolinska Institutet

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Mitochondria targeting and its consequences for cell death in neuroblastoma

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

Kadri Valter

Principal Supervisor:
Vladimir Gogvadze
Karolinska Institutet
Institute of Environmental Medicine
Division of Toxicology

Co-supervisors:
Professor Boris Zhivotovsky
Karolinska Institutet
Institute of Environmental Medicine
Division of Toxicology

Professor Marie Arsenian-Henriksson
Karolinska Institutet
Department of Microbiology, Tumor and Cell Biology

Opponent:
Professor Hans-Uwe Simon
University of Bern
Faculty of Medicine
Institute of Pharmacology

Examination Board:
Professor Helena Jernberg Wiklund
Uppsala University
Department of Immunology, Genetics and Pathology

Professor Lars-Gunnar Larsson
Karolinska Institutet
Department of Microbiology, Tumor and Cell Biology

Professor Ralf Morgenstern
Karolinska Institutet
Institute of Environmental Medicine
Division of Biochemical toxicology

The public defense of this thesis will take place in Petrénsalen, Nobels väg 12b, Karolinska Institutet, Solna

Friday, 18th of January, 2019 at 9.30 AM

If there's a will, there's a way

Because life's too short

ABSTRACT

Mitochondria are one of the central regulators of several physiological and pathological processes. For instance, homeostatic glycolysis, citric acid cycle, and oxidative phosphorylation are all regulated by mitochondria. In addition, these organelles play a pivotal role in apoptosis, a process of programmed cell death. In detail, the permeabilization of the outer mitochondrial membrane and the release of pro-apoptotic proteins from the intermembrane space are considered as an initiating and the no return step of apoptosis. Therefore, apoptosis induction and targeting mitochondria of cancer cells is a promising strategy for tumor cell elimination. During the studies of this PhD project mitochondria targeting and its consequences for cell death in neuroblastoma (NB) were investigated.

In **Paper I** we demonstrated how low doses of the mitochondrial Complex II inhibitor thenoyltrifluoroacetone (TTFA) can sensitize a panel of chemoresistant NB cells for cisplatin treatment. Increase in cell death was due to TTFAs specific inhibition of the ubiquinone-binding site of succinate dehydrogenase (SDH) that led to the formation of reactive oxygen species (ROS). Excessive ROS production resulted in the cytochrome *c* release from the intermembrane space of mitochondria and activation of apoptosis. However, the co-administration of TTFA and cisplatin increased cell death only in NB cell lines with functionally active SDH. These results revealed how mitochondria targeting can be used to raise treatment efficacy, but previous testing of SDH activity is needed for successful outcome.

Many of therapeutic drugs damage DNA, but their ability to target mitochondria is unknown. The effect of conventionally used chemotherapeutic drugs on mitochondria was tested in **Paper II**. Obtained results revealed etoposide-triggered suppression of Complex I respiration. Blockage of the electron chain led to the ROS formation, which did not induce apoptosis. However, cell death was detected after removal of glutamine, a precursor of the antioxidant glutathione. Therefore, etoposide also sensitizes mitochondria and can direct the mitochondrial membrane properties to apoptosis.

Several tumors, such as NB, are dependent on glutamine and targeting glutamine metabolism offers a rationale for treatment improvement. The effect of glutamine withdrawal on anti-cancer drug treatment of NB cells was studied in **Paper III**. We discovered a contrasting effect of drugs in glutamine deprived environment. Glutamine removal inhibited etoposide-induced, but significantly increased cisplatin-induced apoptosis, suggesting the activation of distinct mechanisms. Nevertheless, targeting glutamine metabolism could be considered as part of anti-cancer therapy, but further studies to understand the mechanism of this finding are needed.

Taken together, the findings of this PhD thesis have given new insights into mitochondria targeting for tumor cell elimination. We found new sensitizing effects of TTFA and etoposide that target respiratory chain complexes. Furthermore, the PhD project elucidated the role of glutamine metabolism in the outcome of NB treatment, which can be used as a basis for further studies.

LIST OF SCIENTIFIC PAPERS

I. Kruspig B, **Valter K**, Skender B, Zhivotovsky B, Gogvadze V (2016). **Targeting succinate:ubiquinone reductase potentiates the efficacy of anticancer therapy.** Biochimica et Biophysica Acta, 1863: 2065-2071

II. **Valter K**, Maximchik P, Zhivotovsky B, Gogvadze V. **Distinct effects of etoposide on glutamine-addicted neuroblastoma.** (manuscript, submitted)

III. **Valter K**, Chen L, Kruspig B, Maximchik P, Cui H, Zhivotovsky B, Gogvadze V (2017). **Contrasting effects of glutamine deprivation on apoptosis induced by conventionally used anticancer drugs.** Biochimica et Biophysica Acta, 1864: 498-506

Additional publications (not included in the thesis)

Valter K, Zhivotovsky B, Gogvadze V (2018)
Cell death-based treatment of neuroblastoma
Cell Death and Disease 9:113

Viil J, Klaas M, **Valter K**, Belitškin D, Ilmjärv S, Jaks V (2017)
A label-retaining but unipotent cell population resides in biliary compartment of mammalian liver
Scientific Reports 7:4032297

CONTENTS

LIST OF ABBREVIATIONS

2-DG	2-deoxy-D-glucose
3-BrP	3-bromopyruvate
AIF	apoptosis inducing factor
ALK	anaplastic lymphoma kinase
AMC	7-amino-4-methylcoumarin
ANT	adenine nucleotide translocase
AOA	aminooxyacetate
Apaf-1	apoptotic protease activating factor-1
ATF4	activating transcription factor 4
ATG	autophagy-related genes
ATRX	encoding the RNA helicase
BAK	BCL-2 antagonist or killer
BAX	BCL-2-associated X protein
BCL-2	B-cell lymphoma 2
BH	BCL-2 homology
BID	BH3 interacting-domain death agonist
BITC	benzyl isothiocyanate
BPTES	bis-2-[5-phenylacetamido-1,2,4-thiadiazol-2-yl] ethyl sulfide
BSO	buthionine sulfoximine
CAT	catalase
CCCP	carbonyl cyanide m-chloro phenyl hydrazine
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
CTL	cytotoxic T-cell
dATP	deoxyadenosine triphosphate
DCA	dichloroacetate
DIABLO	direct inhibitors of apoptosis proteins binding protein
DIM	3,30-diindolylmethane
DISC	death-inducing signalling complex
DR	death receptor
DTNB	5,5'-dithiobis-(2-nitrobenzoic acid)
DTT	dithiothreitol
EGCG	epigallocatechin-3-gallate
EGTA	ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid
EndoG	Endonuclease G
ER	endoplasmic reticulum
ETC	electron transport chain
FADD	Fas-Associated Death Domain
$FADH_2$	flavin adenine dinucleotide
FBS	fetal calf serum
FDG-PET	fluorodeoxyglucose positron-emission tomography
FLIP	FADD-like interleukin-1β–converting enzyme (FLICE)-like inhibitory protein
GALNT14	polypeptide N-acetylgalactosaminyltransferase 14
GAPDH	glyceraldehyde 3-phosphate dehydrogenase

GDH	glutamate dehydrogenase
GLS	glutaminase
GM-CSF	granulocyte macrophage colony stimulating factor
GPT	glutamic-pyruvate transaminase
GPx	glutathione peroxidase
GR	glutathione reductase
GSAO	4-(N-(S-glutathionylacetyl) amino) phenylarsenoxide
GSH	glutathione
GSSG	glutathione disulphide
GST	glutathione S-transferase
H_2O_2	hydrogen peroxide
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIF-1	hypoxia-inducible factor 1
HK	hexokinase
HO_2	peroxyl radical
HTRA2	mammalian homolog of the bacterial high temperature requirement protein
HygroB	hygromycin B
IAP	inhibitor of apoptosis proteins
ICAD	inhibitor of caspase-activated DNase
IL-2	interleukin-2
IMM	inner mitochondrial membrane
LDH	lactate dehydrogenase
LIN28B	encoding lin 28 homolog B
MCL-1	myeloid cell leukemia-1
MES	2-(N-morpholino)ethanesulfonic acid
MOMP	mitochondrial outer membrane permeabilization
MPTP	mitochondrial permeability transition pore
NAC	N-acetylcysteine
NADH	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
NB	neuroblastoma
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
O_2^-	superoxide anion
OCR	oxygen consumption rate
$OH^\bullet$	hydroxyl radical
OMM	outer mitochondrial membrane
OXPHOS	oxidative phosphorylation
PARP	poly(ADP-ribose) polymerase
PBR	peripheral benzodiazepine receptor
PBS	phosphate-buffered saline
PDH	pyruvate dehydrogenase
PHOX2B	paired-like homeobox 2b
PiC	phosphate carrier
PPP	pentose phosphate pathway
P/S	penicillin/streptomycin

ROS	reactive oxygen species
SDH	succinate dehydrogenase
Smac	second mitochondrial activator of caspases
SOD	superoxide dismutase
tBID	truncated BID
TCA	tricarboxylic acid cycle
TERT	encoding telomerase reverse transcriptase
TMRE	tetramethylrhodamine ethyl ester
TNF	tumor necrosis factor
TNFR1	TNF receptor 1
TRADD	TNFR-associated death domain protein
TRAIL	TNF-related apoptosis-inducing ligand
Trx	thioredoxin
TTFA	thenoyltrifluoroacetone
UbQ	ubiquinone
VDAC	voltage-dependent anion channel
αKG	α-ketoglutarate
α-TOS	α-tocopheryl succinate
ΔΨm	mitochondrial transmembrane potential

1 INTRODUCTION

1.1 CANCER

Cancer is an "umbrella" term for a group of approximately 300 malignant diseases, which involve excessive cell growth and their potential capability to spread into surrounding tissue and organs. In comparison, benign tumors also have redundant cell division, but there is no invasion to other parts of the body. Generally, cancer "type" is named after the tissue of origin; however, the classification has become more specific. Every year about 14.1 million people and about 165,000 children under 15 years of age worldwide get cancer diagnosis and altogether 90.5 million people had cancer in 2015. Each year malignant growths cause about 8.8 million deceased people, which corresponds to second leading cause of mortality and 15.7% of all deaths.[1-5] Therefore, malignant tumors are a burden to all human societies.

There are several possible treatments for cancer, for example surgery, radiation-, chemo-, immune- and targeted therapies, and any combination of these. The outcome of the therapy depends on the tumor type and severity of the disease at the start of treatment. At the tissue level, cancer is a complex and heterogeneous disease, which makes a specific diagnosis and efficient treatment challenging. Five-year survival rate for developed world adult is approximately 66% and 80% in children under 15 at diagnosis.[1,2,4,6]

Worldwide the most common type of cancer in males is lung cancer and in females breast cancer. Whereas, in children acute lymphoblastic leukemia and brain tumors prevail. Inherited genetic defects are responsible for approximately 5–10% of tumors.[4,7] Due to better health care people live longer, but the risk of developing cancer rises considerably[8]. The possibility of cancer can be decreased by avoiding tobacco smoking[9], alcohol[10], and excessive body weight[11]. Other factors, such as infections[12], UV radiation[13], and pollutants[13,14], are more difficult to control and can nevertheless introduce genetic changes in the cells. However, many genetic alterations are necessary to develop a malignant outgrowth[3].

Cancer development is a multistep process that occurs over a longer time period and involves several genetic mutations and dysfunctions of cellular programs, such as activation of oncogenes, cell proliferation, and inactivation of tumor-suppressor genes, immune system, DNA repair, and cell death in the pre-malignant cell. Development of metastasis, also known as spreading within the body, which meddles physiological functions and strains the tissue, is the main reason for malignancy-related deaths.[3,15-17]

Hanahan and Weinberg have summarized the Hallmarks of Cancer that in detail describes the processes that regulate tumor initiation, development, and progression. These processes are allowing replicative immortality, supporting angiogenesis, refraining from growth suppressors, maintaining proliferative signalling, triggering invasion and metastasis, and avoiding cell death. Furthermore, appearing hallmarks are resisting immune destruction and unbalancing cellular energetics. Supporting characteristics are tumor-stimulating inflammation, and genome instability and mutation.[3]

Cancer in adults can be seen as an age-related spontaneous and/or environment caused disease, whereas early developmental defects are the main reason for childhood tumors[18,19]. Infant malignancies demonstrate an aggressive phenotype and are generally diagnosed at more progressed stages compared to adult disease. Furthermore, besides differences in paediatric and adult cancer development and diagnosis, there are also peculiarities in therapeutic approaches, effects, and consequences of therapy.[20] For example, chemotherapy targets highly proliferating cells and since children's bodies are actively growing, there are more severe side effects from anti-cancer drugs than in adults. The majority of side effects are temporary, but delayed growth, cognitive problems, and formation of secondary tumors later in life are the main issues in paediatric cancer treatment.[21]

1.1.1 Neuroblastoma

The most common extracranial solid cancer in childhood is neuroblastoma (NB), which originates from primitive cells of the sympathetic nervous system[22]. The leading initiators of NB development are defects in neural crest cells originating sympathoadrenal cells. The NB can develop anywhere in the sympathetic nervous system, but the main sites are in the abdomen along the sympathetic chain and in the adrenal gland medullary region.[23] The NB has high genetic and biological heterogeneity and is, therefore, a complex disease that is under thorough investigation to better understand its origin and progression[24,25]. In Sweden, the prevalence of NB in children under 15 years is 0.7/100,000 and every year about 20 children are diagnosed with NB. Most of the incidences are diagnosed in infants under one year of age, who also have a good response to the treatment, whereas older patients have more severe outcomes. In addition, boys seem to be more affected than girls (M/F 1.22). Even though over 99% of localised tumors regress after therapy or recover spontaneously, overall survival rate five years after metastatic malignancy is still rather low – 40%. Moreover, 15% of childhood cancer-related mortality is caused by NB.[22,23,26,27]

To improve NB risk assessment and diagnosis, several classification systems have been developed. For instance, the prognosis can be assigned by investigating the degree of differentiation, the status of stroma, and the mitosis-karyorrhexis index[28]. Even more precise diagnosis can be determined by considering NB stage, patient age, histologic category, grade of differentiation, *MYCN* oncogene status, chromosome 11q status, and DNA ploidy. These factors helps therapists to designate NBs into localized (L1 and L2) and metastatic disease (M and MS)[29].

Studies have identified several germline and sporadic genomic abnormalities in *ALK* (anaplastic lymphoma kinase)[30], *GALNT14* (polypeptide N-acetylgalactosaminyltransferase 14)[31], *LIN28B* (encoding lin 28 homolog B)[32], *MYCN*[33], and *PHOX2B* (paired-like homeobox 2b) genes of NB patients[34]. In addition, excessive cell growth can be also supported by rearrangements in the *TERT* (encoded protein is telomerase reverse transcriptase)[35,36] promoter (25% of the patients) and in the transcriptional regulator *ATRX* (encoded protein is RNA helicase) (10% of diagnosed children)[37]. Furthermore, almost all NBs have chromosomal copy number alterations, for instance, more than 50% have gain of 17q (ref. 38) and 30% have loss

of 1p36 and/or 11q1 (ref. 39). All mentioned chromosomal abnormalities are summarized in **Table 1**.

Table 1. Frequency of germline and sporadic genomic rearrangements in NB[40]. Republished under CC BY 4.0.

Gene or regioon	**Function of the encoded protein**	**Modification**	**Frequency (%)**	**Reference**
ALK	Receptor tyrosine kinase	Mutation Amplification	1	30,41
MYCN	Transcription factor	Overexpression Amplification	20	33,42,43
LIN28B	Suppressor of miRNA biogenesis	Overexpression Amplification	NA	32
TERT	Telomerase reverse transcriptase	Rearrangements	25	35,36
ATRX	RNA helicase	Rearrangements	10	37
17q	NA	Gain	50	38
1p36	NA	Deletion	30	39
11q1	NA	Deletion	30	39

About 2% of NBs have hereditary background and the first determined gene responsible for innate NB was *ALK*[30,41]. In addition, approximately 20% of NBs have amplification of the *MYCN* proto-oncogene, which is particularly prevalent in treatment resistant and poor outcome patients[33,42,43]. There have been attempts to discover even more NB driver mutations and genes by the help of whole-genome sequencing, but without great success[37,44]. Therefore, the main trigger of NB is *MYCN* amplification, and cancer aggressiveness is further maintained by other supporting mutations. Thus, the status of the *MYCN* gene amplification is examined as a compulsory step for therapy specification[45].

The *MYCN* gene was discovered in 1983 as a homologous amplification of the *MYC* gene and thereby belongs to the *MYC* family of regulators and proto-oncogenes. The *MYC* family proteins are transcription factors (MYC, MYCL, MYCN) that regulate several normal cellular processes like glycolysis, glutaminolysis, mitochondrial function and biogenesis[33,46-48]. Expression of the MYCN is tissue restricted and necessary for normal embryonic and postnatal development[49]. The *MYCN* gene is located on 2p24 locus, although when amplified, copies can be also found in other regions. Amplifications of the *MYCN* gene are identified as the inducers of tumor progression and proliferation.[50,51] In addition, deregulation of MYC expression promotes apoptosis, although reduced p53 activity, overexpression of anti-apoptotic proteins, or downregulation of pro-apoptotic proteins inhibit the activation of controlled cell death[52,53]. Therefore, cancer progression is supported by MYC-driven proliferative signals and suppression of apoptosis. For studying *MYCN*–induced NB formation and development *in vivo,*

several orthotopic, xenograft and also transgenic NB mouse models have been generated that overexpress MYCN in neural crest-derived cells to induce NB formation.[54-59]

1.1.2 Neuroblastoma therapeutic approaches

Low-risk NB patients, who are often less than 6 months old, have a better than 90% survival rate and this gives a possibility to identify the subgroups where lower doses of treatment can be used or therapeutics could be even avoided due to spontaneous regression[60-62]. In contrast, high-risk patients who are older and have *MYCN* amplification need more drastic approaches involving chemotherapy, surgery, radiotherapy, biologics (cis-retinoic acid) and immunotherapy[63]. The most commonly used regimen for NB treatment contains combinatory cycles of chemotherapeutic drugs, such as cisplatin, etoposide, vincristine, carboplatin, topotecan, and cyclophosphamide[64-66]. Following treatment also involves surgery and myeloablative therapy combined with stem cell and radiotherapy. Remaining cancer cells are targeted by retinoic acid, granulocyte macrophage colony stimulating factor (GM-CSF) and interleukin (IL)-2 to pursue differentiation.[63,67-69] However, insufficient treatment, variety of side effects, individual variation of pharmacokinetic features and long-term toxicity are still a challenge. Therefore, novel ways to improve NB therapy are under investigation.

One option is to directly target the MYCN protein. Normally, MYCN has relatively short half-time, but amplification-caused high expression will increase the amount of active MYCN/MAX heterodimers. The dimerized complex can bind to the DNA and support continuous cancer progression as a transcriptional factor[70]. Thus, suppression of MYCN signalling could be used to stimulate apoptosis, neuronal differentiation and/or reduce proliferation[71]. For instance, MYCN downregulation by antisense oligonucleotides[72] and RNAi[73-75] has successfully decreased cellular proliferation, migration, and invasion. Described approach has been successful *in vitro;* however, clinical delivery of these compounds to the site of the tumor is still problematic[71,76]. Interestingly, there are also NB cell lines that exhibit a high level of *MYCN* mRNA or protein without the amplification of the gene itself. This effect can be caused by the overall disturbances in protein degradation, which does not require abnormalities in the *MYCN* gene.[77,78]

Another approach for MYCN targeting is to block the heterodimerization of MYCN and MAX, which will lead to the homodimerization of unbound MAX and NB differentiation[79]. Compounds, such as 10058-F4 (ref. 80,81) and 10074-G5 (ref. 80), which block MYCN/MAX interaction, have demonstrated cell cycle arrest, apoptosis, differentiation, and improved efficacy of treatment was seen *in vitro* and in *MYCN* transgenic mice. In addition, drug-like molecules, such as JQ1 (ref. 82), OTX015 (ref. 83), or I-BET762 (ref. 84), have been developed to suppress bromodomain and transcription-regulating proteins and thereby prohibit *MYCN* transcription and NB cell growth *in vivo*. This approach can be beneficial for *MYCN*–driven high-risk NB patients, but less toxic compounds need to be developed for clinical research.

1.2 CELL DEATH

Excessive injury can be fatal to the cell, but depending on the cause and the severity of the damage, the cell tries to repair it or dies. Process of cell death was first described in 1842 by Carl Vogt. Since the initial discovery, different cell death modes and their associated morphological features have been identified and studied. However, for a long time cell death was considered to be only an accident and its regulatory processes remained unknown.[85] Thorough studies have revealed that cell death is an important and often regulated process that helps to maintain tissue homeostasis in multicellular animals. Programmed cell death prevents abnormal and malfunctioning cells from replicating in multicellular animals, thereby eliminating potential pathological conditions. Damaged or potentially harmful cells can be eliminated by different cell death modes after stimulation of specific cellular signaling.

Historically cell death was divided into three major and morphologically distinct types: apoptosis (type I), autophagy (type II), and necrosis (type III). All these types are activated after unique signaling stimuli, which in turn activate type specific, sometimes overlapping, signaling pathways.[86,87] Even though this morphological classification is still widely in use, several other controlled cell death modes have been described. For example, entotic cell death, ferroptosis, senescence, immunogenic cell death, mitochondrial permeability transition (MPT)-driven necrosis, necroptosis, lysosome-dependent cell death, pyroptosis, parthanatos, and mitotic catastrophe[88]. These pathways are activated after specific signaling and also depend on cellular status. All of the pathways also demonstrate a substantial amount of interconnectivity and can result in an entire spectrum of morphological cell death features.

The term apoptosis was first introduced in 1972 by John Kerr, Andrew Wyllie and Alastair Currie who broadened the terminology and thoroughly described this process. Development of multicellular organisms is dependent on apoptosis[89,90]. For example, forming of the limb digits and formation on neural tube of the central nervous system in higher vertebrate development are the result of apoptotic cell death[91,92]. Apoptosis is characterized by cell shrinkage, membrane blebbing, nuclear condensation and fragmentation that lead to the formation of apoptotic bodies, which will be thereafter engulfed and eliminated by macrophages.[87] Further studies on the molecular mechanisms have revealed that apoptosis can be initiated either by a ligand binding to the cell-surface death receptor (the extrinsic pathway) or as a result of mitochondrial outer membrane permeabilization (MOMP) (the intrinsic pathway) (ref. 89,93).

Autophagy is a catabolic “self-eating” process that is triggered in metabolic stress situations. For instance, starvation can initiate autophagy to supply the cell with metabolites. In some situations, it is more beneficial to extend survival by reducing and reusing some of the cellular content, instead of generating new nutrients. Another purpose of autophagy is to eliminate damaged organelles, protein aggregates and infecting organisms.[94,95] There are three types of autophagy, such as microautophagy, Chaperone-mediated autophagy, and macroautophagy, which the last one is the most commonly addressed mechanism. During autophagy cytoplasmic macromolecules and specific organelles are covered with a double membrane to form a large intracellular autophagosome, which fuses with lysosomes to degrade the engulfed material[96,97].

This process is thoroughly regulated by autophagy-related (ATG) proteins[98]. Furthermore, extensive autophagy can be related to cell death, but the mechanism and its purpose is still under investigation[99-101].

Necrotic cell death is usually considered a more passive mode of cell death that happens in extremely harsh conditions and ends with swelling, loss of plasma membrane integrity, and rupture of the dying cells. It will result in leakage of intracellular content into extracellular space, triggering the activation of the immune system. Recent developments in the necrosis fields have, however, discovered that necrosis can also be regulated by tumor necrosis factor (TNF)-α-mediated signalling, protein kinase RIP3, activated cell-surface receptors, DNA damage, or virus infection, which will cause the controlled process called necroptosis.[102-104] Furthermore, as well as apoptosis, necrosis plays a vital role in organism development and adult tissue homeostasis[105,106].

There is also a connection between the abnormalities in the described cell death modes, cancer development, and treatment resistance[107]. For example, apoptosis can be responsible for spontaneous tumor regression and is, hence, used as a strategy for cancer treatment; however, defects in apoptosis machinery can contribute to cancer formation and resistance to therapy[3]. In addition, autophagy can help cells to survive in lack of substrates and, thus, be beneficial for tumor progression[108]. Furthermore, necrosis-driven inflammation may promote cancer growth, but it can be also used to eliminate malignant cells[109]. Therefore, studies of these cell death modes help us to understand the underlying regulatory mechanisms and how they can be used for anti-tumor therapy.

1.3 APOPTOTIC PATHWAYS

Apoptosis is the most studied subject in cell biology[110]. Apoptotic cell death is an important part of multicellular organisms' physiological and pathological processes. Cells undergoing apoptosis have very distinctive characteristics, such as aforementioned cell shrinkage, nuclear condensation and fragmentation, formation of apoptotic bodies *etc*.[87,111] Apoptosis is a genetically controlled and evolutionarily conserved process, which is triggered by various signaling pathways when cellular damage is irreparable. As mentioned above, the two core apoptotic pathways, the extrinsic (death receptor) and the intrinsic (mitochondrial), are cross-linked to increase the apoptotic signal and both of them lead to the activation of caspases (cysteine-aspartic proteases), key enzymes of apoptosis, that cleave proteins to stimulate cell death (**Figure 1**). Caspases are usually present in inactive pro-enzyme form and after activation or auto-cleavage can in turn activate other pro-caspases, resulting in protease cascade, amplification of the apoptotic signal and rapid cell death.

Most of the caspases are pro-apoptotic, but several also take part of inflammatory processes (caspase-1, -4, -5, and -11)[112] or are involved in keratinocyte differentiation (caspase-14)[113]. Some of the caspases are specific for other species, such as endoplasmic-specific apoptosis mediating caspase-12 (rodent specific)[114], or bovine specific caspase-13[115]. There are two groups of apoptotic caspases – initiators (caspase-2, - 8, -9, and -10) and effectors (caspase-3,

-6, or -7). The initiator caspases, as the name requests, activate other complexes and effector caspases. The effector caspases, for example caspase-3, cleave anti-apoptotic proteins, such as inhibitor of caspase-activated DNase (ICAD)[116] or B-cell lymphoma 2 (BCL-2)[117], and cellular structures, such as lamins and cytoskeletal-regulatory proteins[118]. Caspase activity will result in activation of DNA fragmentation and shrinkage of the cell. Another important characteristic of apoptosis is the exposure of phosphatidylserine on the surface of the cell to attract macrophages that endocytose apoptotic cells.[87,119,120]

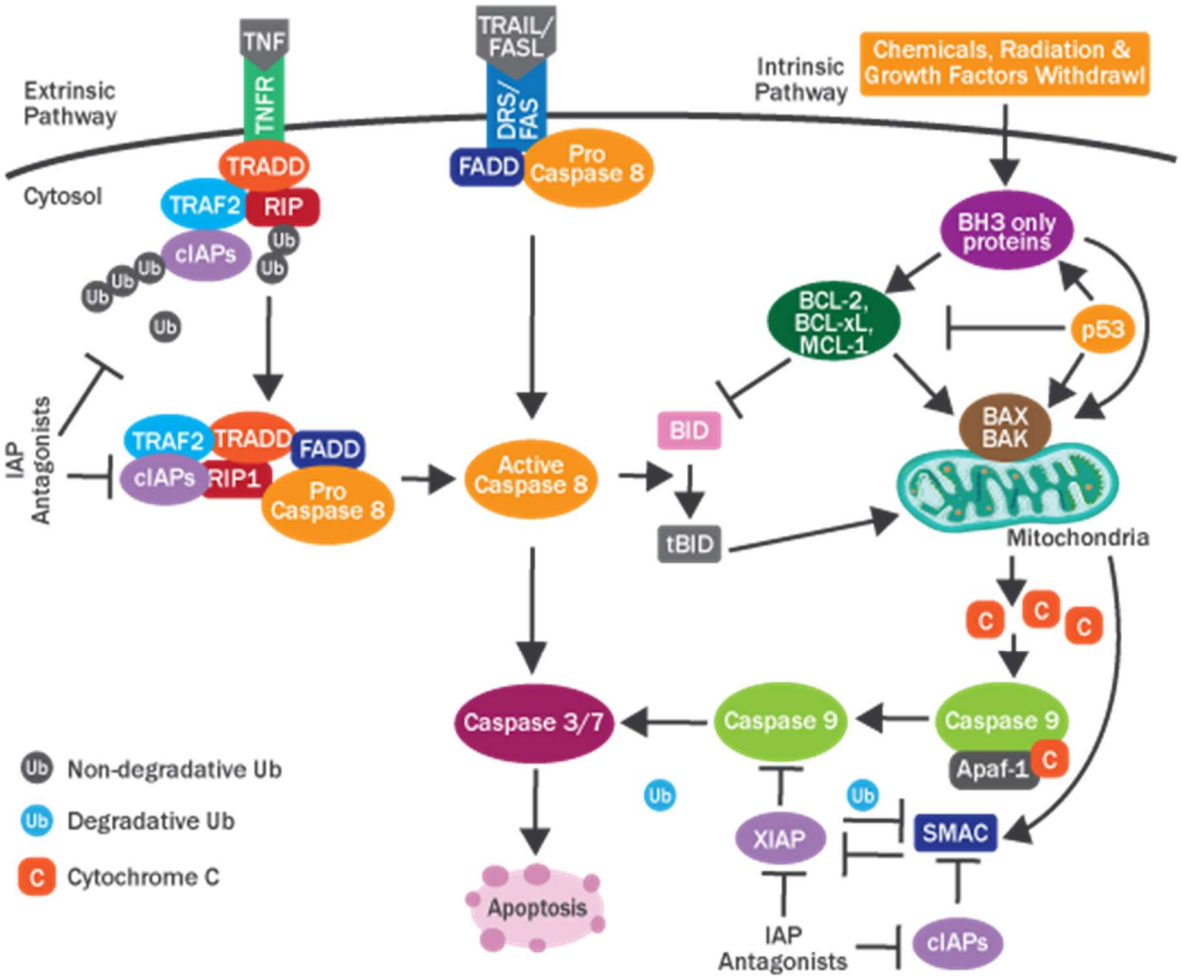

Figure 1. Extrinsic and intrinsic pathways of apoptosis. ©2018 Novus Biologicals

1.3.1 Intrinsic apoptotic pathways

The intrinsic (mitochondrial) pathway of apoptosis is activated by intracellular signals, such as extensive DNA damage (cytotoxic drugs, defective DNA repair, irradiation, reactive oxygen species (ROS)), endoplasmic reticulum (ER) stress (unfolded protein response), hypoxia, hyperthermia, viral infections, and growth factor deprivation. For example, DNA damage-induced apoptotic signals activate the tumor suppressor p53 that directs the signal *via* pro-apoptotic proteins to the mitochondria. All the intracellular signals lead to the loss of the mitochondrial transmembrane potential ($\Delta\Psi m$) and permeabilization of the outer

mitochondrial membrane (OMM) that results in the release of pro-apoptotic proteins from the intermembrane space to regulate and pursue apoptosis (**Figure 1**).[89]

Some of these released proteins are: second mitochondrial activator of caspases (Smac)/direct inhibitors of apoptosis proteins (IAP) binding protein (DIABLO), apoptosis inducing factor (AIF), mammalian homolog of the bacterial high temperature requirement protein (HTRA2/Omi), Endonuclease G (EndoG), and cytochrome *c*. Release of cytochrome *c*, an important protein of the electron transport chain (ETC), from the mitochondrial intermembrane space to the cytoplasm is considered to be a key event in apoptosis induction. Free cytochrome *c* associates with the apoptotic protease activating factor-1 (Apaf-1), pro-caspase-9, and in presence of deoxyadenosine triphosphate (dATP) forms the apoptosome complex. This complex triggers caspase-9 that thereafter initiates effector caspases-3/-7. At the same time, Smac/DIABLO and HTRA2/Omi bind to the IAPs to block their inhibiting effect on caspase-3/-7-/9.[121-128] Furthermore, caspase activity also causes further damage to the mitochondria by cleaving Complex I and II subunits and triggering ROS production[129]. Taken together, pro- and anti-apoptotic proteins are in constant dynamic equilibrium, where minor damage to the mitochondria does not activate caspase cascade and apoptosis, but more severe injury will cause increase apoptotic caspases activity.

The mitochondrial apoptotic pathway and permeabilization of the OMM can be regulated by BCL-2 family proteins that can be pro- and anti-apoptotic. Members of this family include BCL-2-associated X protein (BAX) and BCL-2 antagonist or killer (BAK) proteins that, upon intrinsic signal, oligomerize and form pores in the OMM to induce MOMP.[130] Another way to initiate the caspase cascade is through ER stress mediated caspase-12 activation, which activates pro-caspase-9[131]. This pathway is operated in rodents and not in human cells.

Furthermore, the mitochondrial pathway can also be activated independently of caspases when EndoG and/or AIF are released from the mitochondria. These proteins will move to the nucleus where they take part of chromatin condensation and fragmentation of the DNA (50-200Kb fragments)[132,133]. Nuclear AIF is responsible for fragmentation of DNA into large sections and peripheral chromatin condensation[134]; and translocated EndoG cleaves nuclear chromatin[123]. Moreover, besides blocking IAPs activity, HTRA2/Omi has also protease activity and can degrade XIAP, cIAP1, cIAP2 and Apollon. This is also confirmed by lower apoptosis activity after decreased levels of HTRA2/Omi.[135,136] In addition, the mitochondrial pathway can be indirectly activated by p53, which initiates the expression of the pro-apoptotic BCL-2 proteins, such as BH3 interacting-domain death agonist (BID), PUMA, and/or NOXA[137-139]. Furthermore, p53 can directly induce MOMP by activating BAX or BAK or by binding and inactivating anti-apoptotic BCL-2 proteins[140,141].

1.3.2 Extrinsic apoptotic pathways

Extrinsic (receptor mediated) apoptosis is triggered when specific ligands bind to cell surface death receptors (DR). The DR family consists of 8 members, which belong to TNF receptor superfamily. These receptors have cysteine-rich extracellular domains and approximately 80

amino acid cytoplasmic "death domain" that transfers the extracellular death signal into intracellular.[142] The most known DR is the tumor necrosis factor receptor 1 (TNFR1/DR1) that binds TNFα. In addition, CD95 (DR2/APO-1/Fas) binds CD95L and TNF-related apoptosis-inducing ligand receptors 1 and 2 (TRAILR1/TRAILR2; also DR4/DR5) binds TRAIL.[143,144]

The biophysical status of the ligand can also regulate the outcome of the intracellular signal. For instance, TNR-R1 binding to the soluble TNFα activates the NF-κB pathway but binding to membrane-displayed TNFα activates the extrinsic apoptotic pathway[145,146]. After ligand binding, receptors form trimeric homo-oligomers and recruit death domain-containing protein (Fas-Associated Death Domain (FADD) or TNFR-associated death domain protein (TRADD)) and pro-caspase-8/-10 to form death-inducing signalling complex (DISC). Pro-caspase-8 is a monomeric cytoplasmic protein and forms the dimeric confirmation only after recruitment to the DISC. As part of the DISC, pro-caspase-8 undergoes auto-proteolytic cleavage that triggers its catalytic activity, which in turn activates effector capases-3/-7, to perform cell death.[147-151] The scheme describing the extrinsic apoptotic pathways is illustrated in **Figure 1**.

Extrinsic apoptosis can be divided into two prototypic cell types. Type I cells have sufficient caspase-8 activation to directly activate downstream effector caspases, whereas type II cells have less DISC-activated caspase-8 and cell death signal is amplified through intrinsic mitochondrial pathway.[152] More specifically, in addition to the cleavage of effector caspases, caspase-8 can cleave the pro-apoptotic protein BID, which truncated form (tBID) assists to oligomerize pro-apoptotic proteins BAX and BAK to form pores in the OMM. After pore establishment, proteins from the mitochondrial intermembrane space are released into the cytosol and mitochondrial apoptosis pathway is activated. Thus, the external apoptotic signal can be amplified by mitochondrial pathway.[87,152]

Caspase-8 activation can be regulated by the FADD-like interleukin-1β–converting enzyme (FLICE)-like inhibitory protein (FLIP) that, due to structural similarity, can replace caspase-8 in the DISC complex and inhibit apoptotic activity.[153] The main caspase-8 activity regulator is cFLIP that has two primary mRNA splice variants, cFLIPL (long) and cFLIPS (short), which can both dimerize with caspase-8/-10 and/or DISC. However, the role of cFLIP isoforms is not clear and complicated balance between the variants and the pro-caspase-8 seems to determine the outcome of the signaling.[154-156]

1.3.3 Perforin/granzyme B apoptotic pathway

The perforin/granzyme B apoptotic pathway is mediated by cytotoxic T-cells (CTLs), which release the pore-forming perforin and cytoplasmic granules containing serine protease granzyme B. Secreted perforin forms transmembrane pores that allow granules into cytoplasm. Granules contain granzymes, such as granzyme A and B, that upon release can cleave over 300 apoptosis triggering and supporting substrates, including BID, ICAD, initiator caspase-8 and -10, and executioner caspase-3 and -7.[157-160] Furthermore, granzyme B increases the mitochondrial ROS levels that contributes to the cell death activation, target nuclear PARP and DNA PK proteins, and extracellular matrix proteins[157,161]. Altogether, this described caspase-

independent apoptotic pathway is facilitating elimination of tumor cells and virus infections, which often suppress caspase activity and apoptosis[162].

1.3.4 Apoptosis and cancer

The majority of anti-tumor drugs initiate the intrinsic apoptotic pathway. However, one of the reasons of therapy resistance are frequent mutations in mitochondrial apoptosis pathway genes. For example, mutations in the *p53* are the most prevalent in human malignancies, detected in 80% of tumor cell lines and 40% of human cancers[163], and thereby also affect activity of the intrinsic apoptotic pathway. In addition, translocation and overexpression of the *BCL-2* oncogene is associated with about 85% of human follicular lymphomas[164]. Moreover, somatic mutations in the *BAX* gene, such as single nucleotide substitutions or frameshift mutations, have been identified in solid and hematological malignancies[165,166].

In addition, cancer cells have high levels of pro-apoptotic proteins; although, their activation is inhibited in several ways. For instance, intrinsic apoptosis can be suppressed by overexpression of IAPs.[167] Therefore, suppressing IAPs is a promising way to selectively target tumor cells. For example, overexpression of Smac/DIABLO or administration of IAP-binding peptides, such as Smac-N7, can sensitize cells for treatment[168-170]. However, IAPs do not seem to be the main regulators of apoptosis induction. Another way to regulate apoptotic proteins is on the transcriptional or post-transcriptional level. For instance, anti-apoptotic BCL-2 family proteins are transcriptionally regulated by nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB)[171].

Cancer cells have also developed several strategies to defeat extrinsic apoptotic signals. Mostly cell death is avoided by an increase in anti-apoptotic molecules or by a decrease or defective function in pro-apoptotic proteins. For instance, tumor cells can have mutations in the receptor gene [172-174], downregulate the expression on the cell surface death receptors [175], prevent receptor transportation from ER to the cell surface [176], or express decoy receptors [177]. In addition, death receptor signaling in tumor cells can also be prevented by affecting the activity of their cytoplasmic domains. One of these regulators is aforementioned caspase-8 inhibitory protein FLIP, which is highly expressed in many cancer cells[156,178,179].

Numerous chemotherapeutic drugs induce CD95L expression and extrinsic cell death via CD95 receptor[180-182]. This is a p53-independent process and is especially helpful for patients with deleted/mutated/inactivated p53[183]. However, CD95L and TNFα have shown serious side effects and, therefore, their clinical use is questionable[184], whereas TRAIL has been more promising candidate[185,186]. The synergistic effect of TRAIL and chemotherapeutic drugs results in a downregulation of anti-apoptotic proteins[187] and upregulation of pro-apoptotic molecules[188], which altogether leads to the activation of both the intrinsic and extrinsic apoptotic pathway.

1.4 MITOCHONDRIA AND CANCER

Mitochondria are known as the powerhouses of the cell, but they are also crucial for normal cell function by regulating overall homeostasis. Cells have hundreds of mitochondria, which can be a mixture of wild-type and damaged organelles. Mitochondria have a 16Kb DNA genome (mtDNA) that encodes certain respiratory proteins. However, due to co-evolution, most mitochondrial proteins are nuclear encoded. The role of mutations of the mtDNA are less distinct and, unfortunately, quite often sequencing studies do not include the mtDNA. Nonetheless, relatively low mutation rate has been found in cancer mtDNA, indicating to a regulative mechanism involved in mitochondrial quality control to support cellular metabolism. For example, defects in autophagy cause impaired mitochondrial activity and increase cell death[189-191]. Thus, autophagy works as a quality control, removing damaged organelles, but also provides cells with metabolic substrates and aids malignant growth[191,192].

Besides mitochondria role in energy production and apoptosis, these organelles also regulate metabolic cell signaling, lipid homeostasis, steroid and heme synthesis, and cytosolic calcium ions (Ca^{2+}) homeostasis.[193-195] More specifically, mitochondrial citric acid/Krebs/tricarboxylic acid cycle (TCA cycle) produces nicotinamide adenine dinucleotides (NADH) and flavin adenine dinucleotides ($FADH_2$) that donate electrons to the ETC. Electron flow through the ETC produces a proton gradient on the inner mitochondrial membrane (IMM). This proton gradient is used by the ATP synthase to produce ATP. However, the electron flow through ETC also generates ROS, which are involved in regulation of various metabolic reactions, including MAP kinase and HIF transduction pathways[196,197] and can even lead to cell death. Furthermore, as mentioned above, OMM related BCL-2 family proteins regulate apoptosis, when pro-apoptotic proteins BAX and BAK form channels that aid the release of cytochrome *c* from the mitochondrial inter membrane space[198,199]. Therefore, the interaction between the cell and the mitochondria regulates wide range of cellular functions, such as metabolism, growth, and survival.

Mitochondrial dysfunction can lead to various illnesses, including type 2 diabetes, neurodegenerative diseases, and cancer[3,200,201]. These disorders may be caused by maternally inherited defective mitochondrial genome or mutations in nuclearly encoded mitochondrial proteins. One of the deregulators of this organelle is proto-oncogene *MYC*, which is known to regulate cellular metabolism and mitochondrial function[202,203]. While this organelle can cause or be part of many pathologies, they are also seen as potential targets for therapy. For example, several tumor therapeutics destabilize mitochondria to activate apoptosis.

1.4.1 BCL-2 family

BCL-2 family consists of over 20 proteins, which are either pro- or anti-apoptotic and divided into groups depending of their arrangement of up to four different BCL-2 homology (BH) domains[204,205]. The balance between these proteins will determine cell survival or apoptosis. For instance, anti-apoptotic proteins BCL-2, BCL-xL, and myeloid cell leukemia (MCL)-1 that contain all four variants of the BH domains obstruct MOMP by binding and prohibiting pro-

apoptotic proteins. Apoptosis supporting BCL-2 family proteins consist of BH-3 only and BH1-3 containing effector proteins. The BH-3 only proteins (BID, BAD, BIM, NOXA, PUMA) react to intracellular cell death stimuli, such as DNA damage or ER stress, and are activated in diverse ways. For example, PUMA and NOXA by transcriptional upregulation, BAD by posttranslational modifications, and BID by proteolytic cleavage. Regardless of their activation mechanism, these pro-apoptotic proteins suppress anti-apoptotic BCL-2 proteins activity. In addition, the BH-3 only proteins trigger the effector proteins (BAK, BAX) to form OMM pores, which release cytochrome *c* from the intermembrane space and activate apoptosis.[198,204,206] The classification on BCL-2 proteins is summarized in **Figure 2**.

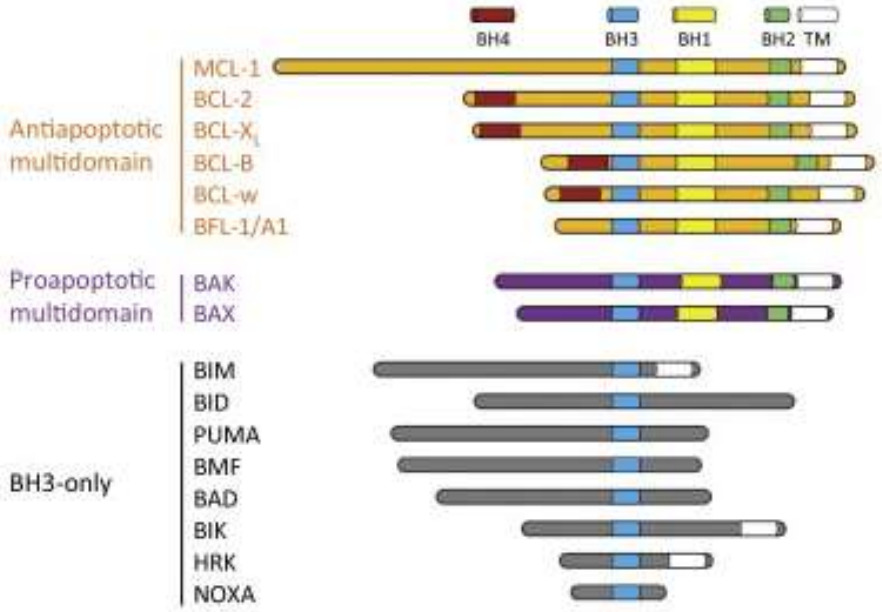

Figure 2. Classifications of BCL-2 proteins according to conserved BCL-2 homology (BH) domains. Abbreviation: TM, transmembrane domain.[207]

Usually is BAX located in the cytosol and BAK, together with the other BCL-2 proteins, connected to the OMM by its carboxy-terminal anchor[208]. There are several ways how BAX and BAK may be activated. For instance, after apoptotic stimuli constantly active BAX/BAK lose their association with the anti-apoptotic proteins (BCL-2, BCL-xL, MCL-1)[209-211]. Another model suggests that BAX/BAK need direct activation via N-terminal conformational change by the BH-3 only proteins tBID and BIM[212,213]. In addition, activation by the interaction with the mitochondrial lipid bilayer is also suggested. It is proposed that tBID inserts into the OMM and recruits BAX, which enrolls more BAX to undergo oligomerization and MOMP[214-216]. In conclusion, the balance between the BCL-2 family pro- and anti-apoptotic proteins regulates MOMP induction.

Usually NB does not have mutations in *BCL-2* but its overexpression and dysregulation are common[217-219]. Moreover, a link between MYC and BCL-2 expression has been discovered. The *MYC* overexpression frequently occurs together with mutations in *BCL-2* genes that assist proliferation and decrease apoptosis.[220,221] Thus, drugs affecting the equilibrium between pro-

and anti-apoptotic proteins are studied for treatment improvement. This can be achieved by using anti-cancer drugs in combination with inhibitors of anti-apoptotic BCL-2 proteins (e.g. ABT-199), which are structurally or functionally similar to BH3-only proteins[222,223]. However, modest outcome, side effects[222], and resistance in relapsed NBs[224] make this approach challenging. These drawbacks are due to suppression of apoptosis by the compensatory upregulation of the anti-apoptotic MCL-1 protein. This can be avoided when ABT-199 is used simultaneously with MCL-1 inhibitor (e.g., A-1210477), which will cause NB cell death[225]. Another way is to directly activate BAX oligomerization with a small molecule BAM7 to promote MOMP and to make cells more sensitive to treatment[226].

1.4.2 Mitochondrial permeability transition pore (MPTP)

Release of mitochondrial content, such as pro-apoptotic proteins like cytochrome *c* or AIF, into the cytosol can be also induced through mitochondrial permeability transition pore (MPTP), a non-specific Ca^{2+}-dependent and ROS sensitive pore in the IMM. Opening of this pore leads to mitochondrial osmotic swelling, vanishing of their $\Delta\Psi m$, and breaking of the OMM.[227,228] The main MPTP components are voltage-dependent anion channel (VDAC), adenine nucleotide translocase (ANT), and cyclophilin D; however, it is not fully clear which other proteins are involved in the pore formation[229,230]. For instance, ATP synthase F0 (ref. 231) subunit c and phosphate carrier (PiC)[232] can also take part of the pore formation. The function of MPT in apoptosis is still not clear but it is suggested that not all of the mitochondria are subjected to MPT and recovery from the damage is possible[233].

Inducing MPTP formation is also considered as a potential strategy to activate apoptosis in tumor cells with high level of anti-apoptotic proteins. For example, MPT can be induced by depleting its endogenous inhibitors, such as glucose, ATP, glutathione (GSH), or creatine phosphate. Another attractive strategy is to use drugs that stimulate ROS production, increase intracellular Ca^{2+} concentrations, or induce detachment of hexokinase (HK) from mitochondria to consequently activate MPT.[229,234,235] There are also several drugs that directly affect MPT pore. Ligands that bind to ANT, such as ANT-cross linker 4-(N-(S-glutathionylacetyl) amino) phenylarsenoxide (GSAO), and inhibit its ATP/ADP antiporter activity induce tumor cell death[236]. The MPT is also promoted by retinoic acid derivatives, for instance all-trans-retinoic acid or ST1926, that are besides that supporting differentiation of NB and leukaemia. Their main mechanism is to increase cytosolic calcium levels for MPT induction.[237,238] Furthermore, regulating the actions of the MTP pore-interacting peripheral benzodiazepine receptor (PBR), which is often overexpressed in cancers, may have anti-tumor effects. The PBR is also suppressing BCL-2 anti-apoptotic proteins and affecting this interaction can activate both types of OMM permeabilization. This has been demonstrated by PBR ligands PK11195 and RO5-4864 that sensitize tumor cells to chemotherapeutic drugs-induced cell death.[239-241]

1.4.3 Electron transport chain (ETC)

Mitochondrial ETC consists of four enzyme complexes (I, II, III, and IV) that are located in IMM together with cytochrome *c* and ATP synthase (Complex V) (**Figure 3**). These proteins

are responsible for electron transfer from TCA substrates to oxygen. Complex I (NADH ubiquinone reductase) accepts and transfers electrons from NADH and Complex II (succinate dehydrogenase) gets electrons from succinate, through $FADH_2$. Thereafter, both of these complexes donate their electrons to Complex III (Ubiquinol-cytochrome *c* reductase), which transfers these *via* cytochrome *c* to Complex IV (cytochrome *c* oxidase) and then oxygen to form water. Described electron movement results in transportation of protons from the matrix to the intermembrane space of mitochondria causing the formation of the mitochondrial membrane potential. This electrochemical gradient is finally used to produce ATP from ADP by transporting protons back to the mitochondrial matrix by ATP synthase.[242-244]

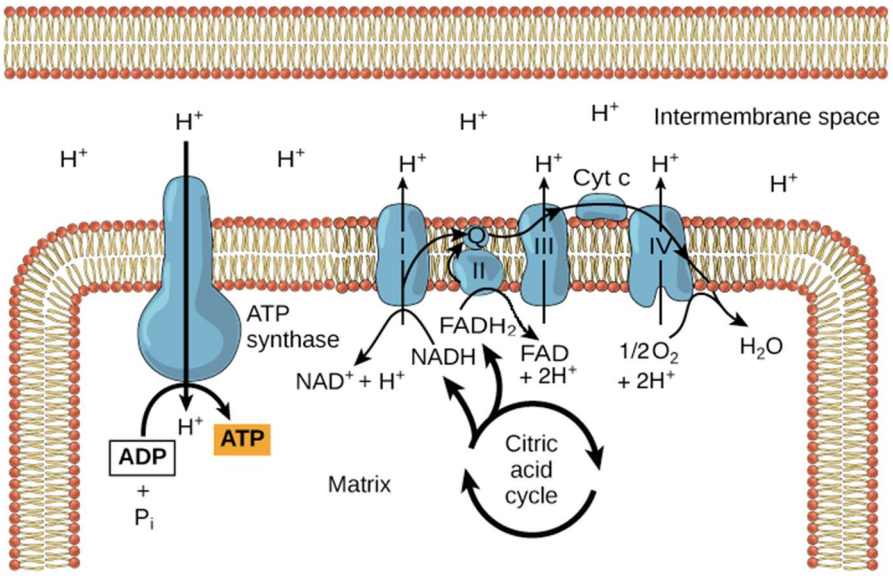

Figure 3. Mitochondrial electron transport chain and oxidative phosphorylation. Republished from "Oxidative phosphorylation: Figure 3," by Openstax College, Biology (CC BY 3.0).

Cancer cells are rapidly dividing, which can make them hypoxic and change their energy supply from OXPHOS to glycolysis. Furthermore, tumor cells have a tendency to have lower amount of mitochondria and/or abnormalities in OXPHOS genes[245-247]. On the other hand, relapsing cancer cells have demonstrated elevated levels of OXPHOS[248-250]. For example, MYC can induce the expression of mitochondrial complexes and support respiration[47,251]. Described changing of the metabolic organization of the cell assists them to survive in poor nourishment environment. Thus, resistant tumor cells may be eliminated by combined use of chemotherapeutic drugs and ETC inhibitors. Cell death caused by excessive ROS production has been shown to derive mostly from Complex I and III, but there is also evidence demonstrating ROS production from Complex II[252-255].

There are numerous Complex I inhibitors out of which rotenone is the most common example[256]. However, acute neurotoxicity as a side effect is reducing their potential use for treatment[257]. On the other hand, inhibiting Complex I by metformin or tamoxifen[258-260] or blocking Complex II by α-tocopheryl succinate (α-TOS) or thenoyltrifluoroacetone

(TTFA)[261,262] to promote electron leakage and ROS formation are used to improve outcome of the therapy. For example, TTFA attaches to the Complex II ubiquinone (UbQ)-binding site to suppress electron flow, resulting in electron escape and ROS formation[263]. In addition, using non-toxic doses of TTFA and cytotoxic drugs in combination can induce ROS production and thereby improve the treatment outcome in NB cell lines[262].

Complex III that accommodates electrons form both Complex I and II is also known as coenzyme Q: cytochrome *c* – oxidoreductase and can be inhibited by antimycin A to increase ROS production and inhibit anti-apoptotic proteins in tumor cells[264-266]. Furthermore, successful results from xenograft models and clinical trials with resveratrol[267] and benzyl isothiocyanate (BITC)[268] show a clear potential for Complex III blockers as tumor eliminating drugs. Complex IV is mostly inhibited by transcriptional regulation by fenretinide[269] or doxorubicin[270]. In addition, apoptotic cell death *via* ATP synthase blocking may be facilitated by oligomycin[271] or 3,30-diindolylmethane (DIM)[272]. The DIM has shown to also have cell cycle, angiogenesis inhibition and pro-apoptotic activity abilities[273], which are now tested in clinical trials.

1.5 OXIDATIVE STRESS AND CANCER

Oxidative stress is caused by an imbalance between ROS production and the ability of cells to take care of the free radicals or to repair the damage. Some of the most known free radicals are superoxide anions (O_2^-), hydroxyl ($OH^\bullet$), hydrogen peroxide (H_2O_2), and peroxyl radicals (HO_2)[274]. ROS production is mainly located in the mitochondria and it is considered to be a normal cellular process that takes place during oxygen metabolism. It is caused by imperfections in the ETC electron flow, where the leakage of electros from complex I and III forms ROS. Other sources of internal ROS are peroxisomes, enzymes, xanthine oxidase, and the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complexes. These free radicals participate in normal physiological cell signalling to regulate proliferation, differentiation, and cell survival. Most of the ROS is produced in the mitochondria, whereas external ROS is usually induced by UV radiation or chemical compounds. Excessive amount of ROS can cause damage to the cells and generate "secondary" ROS by interacting with other compounds and cause severe cell damage.[275-278]

There are several cellular defence systems that are responsible for ROS elimination and regulation. Oxidative damage can be avoided by preventing the formation of ROS or by using enzymatic or non-enzymatic antioxidants to neutralize the radicals. The most known antioxidants are dietary antioxidants (*e.g.* vitamins A, C, and E), endogenous antioxidant enzymes (*e.g.* superoxide dismutase (SOD), catalase (CAT), GSH peroxidase (GPx), GSH reductase (GR), and peroxiredoxins), and antioxidant molecules (*e.g.* GSH), coenzyme Q, ferritin, and bilirubin).[279]

Abnormal levels of cellular antioxidants, such as decrease in GSH, a three amino acid thiol protein consisting of glutamine, cysteine, and glycine, makes cells more sensitive to oxidative stress and induce oxidative damage on lipids, DNA, and proteins. ROS mediated damage is

associated with various illnesses involving neurodegenerative and age-related metabolic disorders, inflammatory disease, cardiovascular disease, allergies, immune system dysfunctions, diabetes, and cancer. For example, cancer initiation and development are connected to ROS-induced DNA mutation and damage, genome instability, angiogenesis, and proliferation. Tumor cells with already elevated ROS levels also have an increase in GSH formation and GSH S-transferases (GSTs) to enhance their resistance to free radicals. In addition, circulating tumor cells seem to have increased supply of NADPH to cope metastatic stress induced increase of ROS. Moreover, introducing antioxidants to high ROS producing cells can stimulate metastasis, instead of prevention. Malignant tumors sustain cellular ROS level that is supporting proliferation without inducing cytotoxicity. Different ROS levels can cause distinct outcome based on the cells, tissues, ROS production site and concentration. Furthermore, excessive ROS production in cancer cells can be in turn used to increase the efficiency of the treatment by using ROS activated pro-drugs that induce apoptosis.[280-286]

Furthermore, keeping in mind the importance of GSH in induction and development of tumors, blocking the GSH-dependent defense system is another promising approach for anti-cancer therapy[286]. The GSH protects proteins by being oxidized into GSH disulphide (GSSG) by reducing cysteine disulphide bonds. The GSSG is in turn reduced by GSH reductase back into GSH.[287] Studies using buthionine sulfoximine (BSO) to block GSH synthesis have shown promising results by inhibiting the formation of several cancers (*e.g.* lymphomas, sarcomas, and breast tumors). Unfortunately, this approach does not work with metastasized cancers, which have upregulation of thioredoxin (Trx) to compensate the lack of GSH. However, in some tumors where BSO is used together with melphalan, synergistic effects can be seen.[288-290] Targeting other mitochondrial redox regulating mechanisms has been also successful. For example, the amount of ROS can be increased by inhibiting glutamate dehydrogenase 1 (GDH1) to reduce fumarate levels and thereby decrease antioxidant GPx[291], which normally reacts with H_2O_2 and organic hydroperoxides.

1.6 METABOLISM AND CANCER

The extensive proliferation of the cancer cells also increases their biosynthetic demands, which raises their need for higher uptake of nutrients from the environment. There are two main substrates of energy in mammalian cells – glucose and glutamine. These substrates are catabolized to provide cells with carbon intermediates for macromolecule formation. One hallmark of cancer is metabolic reprogramming that supports tumor cell macromolecule synthesis, energy requirement, and survival[292]. As previously described, normal cells acquire most of the ATP from OXPHOS (**Figure 4**a), whereas in tumors, as a direct and/or indirect consequence of oncogenic mutations, mitochondrial metabolic reactions are changed. For example, tumors direct glycolytic intermediates instead of mitochondrial oxidation to pentose phosphate pathway (PPP), serine biosynthesis, and lipid biosynthesis. Furthermore, the PPP produced NADPH is used to reduce oxidative stress, mitochondrial ROS production, and support survival in fluctuating oxygen conditions.[293-295]

This is accomplished by restricting glycolytic enzyme activity[296] or glycolysis end-product pyruvate consumption by mitochondria[297]. Furthermore, instead of pyruvate, tumor cell TCA cycle is supported by upregulated glutaminolysis, fatty and amino acids, at the same time maintaining aerobic glycolysis (**Figure 4**b,c) (ref. 297-299). This is a clear example how cancer cells are able to change their metabolism to meet their needs. These hyperglycolytic cells can be targeted by inhibition of glycolytic pathway. For instance, suppressing ATP citrate lyase, lactate dehydrogenase A, or acetyl-CoA carboxylase and fatty acid synthase will all result in decreased tumor growth. Therefore, targeting metabolic enzymes could effectively attenuate cancer development. However, not all of the tumors possess this phenotype and instead produce ATP through OXPHOS pathway. This makes targeting their metabolism challenging and inhibition of glycolysis together with mitochondrial respiration would be more efficient.[293,294]

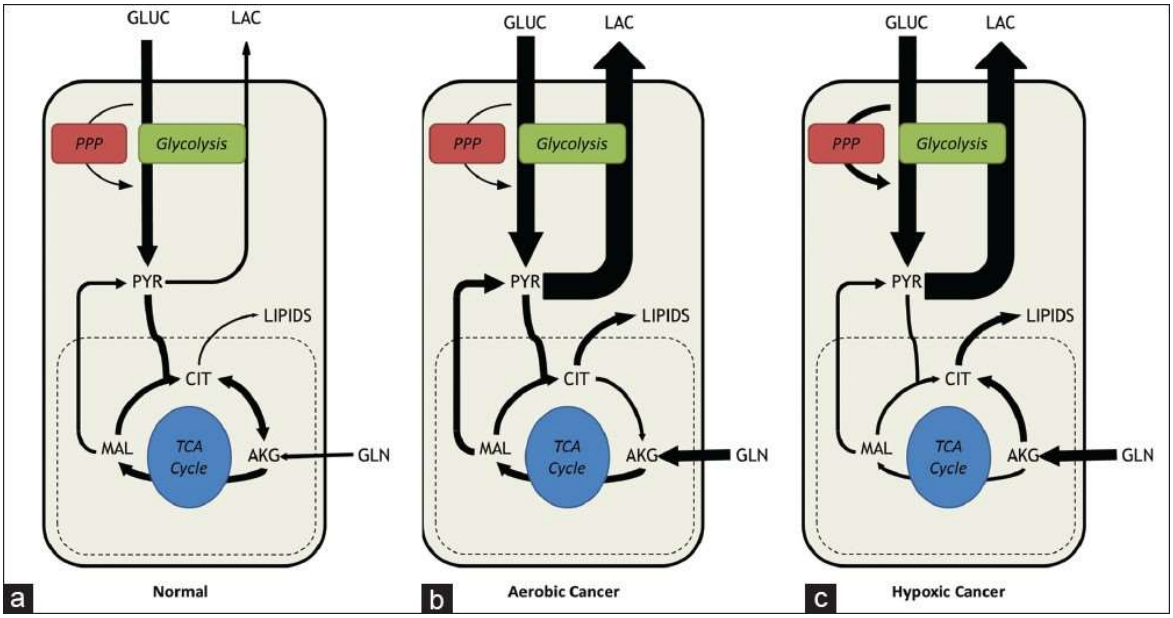

Figure 4. Normal and cancer cell metabolic phenotypes. Abbreviations: glucose (GLUC), lactate (LAC), pentose phosphate pathway (PPP), pyruvate (PYR), citrate (CIT), α-ketoglutarate (AKG), glutamine (GLN), malate (MAL).[300] Republished under CC BY-NC-SA.

1.6.1 Glucose dependency

The aforementioned cancer cells glucose dependency was discovered by Otto Warburg. These changes are even seen in aerobic conditions, despite resulting in less ATP than from OXPHOS (2 vs 36), because ATP production through aerobic glycolysis is quicker than in OXPHOS.[301,302] This metabolic change makes cells less dependent on oxygen and helps them to survive in hypoxic environment[303]. The phenomenon of high glucose uptake by cancer cells is also nowadays widely used in clinics for tumor imaging by fluorodeoxyglucose positron-emission tomography (FDG-PET)[304].

Normally pyruvate is metabolized in mitochondria by pyruvate dehydrogenase (PDH) to support TCA and OXPHOS. Whereas in cancer cells PDH activity is suppressed resulting in decreased OXPHOS and pyruvate conversion to lactate by lactate dehydrogenase (LDH).

However, aerobic glycolysis is not prevalent in all tumors and now it is known that mitochondrial respiration defects are not causing the effect, because most cancers actually maintain mitochondrial function and respiration, which are regulated by strict cellular signaling[305]. These metabolic modifications are conducted by oncogenes/tumor suppressor genes that are also responsible for proliferation upregulation, such as *p53*, *MYC* and hypoxia-inducible factor 1 (*HIF-1*)[306,307].

The *MYC* oncogene stimulates glucose import (*e.g.* GLUT1), glycolysis (*e.g.* HK2, PDK1), and mitochondrial biogenesis to provide cancer cell with metabolic intermediates that are needed for excessive cell growth[308-310]. These tumor cell peculiarities make them also more vulnerable for glucose transportation and glycolysis specific targeting[311,312]. One possible approach is to target HK2 activity. For instance, the synthetic analogue of glucose 2-deoxy-D-glucose (2-DG) cannot be metabolized after phosphorylation by HK2, which causes 2-DG-6-P accumulation, leading to the inhibition of glycolysis and cancer growth[313-315]. Tumor elimination by 2-DG has been demonstrated in numerous NB cell lines[316] and xenograft models[317], irrespectively of cancers MYCN expression, suggesting promising clinical significance. Moreover, the clinical outcome of the 2-DG treatment is improved when co-treated with cytotoxic drugs in breast[318], head and neck[319], and ovarian[320] cancer cell lines. In addition, HK inhibitor lonidamine has been also tested in clinical trials for combinatory treatment and showed promising results in ovarian cancer clinical trial[321]. The glycolytic shift can also be switched by leading pyruvate into mitochondria to reinforce the typical phenotype of non-malignant cells. This could be facilitated by downregulation of LDHA or by pyruvate dehydrogenase kinase (PDK) inhibitors, for example, PDK1 inhibitor dichloroacetate (DCA)[322-324]. For instance, the LDHA inhibitor FX11 suppresses aerobic glycolysis and thereby proliferation of NB cell lines[325].

Some drugs can equally effectively inhibit glycolysis and OXPHOS resulting in better outcome than single targeted medications. For example, bezielle (BZL101) that is in clinical trials for advanced breast cancer[326,327]. In addition, 3-bromopyruvate (3-BrP) suppresses ATP production by inhibiting HK, glycolytic glyceraldehyde 3-phosphate dehydrogenase (GAPDH) enzymes, and OXPHOS. The combined targets of 3-BrP result in promising results in hepatic and pancreatic cancers[328]. Moreover, the 3-BrP has demonstrated even better results when used together with other inhibitors (e.g., rapamycin[329,330]) or cytotoxic drugs (e.g., platinum-based agents[331] and doxorubicin[332]) for NB, leukemia, breast, lymphatic, colon, and hepatic cancers. To decrease the 3-BrPA effect to healthy cells, an adjusted form of 3-BrP named 3-bromo-2-oxopropionate-1-propyl ester has been developed that has showed potent effect on GLUT1- and MKI67-expressing NB cells[333].

1.6.2 Glutamine dependency

Many tumors show signs of increased glucose metabolism or glutamine dependency (**Figure 4**), regardless of the fact that glutamine is a non-essential amino acid that can be synthetized from glucose. Glutamine is the highest concentrated amino acid in plasma and cancer cells utilize it more than any other amino acid.[334-336] Amide nitrogen from glutamine is used in

nucleotide and amino acid synthesis and carbons in GSH, amino acid, and lipid synthesis. Glutamine and its metabolites are known to regulate cellular energy status, redox state, cell signaling, nucleotide and amino acid synthesis. For instance, glutamine is important for the regulation of oxidative stress in the cell, by being used as a precursor for GSH synthesis. Thus, tumor cells fast proliferation and better survival in challenging environments is supported by glutamine administration.[337-339] Based on the aforementioned FDG-PET tumor detection method, glutamine based tracers are also tested in preclinical and early clinical studies[340,341]. This approach gives crucial information about cancers in difficultly analyzed heavy glucose consumption sites, such as the brain, and tumors that use glutamine as their primal energy source instead of glucose.

In cancer, stimulation of glutaminolysis is mainly induced by MYC in low glucose and oxygen conditions. Increase in glutamine uptake is achieved by higher expression of glutamine transporters (SLC1A5, SLC5A1, SLC7A1, SLC7A25) and glutaminases (GLS1/2). Mammalian cells have two types of GLS, kidney-type (GLS1) and liver-type (GLS2), that catalyze the transformation of glutamine to glutamate and ammonia.[342-344] Glutamate can be additionally deaminated by GDH, such as GLUD1, into α-ketoglutarate (αKG), which can be used to maintain the TCA cycle and biosynthesis. Glutamate can also be transaminated by aminotransferases (GOT1/2, GPT), which results in the synthesis of other amino acids, such as aspartate and proline.[345,346] It has been shown that glutamine addiction by tumor cells is diminished after *MYC* knockdown[344]. In addition, glutamine deprivation can result in cell death of glutamine-dependent cells, whereas administration of oxaloacetate, pyruvate, and αKG instead of glutamine can avoid cell death, proposing that *MYC*-driven glutamine consumption is the one providing the TCA cycle with carbon source[347,348]. Therefore, *MYC*-driven tumors and their glutamine metabolism could be targeted to increase the efficacy of cancer therapy.

Glutamine depletion will lead to the activating transcription factor 4 (ATF4)-dependent, but p53-independent, apoptosis *via* activation of expression of the pro-apoptotic BCL-2 family proteins PUMA and NOXA. Due to this peculiarity, simultaneous treatment with ATF4 agonists and glutaminolysis inhibitors will cause apoptosis activation and reduction of NB tumor size.[347] The GLS1 inhibitors, such as small molecule 986 and bis-2-[5-phenylacetamido-1,2,4-thiadiazol-2-yl] ethyl sulfide (BPTES), suppress *MYC*-overexpressing tumor cell growth migration, invasion, and resistance to oxidative stress[349-352]. Although, *MYCN*–amplified NB cells with overly excessive GLS2 expression may be less susceptible to this approach[347,349]. In addition, glutamate dehydrogenase (epigallocatechin-3-gallate (EGCG)) or aminotransferase (aminooxyacetate (AOA)) inhibitors are used for consecutive suppression of glutamate processing[353-355]. However, determining the predominant glutamine metabolism pathway for the particular tumor makes it challenging to anticipate the cancer cells response to these drugs.

2 AIMS

The main goal of this PhD study project was to find strategies for treatment resistant NB cells elimination. Considering the importance of mitochondria in apoptosis, the specific goal was to explore mitochondrial targeting to enhance anti-tumor treatment efficiency. In addition, our aim was to evaluate the role of MYCN expression in NB treatment and to study promising strategies to remove treatment resistant NB cells. Furthermore, another aim of this study was to understand the mechanisms of NB cell resistance and how to sensitize cells to therapy. The results of the research were summarized as following individual papers.

Paper I – In this study, conventionally used chemotherapeutic drug cisplatin was applied in combination with the mitochondrial Complex II inhibitor TTFA to investigate whether mitochondrial destabilization by TTFA can sensitize NB cells to anti-cancer drugs

Paper II – Here we evaluated the direct effect of widely used therapeutic drugs on mitochondrial activity in combination with glutamine withdrawal, and the possible apoptotic effects of such interaction

Paper III – This article investigated how glutamine deprivation affects NB cell lines response to generally used chemotherapeutic drugs

3 MATERIALS AND METHODS

Methods used in constituent papers are briefly described in following paragraphs, however, in detail protocols can be found in the papers.

3.1 HUMAN CELL LINES

All cell lines used in this study, if not stated otherwise, were cultured in 37 ºC humidified air/CO_2 (5%) atmosphere. Complete RPMI 1640 (Sigma) medium including heat-inactivated fetal calf serum (FBS) 10% (w/v), and penicillin/streptomycin (P/S) (100 U/ml) was used. The HCT116 cell line was grown in DMEM (Gibco®) complete medium. For TET21/N cells, 100 µg/ml hygromycin B (HygroB), and 200 µg/ml geneticin were added to the medium. In addition, when needed, the MYCN expression of the TET21/N cells was switched off by adding 0.1 µg/ml doxycycline to the cell culture medium. Glutamine deprivation was achieved by changing the regular medium to glutamine-free RPMI 1640 24 h prior to treatment. The used cell lines and their specifics is summarized in Table 2.

Table 2. Studied cell lines and their specifics

Cell line	**Origin**	**Culture medium**	**Additions to medium**	***MYCN* status**	***P53* status**
TET21/N ($MYCN^+$)	NB	RPMI 1640	▪ FBS ▪ P/S ▪ geneticin ▪ HygroB	transfected with over-expressing *MYCN*	WT
TET21/N (MYCN–)	NB	RPMI 1640	▪ FBS ▪ P/S ▪ geneticin ▪ HygroB ▪ doxycycline	downregulated by doxycycline	WT
SK-N-BE(2)	NB	RPMI 1640	▪ FBS ▪ P/S	amplification	mutated
IMR-32	NB	RPMI 1640	▪ FBS ▪ P/S	amplification	WT
SK-N-AS	NB	RPMI 1640	▪ FBS ▪ P/S	single copy	mutated
SH-SY5Y	NB	RPMI 1640	▪ FBS ▪ P/S	single copy	WT
SK-N-SH	NB	RPMI 1640	▪ FBS ▪ P/S	single copy	WT
HCT116	colon cancer	DMEM	▪ FBS ▪ P/S	single copy	WT

3.2 WESTERN BLOT ANALYSIS

The western blot technique is also known as immunoblotting. It is used to detect proteins of interest in a lyzed tissue or cellular sample. Denatured proteins are separated by electrophoresis

and incubated with primary antibodies recognizing the protein of interest. Secondary antibodies are used for the protein visualization *via* immunofluorescence or chemiluminescence.

For the thesis research, the samples were collected by directly adding trypsin to the cells adhered to the cell culture dish. Detached cells were collected and centrifuged at 1000 rpm for 5 min, followed by supernatant removal, and resuspended in phosphate-buffered saline (PBS). Protein concentration of collected samples was assessed by Pierce bicinchoninic acid (BCA) colorimetric assay and measured by VersaMax™ microplate reader.

Samples for western blot were mixed with 5x Laemmli's loading buffer and boiled for 10 min. Thereafter, samples were loaded into a 10-15% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) to be separated by their molecular weight. Separated proteins were electroblotted (1.5 h at 120 V) to a nitrocellulose membrane, followed by blocking in 5% non-fat milk in PBS (1 h at room temperature) and washing in PBS (3x, 10 min). The membranes were incubated with primary antibodies overnight at 4 ^{o}C. The following day, rinsed membranes were incubated with secondary antibodies (1.5 h, 1:7000). The proteins were visualized by Licor® Odyssey® CLx Imaging System or X-ray film.

3.3 EVALUATION OF APOPTOSIS

3.3.1 Caspase activation

3.3.1.1 Caspase processing and specific substrate cleavage

In normal cells, caspases are in an inactive pro-caspase form and become activated after specific signaling. Apoptotic signaling-induced caspase processing includes dimerization/oligomerization of pro-caspases and subsequent cleavage into associated small and large subunit. The active caspase is then able to cleave its substrates. In this thesis, the processing of caspase-3, caspase-8, and caspase-9 was analyzed by Western Blot and specific antibodies. In addition, activity of caspases can be studied through detecting cleavage of the fluorogenic or chromogenic substrates. For example, poly(ADP-ribose) polymerase (PARP) is a target of the caspase-3 and detection of cleavage of PARP is considered as a sign of apoptotic cell death.

3.3.1.2 Fluorometric caspase activity assay

Caspase activity can be also measured by a fast and easy assay. This method uses different substrates that are specific to each caspase to indicate apoptotic activity. These caspase specific peptides are conjugated with fluorogenic [7-amino-4-methylcoumarin (AMC)] or chromogenic (p-nitroaniline) groups and added to the lyzed samples. In case of caspase activation, the substrates are cleaved and the fluorogenic agent is released. This can be detected by fluorimeter or a fluorescence microtiter plate reader, which excites samples at 360 nm and detects emitted green 450 nm fluorescence.

Besides specific substrates, different caspases also need specific buffer conditions. For example, optimal pH for caspase activity is between 6.5 and 8, which is achieved by 0.1 M 4-

(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer. However, 0.1 M 2-(N-morpholino)ethanesulfonic acid (MES) buffer is more suitable for caspase-2 and caspase-9. Reaction buffers also contain stabilizing agents (sucrose), detergents for cell lysis (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) and/or NP-40) and reducing agents, such as dithiothreitol (DTT). In addition, caspase-6 and caspase-7 activity is improved in the presence of 0.2 M NaCl and 5 mM Ca^{2+}.

3.3.2 OMM permeabilization and release of cytochrome *c*

OMM permeabilization and cytochrome *c* release, also known as the "point of no return" in apoptosis, can be detected using digitonin-permeabilized samples. Fractionation buffer contains 150 mM KCl, 1 mM $MgCl_2$, 0.2 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA), 0.05% digitonin, and 5 mM Tris. After 15 min of incubation at room temperature, samples are centrifuged at 13,400 rpm for 5 min. Thereafter supernatant and pellet are separated, Laemmli's buffer is added and release of cytochrome *c* is detected by the aforementioned western blotting technique.

3.4 ASSESSMENT OF OXIDATIVE STRESS

3.4.1 Mitochondrial ROS

Mitochondria are considered the main source of ROS, which in excessive amounts can lead to apoptotic cell death. Mitochondrial superoxide can be measured in live cells by using MitoSOX™ Red (Molecular Probes®) dye that due to is positive charge locates into mitochondria. The dye reacts with superoxide and the oxidized product absorbs 510 nm and emits 580 nm light. In all of the papers, BD Accuri™ C6 flow cytometer was used to analyze the percentage of MitoSOX-positive cells and the signal strength. In addition, in the Paper I Zeiss LSM 510 META confocal laser scanning microscope (Zeiss Jena, Germany) was used for time-lapse experiments. For this purpose, cells were grown and treated in the POC-R cell cultivation system (Zeiss, Jena, Germany) at 37 °C and humidified air/CO_2 (5%) atmosphere.

3.4.2 Content of SH groups

The sulfhydryl (SH) groups are part of cellular antioxidant system. Measuring the content of SH groups gives information about cells ability to resist oxidative stress. In Paper II and III, amount of SH groups was measured using Ellman's reagent (5,5'-dithiobis-(2-nitrobenzoic acid) or DTNB). Collected cells were resuspended in Tris buffer (150 mM, pH 8.2) and divided into two separate samples, allowing assessment of the total and soluble SH groups of the same sample. After addition of DTNB and incubation for 30 min, samples absorbance was measured at 412 nm using a VersaMax™ microplate reader.

3.5 MEASUREMENT OF MITOCHONDRIAL OXYGEN CONSUMPTION

Measurements of oxygen consumption provide information about mitochondrial intactness and how the drugs affect ETC. In Paper I and II, oxygen electrode (Hansatech Instruments, Norfolk, UK) and OxygraphPlus software (Hansatech Instruments, Norfolk, UK) were used for oxygen

consumption assessment. Cells were collected and resuspended in 0.01% digitonin supplemented KCl-based buffer. Assessment of the activity of different complexes was performed using digitonin-permeabilized cells, to allow accessibility of exogenous substrates to mitochondria. At first, basal respiration was measured, followed by addition of chemicals of interest. Respiratory activity of separate respiratory complexes and rate of maximum respiration were analyzed using specific mitochondrial substrates and inhibitors. For example, pyruvate and malate support Complex I respiration, and succinate Complex II activity. Specific inhibitors, such as rotenone (Complex I), antimycin A (Complex III), and oligomycin (ATP synthase), can also be used to get information about complexes of interest. The rate of maximum respiration can be measured after adding the uncoupling agent carbonyl cyanide m-chloro phenyl hydrazine (CCCP).

In Paper II, besides oxygraph, respiration studies were also conducted using Seahorse Analyser, which allows assessment of oxygen consumption of attached cells in their normal environment on 96 well plate. Typical Seahorse assay is as follows. Initially, baseline cellular oxygen consumption rate (OCR) is measured, which consists of basal and non-mitochondrial respiration. Thereafter, ATP synthase inhibitor oligomycin is added to detect ATP-linked respiration and proton leak. After that, the uncoupler CCCP is injected to measure maximal respiratory capacity and then antimycin A and rotenone to shut down ETC and to analyze non-mitochondrial respiration. The oxygraph and Seahorse Analyser data was normalized by either cell number or protein concentration.

3.6 MEASUREMENT OF MITOCHONDRIAL TRANSMEMBRANE POTENTIAL (ΔΨm)

The ΔΨm plays a central role in aerobic energy production and other mitochondrial processes, such as Ca^{2+} accumulation or protein import. The proton gradient is a result of ETC activity and is in turn used for ATP production, therefore, changes in the ΔΨm can affect vital mitochondrial functions. Cationic mitochondria-accumulating fluorescent probes, such as tetramethylrhodamine ethyl ester (TMRE), can be used to measure ΔΨm of intact cells.

3.7 MEASUREMENT OF ALTERATIONS IN CYTOSOLIC Ca^{2+}

The Ca^{2+} is part of cellular signaling and regulate the activity of several enzymes, proteins, and ion channels. Sudden increase of cytosolic Ca^{2+} can trigger its accumulation in mitochondria with subsequent rupture of the mitochondrial membrane, due to the stimulation of MPTP, and cell death activation. Cytosolic calcium levels can be analyzed using the live cell-permeable calcium-sensitive fluorescent dye Fluo-4AM (Invitrogen-Molecular Probes, Eugene) and flow cytometer or fluorescent microscope.

3.8 STATISTICS

All the data presented in this thesis are the average of at least three separate repetitive experiments. The error bars represent standard deviation and the statistical significance was

evaluated using Student's t-Test (two-tailed distribution, two-sample unequal variance) or two-way ANOVA (*$p < 0.05$ and **$p < 0.01$).

4 SUMMARY OF THE PAPERS

PAPER I

Kruspig B, **Valter K**, Skender B, Zhivotovsky B, Gogvadze V (2016).

Targeting succinate:ubiquinone reductase potentiates the efficacy of anticancer therapy.

Biochimica et Biophysica Acta, 1863: 2065-2071

Mitochondria are one of the central elements of apoptosis because the release of pro-apoptotic proteins from the intermembrane space of mitochondria into cytosol is considered as a "point of no return". The release of cytochrome *c* can be stimulated by compounds specifically destabilizing mitochondria, such as inhibitors of mitochondrial respiratory complexes. Blocking respiratory complexes stimulates electron leakage with formation of ROS, which contribute to OMM permeabilization. DNA damaging alkylating agents, such as cisplatin, can lose their therapeutic effect due to the development of resistance. Therefore, increasing the cytotoxic efficacy of cisplatin by co-administering a mitochondrial drug can lead to a better treatment outcome. The aim of this paper was to investigate whether mitochondrial destabilization by the mitochondrial Complex II inhibitor TTFA can potentiate the therapeutic effectiveness of cisplatin on a panel of NB cell lines.

In this paper, NB cell lines with different sensitivity towards therapy were used. The effect of the co-administration was analyzed by assessment of mitochondrial function and various apoptotic markers. Our results revealed how simultaneous administration of non-toxic dose of succinate dehydrogenase (SDH) ubiquinone-binding site inhibitor TTFA and harmless dose of cisplatin stimulated cell death in several NB cell lines in a synergistic manner. Further studies revealed that the TTFA-induced mitochondrial ROS production is the underlying mechanism of sensitization to cisplatin treatment. Excessive ROS production led to the permeabilization of the OMM, release of cytochrome *c*, processing and activation of caspase-3, and cleavage of PARP; all markers of an apoptotic cell death. Furthermore, experiments with methyl malonate, an inhibitor of the SDHA subunit, suppressed TTFA-stimulated apoptosis, indicating that TTFA's chemosensitizing effect requires oxidation of succinate.

However, co-administration of TTFA and cisplatin to IMR-32 NB cells had contrasting effects, resulting in suppressed apoptosis. Studies on IMR-32 Complex II revealed its impaired activity, explaining the unexpected outcome. Therefore, fully active SDH is a prerequisite for TTFA-induced sensitization of NB cells to treatment with cisplatin. In conclusion, these findings show the potential use of TTFA as an adjuvant to improve cisplatin treatment of specific types of NB.

PAPER II

Valter K, Maximchik P, Zhivotovsky B, Gogvadze V.

Distinct effects of etoposide on glutamine-addicted neuroblastoma.

(manuscript, submitted)

Tumor cell elimination through stimulation of mitochondrial apoptotic pathways is a promising strategy. Compounds that target mitochondria can suppress mitochondrial activity or contribute to the outer mitochondrial membrane permeabilization leading to apoptosis. Conventionally used chemotherapeutic drugs are DNA-damaging agents but their ability to directly target mitochondria is unknown. In addition, some tumors display addiction to glutamine, a substrate for antioxidant GSH, despite the fact that it is a nonessential amino acid. A subset of highly aggressive cases of NBs is characterized by high MYCN expression, which in turn regulates cellular metabolism, including glutaminolysis. Our aim was to reveal whether chemotherapeutic drugs affect mitochondrial activity, and if this can contribute to apoptosis initiation.

Our results showed that among tested therapeutic drugs, only etoposide caused a rapid decrease in oxygen consumption. Although, the inhibition of respiration was reversible and restored in a time- and concentration-dependent manner. Further studies demonstrated a severe suppression of Complex I after adding etoposide, whereas no immediate effect of the drug on Complex II was detected. The assessment of respiration after 6 h and 18 h etoposide treatment revealed that Complex I activity has recovered to normal level. Blocking the Complex I can cause a leakage of electrons and formation of ROS. Hence, the assessment of mitochondrial superoxide production upon etoposide administration was performed, revealing sudden ROS formation. However, we could not detect strong markers of apoptosis, they arose only after glutamine withdrawal and subsequent attenuation of the level of GSH. Therefore, simultaneous stimulation of mitochondrial ROS production and removal of the antioxidant GSH can lead to the destabilization of mitochondria and subsequent cytochrome *c* release and apoptosis activation. In addition, after 24 hours, apoptosis was stimulated *via* p53-dependent, BID-mediated pathway. The outcome of the treatment with etoposide, hence, will be dependent on the balance between these two processes.

In conclusion, DNA targeting etoposide also interacts and inhibits mitochondrial ETC Complex I that can induce cell death *via* electron leakage and ROS production. At the same time, mitochondrial antioxidant defense system is removing the excess ROS and rescues cells from controlled cell death. Thus, glutamine removal and subsequent depletion of antioxidants can make targeting mitochondria more efficient, enhance and strengthen anti-tumor therapy.

PAPER III

Valter K, Chen L, Kruspig B, Maximchik P, Cui H, Zhivotovsky B, Gogvadze V (2017).

Contrasting effects of glutamine deprivation on apoptosis induced by conventionally used anticancer drugs.

Biochimica et Biophysica Acta, 1864: 498-506

Targeting energy-producing pathways is a promising approach for cell death stimulation and cancer cell elimination. In addition to the glycolytic switch (Warburg effect), various tumors show glutamine dependence even though it is a non-essential amino acid that can be produced from glucose. Attained glutamine is converted by glutaminase into glutamate, which is used as an important pre-substrate for the Krebs cycle, synthesis of GSH, amino and fatty acids to support intensive proliferation of cancer cells. One of such glutamine-dependent tumors is NB, most common solid cancer in childhood. Therefore, specific targeting of cancer cells' glutaminase or withdrawal of glutamine could be a promising therapeutic strategy to enhance the efficiency of NB treatment and overcome drug-resistance. The aim of this work was to study changes in the cellular response to chemotherapeutic drugs under glutamine deprivation.

This was achieved by growing cells in a glutamine-supplemented or glutamine-free medium 24 h prior treatment with cisplatin or etoposide. We found that depending on the drug, glutamine deprivation can lead to contrasting cellular responses. The withdrawal of glutamine consistently suppressed etoposide-induced but stimulated cisplatin-induced apoptosis, thereby exposing a promising strategy for the elimination of otherwise resistant tumor cells. Further analysis revealed that suppression of etoposide-induced cell death correlated with an attenuation of p53, which regulates the expression of various pro-apoptotic BCL-2 family proteins. However, increase in cisplatin-induced apoptosis was due to the ROS-mediated downregulation of FLIP-S, a caspase-8 inhibitor, which in turn allowed caspase-8 activation and apoptotic cell death.

Therefore, tumor cells can be sensitized to therapy by suppressing glutamine metabolism; although, the possibility of unfavorable effects due to the specific anti-cancer drug should be considered.

5 DISCUSSION

Cancer and apoptosis are known as antagonistic processes and, therefore, stimulation of apoptosis is the main strategy in cancer therapy. However, defects of the apoptotic pathways can make cells treatment-resistant.[3] Hence, more specific and effective approaches of cancer cell targeting are being developed to achieve better outcome. For example, mitochondrion, one of the key player of apoptosis, represents an intriguing target for therapy improvement. Besides regulating apoptosis, mitochondria also coordinate cellular metabolism, redox status, Ca^{2+} homeostasis *etc* making them appropriate targets for cancer cell elimination.[193-195,356]

To overcome treatment resistance and severe side effects, sensitizing cells and their mitochondria with adjuvant chemicals to chemotherapeutic drugs is a promising approach. The aim of the **Paper I** was to study whether the ETC Complex II inhibitor TTFA, more specifically SDH ubiquinone-binding site inhibitor, can sensitize a panel of NB cell lines with different sensitivity for clinically used anti-cancer drug cisplatin. Our results demonstrated that TTFA can potentiate the effect of cisplatin in all the NB cell lines, which have a functional succinate oxidation.

Neuroblastoma is the most common extracranial solid tumor in childhood and a subset of highly aggressive NBs are known for their *MYCN* oncogene amplifications[33,42,43]. In addition, MYCN regulates mitochondrial biogenesis and probably can modulate mitochondrial stability in the cell. Therefore, cell lines with different MYCN expression were analyzed. The SK-N-BE(2) and SK-N-AS were included as highly resistant cell lines due to their mutations in *p53* and the epigenetic loss of caspase-8 in SK-N-BE(2). The TET21/N cell line is transfected with overexpressing *MYCN* and represents a cell line with medium response to treatment. In addition, the IMR-32 cell line was chosen for its high sensitivity to anti-cancer drugs.[357,358] In almost all of the tested NB cell lines, TTFA enhanced the apoptotic effect of cisplatin. Importantly, both TTFA and cisplatin were used in concentrations that by itself did not cause any or very little toxicity to the cells. Upon co-treatment, reduced concentrations give the possibility to decrease the occurrence of side effects, at the same time achieving better results.

Based on the results, we revealed, as expected, that the increased cell death was due to the mitochondrial instability, primarily caused by TTFA action on ETC Complex II. The TTFA caused leakage of electrons from Complex II resulting in excessive mitochondrial ROS production that contributed clearly to the increase in cell death. It has been shown previously that mainly Complexes I and III are the sites of ROS production[254,255]. However recent publications, including ours, demonstrated that inhibition of Complex II, especially its ubiquinone-binding site, also leads to ROS formation[252,253]. The sensitization effect vanished in the presence of antioxidant N-acetylcysteine (NAC), confirming the importance of ROS activity in apoptosis induction. Oxidative stress can trigger MPTP opening[359] and oxidization of the cytochrome *c* anchor cardiolipin[360], leading to cytochrome *c* release and apoptosis activation. It is worth noting that for stimulation of the cell death by TTFA, the cells should be

able to oxidize succinate, since, suppression of SDH subunit A, responsible for conversion of succinate into fumarate, repressed stimulation.

However, this effect was not seen in every cell line; the IMR-32 was the only tested cell line, in which apoptosis was not stimulated after TTFA treatment. This finding was unexpected because the IMR-32 cell line itself is very responsive to treatment and 10 times lower concentration of cisplatin had to be used in order to study the mechanism. The analysis of IMR-32 Complex II ability to oxidize succinate revealed its impaired function, explaining the unpredicted effect of TTFA. Some tumors have mutations in SDH, which leads to the accumulation of succinate and thereby support resistance of Complex II targeting. Therefore, prior knowledge about oxygen consumption and possible mutations in tumor cell ETC are crucial for beneficial outcome. Thus, suppression of various complexes of the respiratory chain is a promising strategy for anti-tumor therapy but further studies should be conducted to determine its safety and other potential targets.

Generally, anti-cancer drugs damage nuclear DNA, which leads to the activation of p53 and pro-apoptotic factors. Although, DNA might not be the only target of chemotherapeutic drugs and secondary targets play an important role in treatment efficiency and safety. For example, DNA-targeting drugs can affect mitochondria. This information can be crucial to understand the treatment outcome, increase its efficiency, and decrease side effects. Our aim of **Paper II** was to expand our knowledge about the effects of anti-cancer drugs on mitochondria and its consequences on treatment. To our surprise, among several anti-tumor drugs, only etoposide had a direct and strong effect on mitochondria. We found that upon addition of etoposide, there was a sudden stop in Complex I activity, which led to the electron leakage and ROS formation. However, it was not enough to induce apoptosis after 6 h of treatment; cell death at this early time point was only detected upon removal of glutamine and stimulation of oxidative stress.

Excessive ROS production is an important part of anti-tumor strategy and many drugs are developed to target mitochondria. Although, cancer cells also have mechanisms to remove ROS and thereby reduce oxidative stress caused damage. They have high levels of ROS-detoxifying enzymes to avoid cell death activation.[361] Tumor cells ability to remove ROS can be in turn affected by reducing activity of their antioxidant machinery. For example, reduction of water- and lipid-soluble antioxidant content, such as GSH, supports ROS accumulation, which can cause toxicity and apoptosis.

Glutathione is part of glutamine metabolism that is in turn regulated by *MYCN* oncogene. The MYCN regulates the expression and activity of the glutaminase, which converts glutamine into glutamate, a precursor of GSH.[344] MYC is also a regulator of cell proliferation, differentiation, apoptosis, and stimulates mitochondrial biogenesis and function[301]. Thus, targeting MYCN activity and functions is an appealing strategy for tumor cell elimination. The MYCN regulated metabolism can be affected by glutamine withdrawal, making cells more susceptible to cell death. As expected, our data with etoposide treated and glutamine deprived cells showed a significant increase in apoptosis after 6 h of treatment. However, this effect was reduced after 24 h of treatment, suggesting the existence of different mechanisms activated at different time-

points. More specifically, 6 h etoposide treatment does not affect the level of full-length BID but after 24 h cleavage of BID is detected. Therefore, at early time-points, etoposide triggered suppression of Complex I and ROS production are the main activators of apoptosis, although, after 24 h, p53 and BID activation are responsible for apoptotic cell death.

Downregulation of MYCN in NB could reduce the level of glutaminase and consequently GSH, making cells even more sensitive to oxidative stress. However, our results did not reveal any attenuation of glutaminase content nor GSH depletion after MYCN downregulation nor glutamine depletion. Furthermore, MYCN low expressing cells were more resistant to etoposide treatment. This can be explained by the low basal level of p53 in $MYCN^-$ cells that attenuates cells response to DNA damage. In addition, lower levels of full-length BID were also detected in $MYCN^-$ cells. All contributing to the lower apoptotic response to treatment than $MYCN^+$ cells.

Regardless of aforementioned shortcomings, lack of MYCN did not affect etoposide effect on Complex I. Moreover, as with $MYCN^+$ cells, removal of glutamine stimulated apoptotic cell death after 6 h of treatment. Furthermore, the increase in apoptosis was almost twice as high as with $MYCN^+$ cells. This revealed a promising strategy to overcome $MYCN^-$ cell resistance to treatment. Even though there was also a decrease of apoptosis detected after 24 h, it was still higher than with etoposide alone and the reduction was not as prominent as in $MYCN^+$ cells.

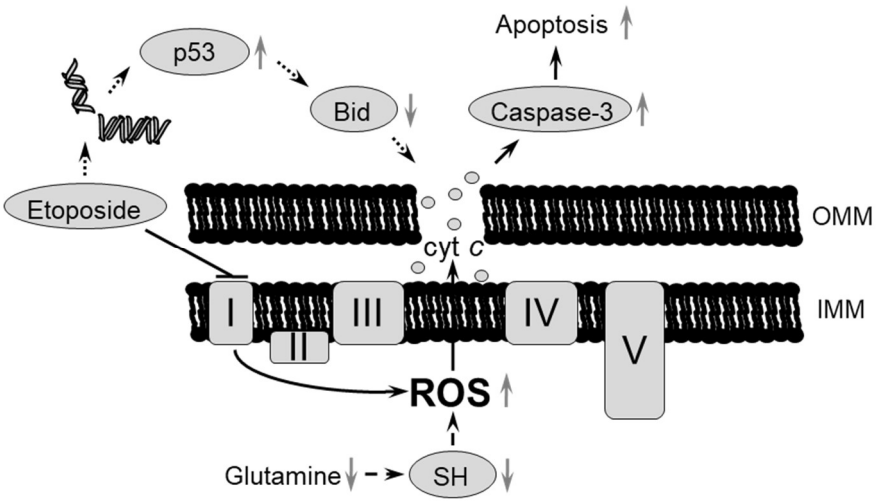

Figure 5. Schematic representation of possible pathways involving etoposide treatment and glutamine deprivation in TET21/N cells. Etoposide has an immediate effect (black arrows) on mitochondrial respiratory chain Complex I that will cause ROS production, cytochrome *c* release and modest apoptosis induction. This effect can be increased by glutamine deprivation (dashed arrows). Long-term (24h) etoposide treatment (dotted arrows) will, however, cause DNA damage-dependent p53 expression and mitochondrial apoptosis activation. OMM – outer mitochondrial membrane; IMM – inner mitochondrial membrane

In conclusion, etoposide-caused apoptosis is a complex process that involves activation of various pathways at different time points. We discovered that besides DNA damage, subsequent stabilization of p53, and apoptosis activation, etoposide also suppresses ETC Complex I activity. This will lead to electron leakage from ETC and formation of ROS.

However, the cellular antioxidant defense systems are very efficient in removing all the excess ROS and thereby avoiding cell death. This can be prevented by reducing the level of cellular antioxidants, for example, by glutamine withdrawal that leads to the reduction of GSH and the increase in ROS levels leading to apoptosis induction. A scheme representing the possible pathways involved in apoptosis stimulation in our experimental model is shown in **Figure 5**. Altogether, relative contribution of oxidative stress and p53 expression determine the outcome of the treatment. Therefore, depletion of antioxidants or inhibition of pathways responsible for cellular antioxidant response can be used to improve tumor treatment.

Metabolism of GSH is an important part of many cancer cells existence and targeting this peculiarity has been an appealing approach for tumor elimination[352,362]. In **Paper III** we investigated the effect of glutamine removal to conventionally used anti-cancer drug treatment on NB cell lines. To our surprise, depending on the anti-tumor drug, glutamine deprivation had contrasting effects. Glutamine withdrawal increased cisplatin-induced cell death but decreased apoptotic response to etoposide, thereby demonstrating an activation of different pathways.

Etoposide and cisplatin are chemotherapeutic drugs that target DNA and stimulate p53 expression. We also detected this in NB cells after etoposide treatment, which led to the expression of p53, DR5, caspase-8 processing, and induction of apoptosis. Although, when glutamine was removed before etoposide treatment adverse effects were seen. Upon treatment with etoposide, removal of glutamine prevented upregulation of p53, DR5 expression, and caspase-8 processing, resulting in suppression of cell death. Transcription factor p53 seems to be the main upstream regulator of the process. Besides regulating DR5, p53 can also activate other pro-apoptotic genes, such as *BID*, *BAX*, and *NOXA* or *PUMA*, that facilitate OMM permeabilization, cytochrome *c* release and apoptotic cell death[138,363,364]. Hence, aforementioned downregulation of p53 can be the reason of apoptosis suppression. However, the connection between glutamine deprivation, etoposide treatment, and p53 is not clear yet and further studies should be conducted to better understand the link.

In case of cisplatin, contrary, glutamine withdrawal further stimulated p53 and DR5 expression. Furthermore, cisplatin is also known to produce ROS[365]. The ROS formation analysis revealed that low dose of cisplatin had a modest effect on ROS production, whereas, removal of glutamine together with cisplatin treatment strongly stimulated formation of ROS. This can be explained by the role of glutamine as a precursor for the antioxidant GSH, therefore, the lack of glutamine results in GSH depletion and increased ROS levels. This was also confirmed by experiments with GSH synthesis inhibitor BSO that had the same effect on the consequences of cisplatin treatment as glutamine removal. The ROS regulates many cellular processes by effecting enzyme activity. Our analysis revealed ROS-facilitated attenuation of protein kinase Akt, phosphorylated Akt, and its target FLIP-S, a caspase-8 inhibitor. Subsequently, the activity of caspase-8 was elevated stimulating apoptotic cell death. Based on these results, the DR5 upregulation and ROS-induced FLIP-S downregulation are both contributing to the caspase-8 activation, which induces apoptosis via mitochondrial BID-mediated pathway followed by apoptosome formation, caspase-9 processing and activation of caspase-3.

Furthermore, the significance of caspase-8 activity for apoptosis stimulation upon glutamine withdrawal in cisplatin-treated NB cells was confirmed by using caspase-8 deficient SK-N-BE(2) cell line. The described increase in cell death in TET21/N cells upon glutamine withdrawal was not detected in SK-N-BE(2) cells, in which caspase-8 gene in epigenetically silenced.

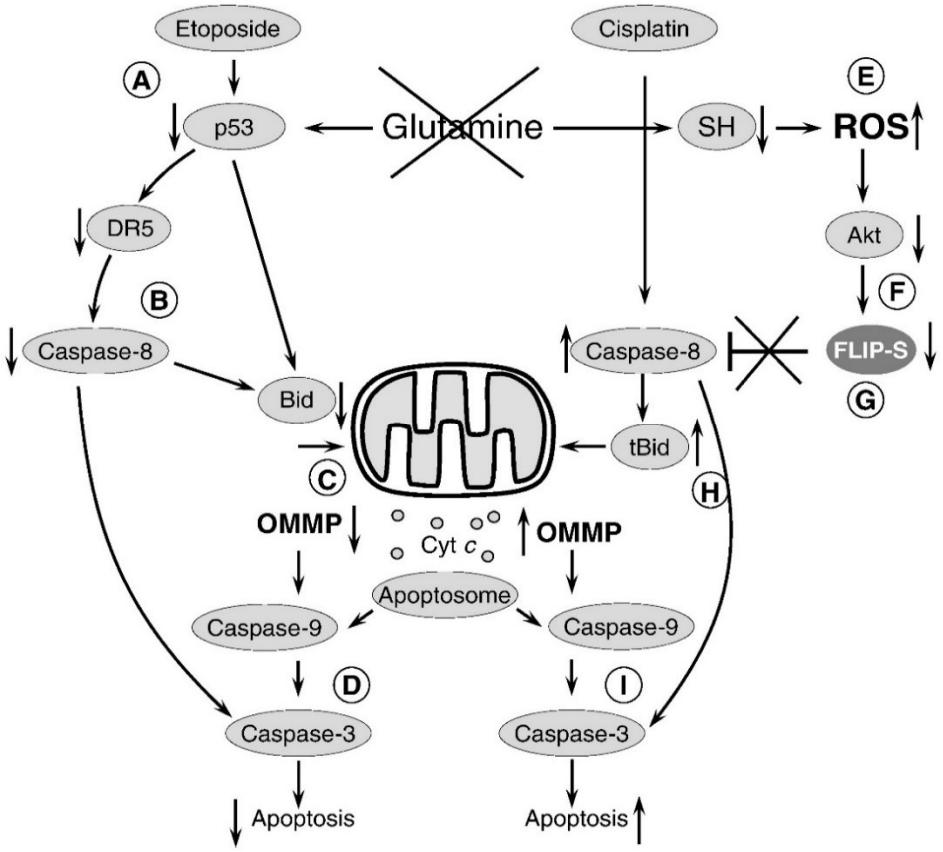

Figure 6. Glutamine withdrawal alters apoptotic response in etoposide or cisplatin treated cells. Withdrawal of glutamine abrogates p53 stabilization in etoposide-treated cells (A). Consequently, DR5 is downregulated, processing of caspase-8 is suppressed and cleavage of BID is attenuated (B). Subsequently, OMM is stabilized towards permeabilization (C). This leads to prevention of caspase-9 processing and inhibition of caspase-3 activity and cell death (D). In case of cisplatin glutamine withdrawal leads to glutathione depletion and stimulation of ROS production (E) causing the suppression of Akt pathway (F). As a result, the level of FLIP-S declines (G), caspase-8 is activated causing cleavage of BID, OMM permeabilization, release of cytochrome *c*, processing of caspase-9, activation of caspase-3, and cell death (I).

The scheme illustrating the ongoing mechanism in NB cells after glutamine removal and treatment with etoposide or cisplatin is shown in **Figure 6**. In conclusion, two separate pathways are activated depending on the anti-cancer drugs. Glutamine deprivation suppresses etoposide-induced apoptosis *via* downregulation of p53 and subsequent events. In contrast, glutamine withdrawal increases cisplatin-induced cell death *via* rise in ROS production and caspase-8 activity. Taken together, targeting glutamine metabolism is a promising approach for treatment improvement, especially in case on cisplatin, where lowering the doses means decreased side effects. However, glutamine deprivation can also suppress apoptosis, which was seen in the case of etoposide. Due to this unexpected outcome, targeting glutaminolysis should

be done cautiously. Further research is needed to undercover the exact mechanisms of action and to find effective inhibitors of glutamine metabolism.

6 CONCLUSIONS AND OUTLOOK

Induction of apoptosis, as a mode of controlled cell death, is one of the main strategies to eliminate cancer cells. Usually, in the process of anti-tumor therapy, apoptosis is activated after administration of DNA damaging chemotherapeutic drugs. These chemicals have shown promising results, but severe toxicity, side effects, and development of resistance make their use challenging. Furthermore, mutations in the apoptotic machinery of cancer cells can suppress cell death. Therefore, additional strategies are needed for treatment improvement. One approach would be to target different steps of apoptotic pathway to increase the effect of the drugs. This can be done, for example, *via* targeting mitochondria that are known as central regulators of apoptosis. Suppression of cellular energy producing pathways was confirmed to be a promising strategy for tumor cell elimination. Inhibition of glycolysis[317], glutamine deprivation[366], or targeting mitochondria can contribute to the improvement of anti-tumor therapy.

In this study we analyzed whether in NB cell lines mitochondrial destabilization by the mitochondrial ETC Complex II inhibitor TTFA can sensitize cells to alkylating-agent cisplatin. We demonstrated how simultaneous use of non-toxic dose of TTFA and low dose of cisplatin can result in significant increase ROS production and subsequent activation of apoptotic cell death of NB cell lines that have fully functional Complex II. These findings clearly demonstrated the potential use of direct mitochondrial targeting as a part of anti-cancer strategy. However, the toxicity of TTFA in animal models is still unknown and should be studied as a next step towards combined therapy. Furthermore, the TTFA sensitizing effect only occurs in functional Complex II cell lines, suggesting the need of preliminary experiments before treatment for successful NB therapy.

Not only specific inhibitors of the mitochondrial respiratory chain, such as TTFA, but also DNA damaging drugs can cause similar effects. In this study, we observed an immediate effect of certain anti-cancer drugs on NB mitochondria. Obtained results revealed a direct effect of etoposide, but not cisplatin or doxorubicin, specifically on Complex I. The activity of Complex I was suppressed and facilitated the leakage of electrons, subsequent ROS formation, and apoptosis in glutamine deprived conditions. These results show that in some cases, the direct effect of anti-tumor drugs on mitochondria should be taken into consideration. The mechanism behind etoposide suppression of Complex I is unclear and studies revealing this action could increase etoposide efficacy. For example, if etoposides respiratory inhibition could be supported and maintained, additional redox manifestations, such as reducing glutamine metabolism as used in the study, may not be needed.

Despite the benefit for anti-tumor treatment, glutamine deprivation should be performed cautiously. As we showed, cellular response to various anti-cancer drugs under glutamine deprivation revealed contrasting outcomes. Glutamine withdrawal stimulated cisplatin-induced apoptosis but suppressed etoposide-induced cell death. To better understand this phenomenon, the connection between glutamine removal, drug activity, and p53 expression should be further

investigated. In our experiments glutamine deprivation was carried out before addition of the drugs, studying the more specific effect of the absence of glutamine on cell metabolism could provide us with additional information. Taken together, targeting glutamine metabolism can sensitize NB cells to treatment and the development of better inhibitors is a promising strategy for tumor cell elimination. However, adverse effects due to the drug action mechanism should be taken into consideration.

In conclusion, this study gained deep understanding about treatment of resistant NB cells lines and their mitochondrial stability under conditions of therapeutic targeting. The results of the doctoral study improved the knowledge about different MYCN expressing and glutamine dependent NB cells lines, giving new possibilities and insight into NB response to treatment. Moreover, several approaches for NB cell sensitization to chemotherapeutic drugs were revealed, which pave the way for more effective treatment in the future.

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