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MITOCHONDRIA AND CELLULAR ENERGY METABOLISM IN PLATINUM CHEMOTHERAPY

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ABSTRACT

Chemotherapy is a major strategy in the treatment of cancer. Unfortunately, treatment of advanced cancers often fails due to tumor resistance to chemotherapeutic drugs. Studies of cell death signaling induced by chemotherapeutic agents are of importance to develop strategies for improved therapy, either by development of new drugs or by potentiation of existing drugs. Discovery of markers for prediction of therapeutic outcome is also of great importance for identification of responsive patients and thereby improved treatment.

This thesis presents results on apoptotic stress-signaling induced by the platinum chemotherapeutic drug cisplatin, and on combining chemotherapeutic drugs with inhibitors of cell metabolism as a potentiation strategy.

MEKK1, a kinase in the MAPK-signaling pathway, was shown to be responsible for cisplatin induced activation of the mitochondrial proapoptotic protein Bak, as seen by conformational change of this protein. However, activation of Bak was not sufficient to induce apoptosis unless signaling from another protein of the MAPK pathway, JNK, was present. Gel filtration experiments revealed Bak complexes of sizes between 80 to 170 kDa in cells with cisplatin-induced onset of apoptosis. By chemical inhibition and gene knock-out, JNK was shown to be crucial for the formation of these complexes.

p53 is involved in responses to platinum drugs. Here, a novel mechanism for negative regulation of p53 translocation to mitochondria is presented. Induction of iNOS after cisplatin treatment was shown to inhibit mitochondrial translocation of p53 as evaluated by chemical inhibition of iNOS. There was no upregulation of iNOS after treatment with oxaliplatin; however, addition of exogenous nitric oxide abrogated mitochondrial translocation of p53 after treatment with this drug.

Seventeen clinical and experimental drugs were screened for potentiation of apoptosis together with glycolysis inhibitor 2-deoxyglucose (DG) and inhibitor of fatty acid β-oxidation etomoxir in HCT116 colon carcinoma cells. DG was more potent than etomoxir in this respect. Cytotoxic responses to combination treatment varied and included apoptosis, necrosis and growth arrest. The combination of cisplatin and DG showed substantial increase in apoptotic response compared to cisplatin alone. Because treatment of ovarian cancer involves platinum drugs, this combination was further studied in two ovarian carcinoma cell lines and primary ovarian carcinoma cells purified from ascites. In the cell lines, higher use of glucose was coupled to increased resistance to cisplatin and carboplatin. In the primary tumor cells, low expression of mitochondrial β-F1-ATPase correlated with reduced cisplatin IC_{50} in the presence of DG.

LIST OF PUBLICATIONS

I. Linda Strandberg Ihrlund, **Emma Hernlund**, Kristina Viktorsson, Theocharis Panaretakis, Gabor Barna, Kanaga Sabapathy, Stig Linder and Maria C. Shoshan
Two distinct pathways of Bak regulation during apoptotic stress signaling: Different roles of MEKK1 and JNK1 *Exp Cell Res. 2006, 312: 1581-1589*

II. **Emma Hernlund**, Özgur Kutuk, Huveyda Basaga, Stig Linder, Theocharis Panaretakis and Maria C. Shoshan
Cisplatin-induced nitrotyrosinylation of p53 prevents its mitochondrial translocation *Free Rad Biol Med. 2009 Jun 15;46(12):1607-13*

III. **Emma Hernlund**, Linda Strandberg Ihrlund, Omar Khan, Yildiz Ö. Ates, Stig Linder, Theocharis Panaretakis and Maria C. Shoshan
Potentiation of chemotherapeutic drugs by energy metabolism inhibitors 2-deoxyglucose and etomoxir *Int J Cancer 2008 Jul 15;123(2):476-83*

IV. **Emma Hernlund**, Elisabet Hjerpe, Elisabeth Åvall-Lundqvist and Maria C. Shoshan
Ovarian carcinoma cells with low levels of β-F1-ATPase are sensitive to combined platinum and 2-deoxyglucose treatment *Mol Cancer Ther. 2009 Jul;8(7):1916-23*

Additional publications:

Ihrlund LS, **Hernlund E**, Khan O, Shoshan MC
3-bromopyruvate as inhibitor of tumour cell energy metabolism and chemopotentiator of platinum drugs *Mol Oncol 2008 Jun;2(1):94-101*

Hellberg V, Wallin I, Eriksson S, **Hernlund E**, Jerremalm E, Berndtsson M, Eksborg S, Arnér ES, Shoshan M, Ehrsson H, Laurell G
Cisplatin and oxaliplatin toxicity: importance of cochlear kinetics as a determinant for ototoxicity *J Natl Cancer Inst. 2009 Jan 7;101(1):37-47*

Berndtsson M**, Hernlund E**, Shoshan MC, Linder S
Conditional screening shows that mitotic inhibitors induce AKT/PKB-insensitive apoptosis
J Chem Biol. 2009 Jun;2(2):81-7

CONTENTS

LIST OF ABBREVIATIONS

AMPK	AMP-activated protein kinase
ANT	adenosine nucleotide translocase
AP-1	activator protein 1
Apaf-1	apoptotic peptidase activating factor 1
ATF-2	activating transcription factor 2
ATM	ataxia telangiectasia mutated kinase
ATP	adenosine 5'-triphosphate
ATR	ataxia telangiectasia and Rad-3 related kinase
Bad	Bcl-2 associated death promoter
Bak	Bcl-2 antagonist killer 1
Bax	Bcl-2 associated x protein
Bcl-2	B-cell CLL/Lymphoma 2
Bcl-xL	Bcl-2 regulated gene, long isoform
BEC-index	bioenergetic cell index
BH	Bcl-2 homology
Bid	Bcl-2 interacting domain death agonist
BMH	1,6-bis-maleimidohexane
CPT-1	carnitine palmitoyltransferase-1
Da	Dalton
DG	2-deoxyglucose
DNA	deoxyribonucleic acid
EGF	epidermal growth factor
ER	endoplasmic reticulum
ERCC1	excision repair cross-complementation group 1
ERK	extracellular regulated kinases
FACS	flourescence-activated cell sorting
$FADH_2$	flavin adenine dinucleotide
FASN	fatty acid synthase
FDA	US Food and Drug Administration
FDG	18-fluoro deoxyglucose
GGR	global genomic NER
HAT	histone acetyl transferase
HDAC	histone deacetyl transferase
HIF	hypoxia inducible factor
HM	heavy membrane
HR23B	human homolog of Rad23 B
Hsp60	heat shock protein 60
IMM	inner mitochondrial membrane
iNOS	inducible nitric oxide synthase
IP	Immunoprecipitation
JNK	c-Jun NH_2-terminal kinase
LDH	lactic dehydrogenase
LKB1	liver kinase B1
M2-PK	pyruvate kinase type M2

MAO	monoamine oxidase
MAPK	mitogen-activated protein kinase
MAP2K	MAPK kinase
MAP3K	MAPK kinase kinase
Mcl-1	myeloid cell leukemia 1
Mdm-2	mouse double minute protein 2
MEF	mouse embryonic fibroblast
MEKK1	MAPK/ERK kinase kinase 1
MKK	MAPK kinase
MMR	mismatch repair
MOMP	mitochondrial outer membrane permeabilization
mTOR	mammalian target of rapamycin
mTORC	mTOR complex
MPT	mitochondrial permeability transition
MPTP	mitochondrial permeability transition pore
mtDNA	mitochondrial DNA
MTS	3-(4,5-dimethylthiazol-2-yl)-2-(4-sulfophenyl)-2-tetrazolium, inner salt
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NAD	nicotine adenine dinucleotide
NFAT	nuclear factor of activated T-cells
NER	nucleotide excision repair
NO	nitric oxide
OA-519	oncogenig antigen 519
OMM	outer mitochondrial membrane
PET	positron emission tomography
PI	propodium iodide
PI3K	phosphatidylinositol 3-kinase
PPP	pentose phosphate pathway
PUMA	p53-upregulated mediator of apoptosis
RNS	reactive nitrogen species
ROS	reactive oxygen species
SAPK	stress-activated protein kinase
SCO2	synthesis of cytochromec oxidase protein
SH3	Src-homology 3
Smac/Diablo	second mitochondria-derived activator of caspase/Direct IAP-binding protein with low pI
SNAP	S-nitroso-N-acetyl-DL-penicillamine
TCA	trichloro acetic acid
TCA cycle	tricarboxylic acid cycle
TCR	transcription coupled NER
TIGAR	TP53-induced glycolysis and apoptosis regulator
TNFα	tumor necrosis factor alpha
TSC	tuberous sclerosis complex
VDAC	voltage dependent anion channel
XPA	*xeroderma pigmentosum* group A
XPF	*xeroderma pigmentosum* group F
XTT	2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-

	5[(phenylamino)carbonyl]-2H-tetrazolium hydroxide
β-F1-ATPase	the β-catalytic subunit of the H+-ATP synthase
4EBP1	4E-binding protein

1 INTRODUCTION

About 50 000 persons are diagnosed with cancer every year in Sweden, and around 20 000 die as a result of this disease. A cancer is a malignant tumor originating from normal tissues of the body. The most common types of tumors are the carcinomas (e.g. breast, prostate, lung colon and ovarian cancers), which originate from epithelial cells. In most cases, the development of a cancer is a slow process that applies to Darwinian rules of selection, where cells with the capability to proliferate are continuously selected for (Foulds 1954). Cancer initiation and progression require gain-of-function alterations in oncogenes and loss-of-function alterations in tumor suppressor genes to override the tightly regulated homeostasis and limitations of replication for normal cells. Genetic alterations include point mutations, deletions, translocations, gene amplification and epigenetic changes.

Together, such alterations lead to some basic features that are common to most cancers. Hanahan and Weinberg have summarized six traits in their popular review "Hallmarks of Cancer": limitless replicative potential, sustained angiogenesis, evasion of apoptosis, self-sufficiency in growth signals, insensitivity to anti-growth signals and tissue invasion and metastasis (Hanahan and Weinberg 2000).

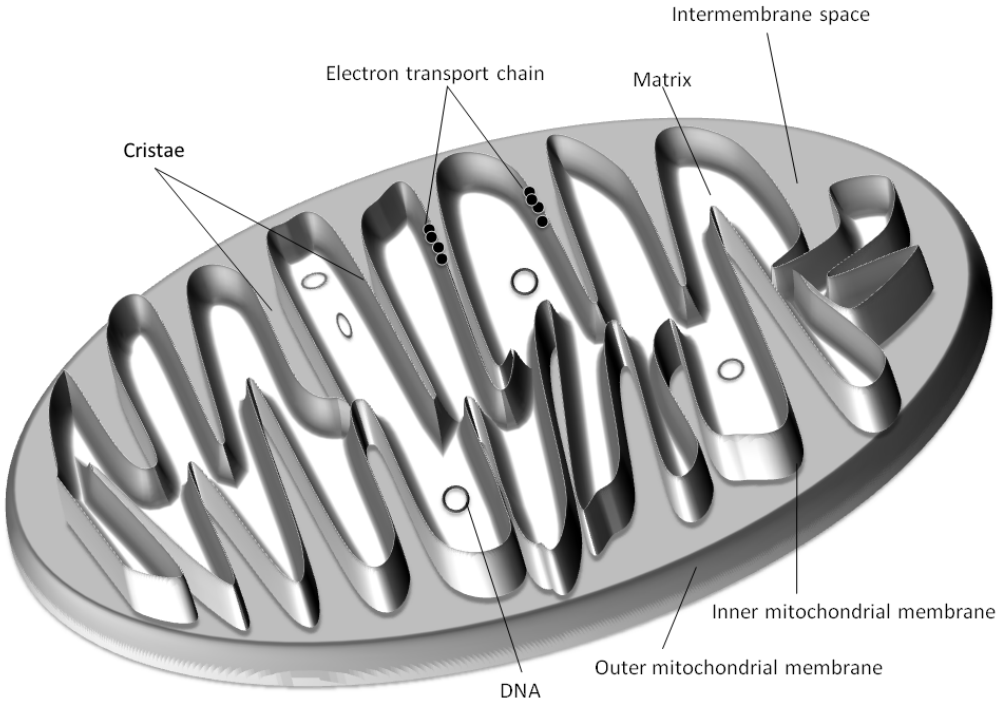
This thesis deals with the apoptotic signaling induced by chemotherapeutic drugs. It also aims to investigate increased anti-tumor effects by exploring what has been proposed to be the "seventh hallmark of cancer" – the metabolic changes of cancer cells. Central to both themes are platinum drugs and effects at the mitochondrion.

1.1 THE MITOCHONDRION

Mitochondria can be found in almost all eukaryotic cells and the number of mitochondria varies widely depending on cell and tissue type. They are thought to be of prokaryotic origin and included in eukaryotic cells in a symbiotic fashion. They carry their own genome as several copies of a single chromosome of circular DNA. Mitochondrial DNA (mtDNA) encodes genes for 37 protein, including 13 subunits of respiratory complexes I, III, IV and V. Also tRNAs needed for translation of mRNA are transcribed from mtDNA (Chan 2006).

Mitochondria consist of two membranes: the outer and inner mitochondrial membranes (OMM and IMM respectively). The OMM has a protein-to-phospholipid ratio similar to that of the plasma membrane and harbors numerous channel forming porins that allow free diffusion of molecules smaller than 5kDa (Distler, Kerner et al. 2008). It is also through the OMM that mitochondria can associate with the endoplasmic reticulum (ER).

The IMM has a much larger area than the OMM and is carefully folded into cristae. This highly impermeable membrane has a very high protein-to-phospholipid ratio, containing high proportions of the otherwise unusual phospholipid cardiolipin, which binds to cytochrome c in the intermembrane space (Orrenius 2007). The mitochondria can through these membranes and their properties be divided into five compartments: the OMM, the intermembrane space, the IMM, the cristae formed by the IMM and the mitochondrial matrix.



Mitochondria normally provide most of the cellular energy production through the tricarboxylic acid cycle (TCA cycle) and the subsequent electron transport chain. The TCA cycle is performed in the mitochondrial matrix while ATP production occurs over the inner mitochondrial membrane at the cristae. The TCA cycle uses acetyl-CoA formed from e.g. glycolytic end-product pyruvate and oxidizes it to carbon dioxide. In this process, reduced cofactors (three NADH and one flavin adenine dinucleotide ($FADH_2$)) are produced. The energy from reduced cofactors is transferred to oxygen in the electron transport chain in the IMM and is then used to synthesize ATP. The electrons are transported in a chain consisting of complexes I through IV while complex V is responsible for ATP synthesis. Complexes I, III and IV also transport protons over the IMM to build up the mitochondrial membrane potential which is the prerequisite for ATP synthesis. The mitochondrial membrane potential is essential for accumulation of calcium, respiration and production of ATP and is commonly used as a measure of cell viability.

The mitochondrial respiratory chain is also a major source of ROS and about 1-2 % of the oxygen consumed during normal cellular respiration is converted into superoxide anion radicals. Superoxide results from leakage of electrons from complexes, mostly from complex I, into the mitochondrial matrix. Superoxide production via complex III occurs at both sides of the IMM (Orrenius 2007). The monoamine oxidase (MAO) located at the OMM is also an important source of mitochondrial produced ROS (Cadenas and Davies 2000). Superoxide can pass freely over mitochondrial membranes. There are enzymatic systems to detoxify cells from superoxide, such as superoxide dismutase which converts it to hydrogen peroxide. Mitochondria are not only producers of ROS – they are also sensitive targets for ROS damage. Especially the superoxide produced in the matrix, due to its close proximity, is harmful to mtDNA which is not protected by histones. ROS-induced damage of mtDNA is proposed to have an important role in aging (Pang, Ma et al. 2008; Santos, Martinez et al. 2008).

The mitochondrial matrix can function as transient storage of calcium through interaction with the primary calcium storage site – the ER. Calcium overload in mitochondria can lead to MPTP opening and loss of mitochondrial membrane potential (Hajnoczky, Csordas et al. 2006).

1.2 CELL DEATH

The cell is a defined space where reactions happen based on the conditions provided by the genomic information. A person is considered dead when the brain activity ceases. By analogy, a cell can be considered dead when the nucleus ceases to function, when no more proteins can be expressed. In addition to this, loss of membrane integrity is a crucial criterion for cell death. In the human body, there is a massive cell turn-over with between 50 and 70 billion of cells dying every day. The death of individual cells is a prerequisite for the well-being of the multicellular organism. Defects in cell death processes are associated with several diseases, such as diabetes, neurodegenerative diseases and cancer.

1.2.1 Apoptosis

Studies of the morphological phenomenon of cell shrinking as a form of cell death have gone in and out of fashion during the last 100 years. A major milestone and attention drawer to the field was the research on the nematode *C. elegans* which in 1972 led to the characterization and introduction of the term apoptosis (Kerr, Wyllie et al. 1972). Apoptosis is characterized by cell shrinkage, chromatin condensation, and nuclear and cell fragmentation into apoptotic bodies that can subsequently be engulfed by neighboring phagocytic cells. It is also a tightly regulated multi-step pathway, importantly consuming ATP and not eliciting an immune reaction. Apoptosis can be executed after signals both from the outside and the inside of the cell.

1.2.2 Necrosis

In contrast to the regulated “on purpose” death necessary for life represented by apoptosis, necrosis may inflict damage to a complex multi-cellular life. Often it is the consequence of external factors, such as heat or cold, mechanical tearing of cells and low blood supply. During necrosis, the cytoplasm and mitochondria of cells swell leading to cell rupture and release of cellular compartments into the surrounding tissue. This will lead to an inflammatory response by the immune system. However, although necrosis has mainly been considered accidental to its nature, recent years’ research has presented data on regulated, or programmed, necrosis. The field originated based on data showing that cells die in a necrotic fashion if apoptosis initiation is stimulated concurrently with inhibition of apoptosis execution. It has been shown that stimuli such as intracellular Ca^{2+} overload, excessive production of reactive oxygen species (ROS) and energy depletion are capable of triggering necrosis (Vanlangenakker, Berghe et al. 2008). However, there is still some debate regarding the relevance of programmed necrosis *in vivo* (Edinger and Thompson 2004).

1.2.3 Additional routes to cell death

With definition of cell death as loss of function of the genome and loss of cell membrane integrity, i.e. the disruption of the boundaries of one life unit, apoptosis and necrosis each

represents a way to disrupt one of each of these vital criteria. However, further subdivisions of cell death modes have been introduced.

Autophagy (Greek for self-eating) is an evolutionary conserved process induced by starvation of cells. During autophagy, double membrane encapsulation of macromolecules and organelles constitutes the formation of a so-called autophagosome which can then fuse with lysosomes to form an autophago-lysosome where the encapsulated material is broken degraded and recycled within the cell (Debnath, Baehrecke et al. 2005). In organisms like yeast and bacteria this process is pro-survival, protecting the cell from starvation. For mammalian cells autophagy is proposed to be important for both cell survival and death (Edinger and Thompson 2004).

In the case of extensive DNA damage with lack of cell-cycle checkpoints or interference with the microtubular system in mitosis, cells can fail to go through the mitotic phase of the cell cycle. This suggested form of cell death has been termed *mitotic catastrophe*. There have been attempts to put morphological definitions on mitotic catastrophe including multiple micronuclei and condensed chromatin (Swanson, Carroll et al. 1995; Ianzini and Mackey 1997), but the question remains whether this is a separate mode of cell death or just a pre-stage to death.

1.3 APOPTOTIC SIGNALING

The complex execution of a cell suicide machinery leaving phagocytosable fragments without an inflammatory response can be triggered both from signaling within the cell and from external mediators. These two main pathways to apoptosis are termed the extrinsic (or death receptor) pathway and the intrinsic (or mitochondrial) pathway. The apoptotic machinery itself can also be divided in two: stimuli sensing proteins and executioner proteins. Executioner proteins are to a large part shared between the intrinsic and extrinsic pathway.

1.3.1 Caspases

Caspases (cysteine-dependent aspartate specific proteases) are the key players in apoptosis. They are constitutively expressed in a procaspase form and regulated posttranslationaly by cleavage. There are two groups of caspases; those that will become active upon death stimuli and autocleavage are called initiator caspases and those that are activated by initiator caspases to cleave a wide variety of intracellular targets are called effector caspases. Initiator caspases are e.g. caspases -9 and -8, and effector caspases -3, -6 and -7 (Thornberry and Lazebnik 1998; Chowdhury, Tharakan et al. 2006). Effector caspases are needed for the breakdown of cytoskeletal and nuclear structures as well as the release of the DNAse responsible for cutting DNA into the for apoptosis typical 200 base-pair ladder.

1.3.2 Mitochondria and apoptosis

Mitochondria are essential in the intrinsic damage-sensing pathway to apoptosis. The initiator caspase of this pathway is caspase-9. It is activated in the apoptosome, a protein complex in the cytosol. It consists of caspase-9, apoptotic peptidase activating factor 1 (Apaf-1), adenosine triphosphate (ATP) and cytochrome c (Riedl and Salvesen 2007). Cytochrome c normally resides in the mitochondria, in the space between the outer and inner membrane. The other constituents of the apoptosome are always available in the cytosol. Hence, it is the

release of cytochrome c that initiates the apoptotic execution phase. Other proapoptotic factors are also released from the mitochondrial inter-membrane space e.g. second mitochondria-derived activator of caspase/direct IAP-bind protein with low pI (Smac/Diablo) (Chipuk and Green 2008). The release of cytochrome c from the mitochondrial intermembrane space is made possible by mitochondrial outer membrane permeabilization (MOMP).

Although there is a debate on the exact mechanism how this release of proapoptotic factors from mitochondria occurs, there is clear evidence that members of the B-cell CLL/Lymphoma 2 (Bcl-2) family are central to this process. There are currently two models of how mitochondrial membranes are permeabilized – both involving opening of protein pores.

1.3.2.1 Mitochondrial permeabilization by Bcl-2 family proteins

The first model involves proteins of the Bcl-2 family which are divided into three groups based on the presence of Bcl-2 homology domains (BH domains). Proteins of the Bcl-2 family can be both promote and inhibit MOMP. The antiapoptotic proteins (e.g. Bcl-2, Bcl-2 regulated gene, long isoform (Bcl-xL) and Myeoloid cell leukemia 1 (Mcl-1)) contain BH domains 1-4 and are usually integrated in the outer membrane of mitochondria. The proapoptotic members can consist of BH domains 1-3 or only the BH3 domain. The proteins containing domains 1-3 have an executive role in induction of MOMP and are Bcl-2 associated x protein (Bax) and Bcl-2 antagonist killer 1 (Bak) (Oltvai, Milliman et al. 1993; Chittenden, Flemington et al. 1995). When activated, these proteins can form multimeric oligomeres in the outer mitochondrial membrane in form of a pore through which cytochrome c and other proapoptotic factors are released. Cells lacking both Bax and Bak show resistance to a variety of stimuli known to cause mitochondria-dependent apoptosis (Wei, Zong et al. 2001). The BH3-only proteins function in response to specific cellular stresses and include e.g. Bcl-2 associated death promoter (Bad), Bcl-2 interacting domain death agonist (Bid), Noxa and p53-upregulated modulator of apoptosis (PUMA) (Huang and Strasser 2000). In order for Bak and Bax to induce oligomerization and permeabilization of mitochondria, these proapoptotic proteins have to be active – i.e. they undergo a conformational change. In one version of this model, this is occurs directly at the site of mitochondria by the action of certain BH3-only proteins (Bid and Bim). The ratio of antiapoptotic Bcl-2 proteins to Bax/Bak in itself would in this theory not be important until the BH3-only proteins (or other direct activators) are inducing Bax/Bak activation. In another version, there is a constant balance between the total amounts of pro- and antiapoptotic members of the Bcl-2 family (Huang and Strasser 2000; Chipuk and Green 2008).

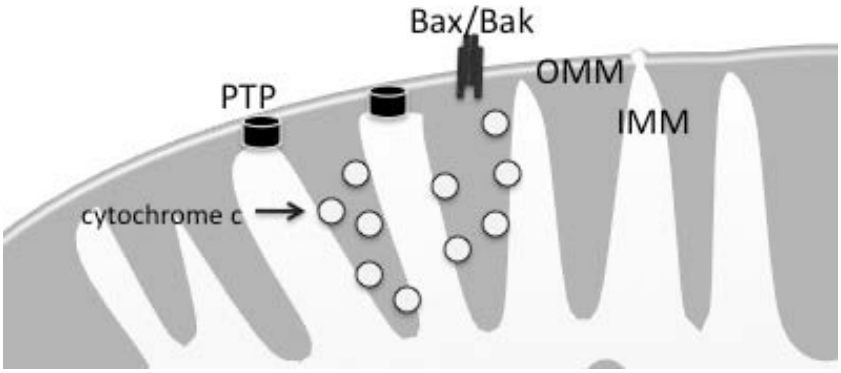


Figure of two models for mitochondrial permeabilization: the Permeability Transition Pore at inner/outer mitochondrial membrane (IMM/OMM) junctions and the Bax/Bak oligomers formed in the outer mitochondrial membrane. Subsequent release of cytochrome c leads to formation of apoptosome proapoptotic complex in the cytoplasm.

1.3.2.2 Mitochondrial permeabilization by the mitochondrial permeability transition pore

The second main model for the mitochondrial release of proapoptotic molecules is through mitochondrial permeability transition (MPT) which involves opening of the mitochondrial permeability transition pore (MPTP). The MPTP consists of adenosine nucleotide translocase (ANT), voltage dependent anion channel (VDAC) and the matrix protein cyclophilin D. The pore forming complexes are located at the junction sites of the inner and outer mitochondrial membrane, thereby allowing contact between mitochondrial matrix and cytosol. Molecules less than 1500 Dalton (Da) are allowed passage through an open MPTP.

MPT can be induced by Ca^{2+} overload, inorganic phosphate, oxidizing agents and certain fatty acids (Zoratti and Szabo 1995). The calcium regulation of MPTP has been attributed to the calcium binding abilities of VDAC (Shoshan-Barmatz and Gincel 2003). Upon opening of the MPTP, osmotic pressure makes the matrix swell, leading to rupture of the outer mitochondrial membrane and release of proapoptotic proteins, including cytochrome c, from the inter membrane space. Also, mitochondrial depolarization through MPT leads to uncoupling of mitochondria (allows protons to pass over the inner mitochondrial membrane without the generation of ATP), a process that makes the mitochondria switch from ATP production to ATP hydrolysis. This leads to a significant drop in ATP levels (Javadov, Karmazyn et al. 2009).

There are suggestions that MPT is important also for cell death by necrosis. In a dose-response manner, only a mild degree of MPT would elicit an apoptotic signaling cascade whereas the more pronounced MPT would lead to necrosis (Honda and Ping 2006; Gogvadze, Orrenius et al. 2009).

1.3.3 Proapoptotic signaling of the mitogen-activated and stress-activated protein kinase pathways

Mitogen-activated protein kinase (MAPK) pathways are evolutionary conserved kinase modules which transfer extracellular signals into cellular responses such as proliferation and differentiation, but which also help regulate apoptosis. These pathways function by activation by phosphorylation in a three kinase cascade where the MAPK is phosphorylated by a MAPK kinase (MAP2K), which in turn is phosphorylated by a MAPK kinase kinase (MAP3K). There are at least 20 different MAP3Ks, to be compared with seven MAP2Ks and eleven MAPKs (Johnson and Nakamura 2007). Many of the MAP3Ks are activated by GTPases, such as the Ras or Rho family. The diversity of proteins as well as possibilities for protein-protein interactions allow for MAPKs to be involved in the response to a diverse range of stimuli. These stimuli include cytokines, growth factors, antigens, cell adhesion and cell-cell interactions, toxins and pharmacological drugs. Stress stimuli like heat, cold and toxicity can also activate the MAP3Ks. The MAPKs can exert their function by phosphorylation of cytoplasmic target proteins or by translocation to the nucleus, where they can directly regulate the activity of transcription factors controlling gene expression.

1.3.3.1 MEKK1

MAPK/ERK kinase kinase 1 (MEKK1) was the first mammalian MAP3K to be cloned, based on homology with the yeast MAP3K proteins Ste11p and Byr2p (*S. cervisiae* and *S. pombe* respectively) (Lange-Carter, Pleiman et al. 1993). Later, MEKK2, 3 and 4 were identified by the same strategy (Blank, Gerwins et al. 1996; Gerwins, Blank et al. 1997). MEKK1-4 all contain an N-terminal regulatory domain and a C-terminal catalytic domain with serine-threonine kinase activity. MEKK1 and 4 both contain proline rich regions and pleckstrin homology domains at the very N-terminal. The pleckstrin homology domains may be responsible for the cellular membrane attachment of these two proteins, while the proline rich sequences are involved in binding proteins containing src-homology 3 (SH3) domains (Hagemann and Blank 2001). MEKK1 was named for its ability to phosphorylate the MAP2K protein MEK; this was previously was attributed only to Raf (Lange-Carter, Pleiman et al. 1993). MEKK1 can be activated by receptor-mediated stimuli like epidermal growth factor (EGF) (Fanger, Johnson et al. 1997), tumor necrosis factor alpha (TNFα) (Ishizuka, Terada et al. 1997) and inter-leukin 1 (IL-1) (Kopp, Medzhitov et al. 1999). Also, cytotoxic agents like DNA- and microtubuli-damaging agents can increase MEKK1 activity (Widmann, Gerwins et al. 1998; Yujiri, Sather et al. 1998). In the case of DNA damage, the protein tyrosine kinase c-Abl has been shown to phosphorylate MEKK1 both *in vitro* and *in vivo* (Kharbanda, Pandey et al. 2000).

However, it was later suggested that the MEKKs primarily activate MAPKs of the c-Jun NH_2-terminal kinase (JNK)/stress-activated protein kinase (SAPK) and also p38 families, in contrast to Raf kinases, which signal only through MEK and extracellular regulated kinase (ERK). Furthermore, MEKK1 can be a caspase substrate. The 196 kDa protein can be cleaved at Asp^{874} which leaves the 91 kDa C-terminal with catalytic activity in the cytoplasm. There, it can amplify the caspase activation and apoptosis (Hagemann and Blank 2001). Its effect is probably independent of JNK signaling but dependent on Bcl-2 (Widmann, Gerwins et al. 1998; Gibson, Widmann et al. 1999). In addition to stress responses through JNK and p38 pathways, MEKK1 has been shown to be of importance for cell adhesion and/or migration (Xia, Makris et al. 2000).

1.3.3.2 JNK

JNKs exist in three isoforms, JNK1-3. JNK1 and JNK2 are expressed ubiquitously while JNK3 is expressed only in brain, testis and heart tissues. Two MAP2K proteins have been shown to activate JNK: MAPK kinase 4 (MKK4) and MAPK kinase 7 (MKK7) (Johnson and Nakamura 2007). In addition to phosphorylation of JNKs, MKK4 is also able to phosphorylate p38 MAPKs and has been shown to be a tumor suppressor in a number of cancers (Su, Hilgers et al. 1998; Yamada, Hickson et al. 2002). To become activated by MKK4 or MKK7, JNK is phosphorylated at threonine and tyrosine residues within the activation loop of the protein. JNKs then activate apoptotic signaling either through the upregulation of proapoptotic genes by transactivation of specific transcription factors or through direct modulation of mitochondrial pro- or antiapoptotic proteins. For transactivation of genes, phosphorylated JNK translocates from the cytoplasm to the nucleus of the cell. Once there, it can phosphorylate and transactivate transcription factors like c-Jun, activating transcription factor 2 (ATF-2), p53, c-myc, Elk-1 and nuclear factor of activated T-cells (NFAT) (Raman, Chen et al. 2007). The nuclear activity of activated JNK can thereby lead to increased expression of proapoptotic genes and/or decreased expression of antiapoptotic genes. c-Jun activation and subsequent formation of activator protein 1 (AP-1) is important for a variety of cell types to induce an apoptotic response to JNK activation. Some of the stimuli that can elicit JNK-dependent apoptosis are genotoxic drugs, microtubule damaging agents, TNF-α, ROS, lipopolysaccharide and endoplasmic reticulum (ER) stress (Dhanasekaran and Reddy 2008).

MAPK pathways show response to a variety of stimuli and can elicit a broad spectrum of cellular responses. This complexity is difficult to map. However, levels of complexity can be explained in terms of e.g. cellular localization, interacting regulatory proteins, scaffold proteins, type of cell, post-translational modifications (e.g. ubiquitinylation and proteolytic cleavage) and binding proteins at all levels of the signaling cascade.

1.3.4 p53 and damage response

p53 is one of the most well studied proteins in cancer research. As “the guardian of the genome”, it is the most commonly inactivated tumor suppressor gene in human cancer. p53 acts in response to diverse forms of cellular stress, e.g. DNA damage, oxidative stress, oncogenic stress and ribonucleotide depletion (Giaccia and Kastan 1998) to mediate a variety of antiproliferative responses. Because of its important function, both cellular expression and the activity of p53 are tightly regulated. Although p53 is constantly transcribed, protein levels are kept low by high proteasomal degradation mediated through the activity of the E3-ligase mouse double minute protein 2 (Mdm2). In this fashion, p53 protein levels can quickly increase in response to cellular stress by decreased proteolysis. The most well studied mechanism for stabilization p53 is decreased recognition by Mdm2 due to phosphorylation occurring at serines of the N-terminal domain of p53. Especially phosphorylation of ser20 has been shown to be of important for the reduction p53-Mdm2 binding (Unger, Juven-Gershon et al. 1999). A broad range of kinases can mediate this posttranslational modification, e.g. ataxia telangiectasia mutated kinase (ATM), ataxia telangiectasia and Rad-3 related kinase (ATR), dsDNA-activated protein kinase (DNA-PK), Chk1, Chk2 and the MAPKs JNK and p38 (Kruse and Gu 2009). ATM and ATR are damage-sensing kinases and can act on p53 directly or indirectly through Chk1 and Chk2 (Kastan and Bartek 2004).

Mdm2-p53 interactions can serve as a further target of regulation. The tumor suppressor protein alternative reading frame (ARF) can affect p53 degradation by directly binding to Mdm2 leading to sequestration in the nucleolus or by inhibiting the ubiquitin ligase activity through binding (Honda and Yasuda 1999; Weber, Taylor et al. 1999). Expression of ARF can be induced by oncogenic stress such as early region 1A (E1A), c-Myc and Ras (Pauklin, Kristjuhan et al. 2005).

In the nucleus, p53 is a transcription factor, primarily inducing growth arrest genes like p21 and regulators of apoptosis. Proapoptotic genes under direct p53 regulation are e.g. Bax, PUMA, NOXA and Apaf-1 of the intrinsic pathway. The extrinsic pathway can also be activated through p53 activation of the genes for Fas and Fas ligand, although the overall contribution of this pathway to p53 induced apoptosis is not well understood (Fridman and Lowe 2003). p53 can also be antiproliferative through actions on survival signaling, e.g. through the inhibition of the PI3 kinase pathway, where p53 can induce the negative regulator phosphatase and tensin homolog (PTEN) (Stambolic, MacPherson et al. 2001). p53 may also transrepress antiapoptotic genes.

1.3.4.1 Transcription independent apoptosis

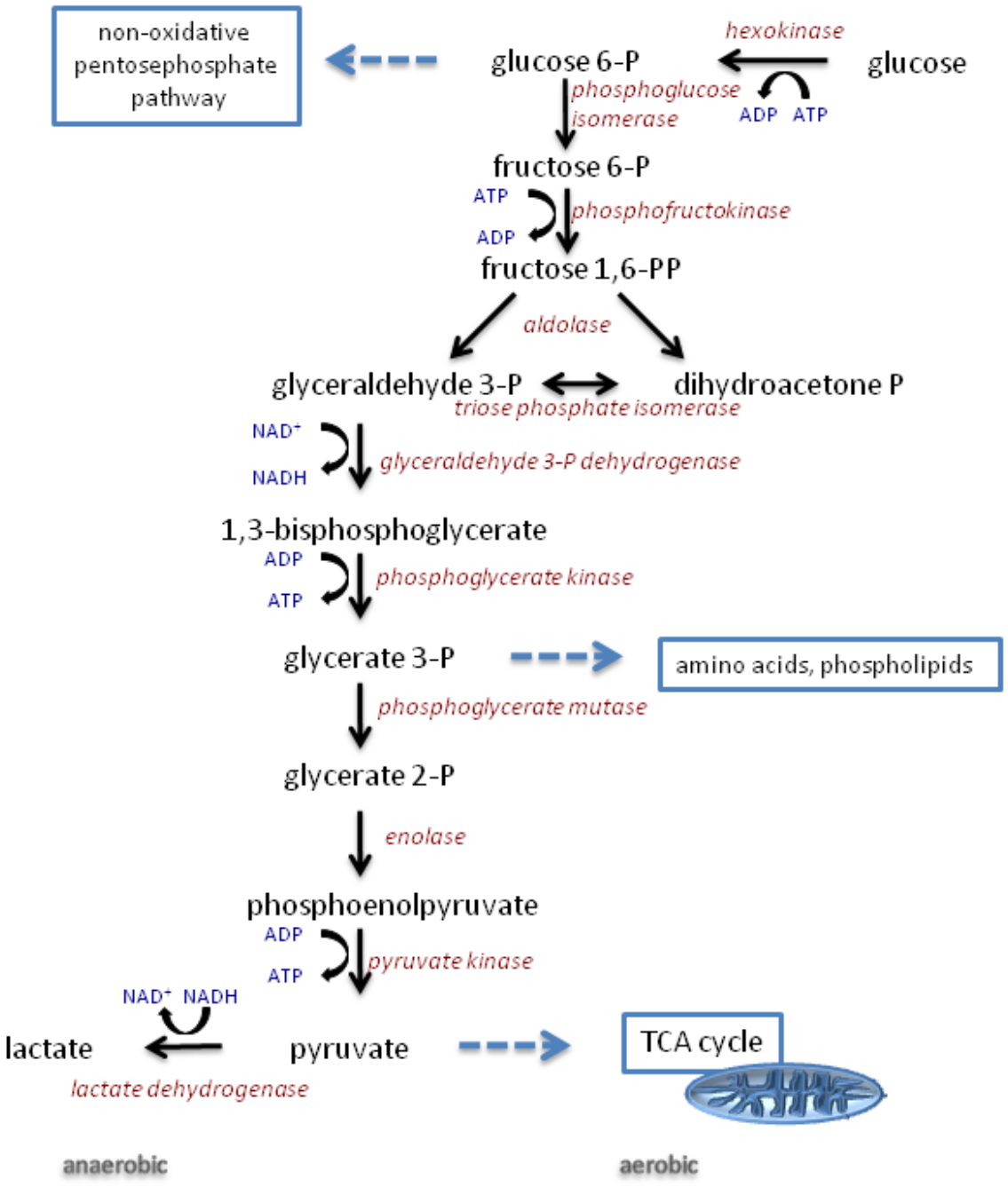
Transcription independent functions of p53 have been described since the mid -90s (Caelles, Helmberg et al. 1994; Wagner, Kokontis et al. 1994). It has been shown that in response to genotoxic stress p53 can rapidly translocate to the mitochondria, triggering an early wave of mitochondrial depolarization and caspase activation (Mihara, Erster et al. 2003; Erster, Mihara et al. 2004). The MOMP induced by wild type p53 (and wild type only) at mitochondria is believed to be due to p53 binding to antiapoptotic proteins Bcl-xL and Bcl-2, thereby releasing Bcl-2 family proapoptotic effector proteins Bax and Bak (Mihara, Erster et al. 2003; Tomita, Marchenko et al. 2006). Albeit with lower binding affinity, mitochondrial p53 has also been shown to directly bind to Bak (Leu, Dumont et al. 2004). There are also suggestions that cytoplasmic p53 has a role involving PUMA and Bax (Green and Kroemer 2009). In this model, cytoplasmic p53 is normally sequestered by Bcl-xL, but upon genotoxic stress and p53 induced transcription of PUMA, PUMA can replace and release cytoplasmic p53 to induce apoptosis at the mitochondria by activating Bax. Hence, in different ways, both models need nuclearly active p53 for the direct activation of MOMP.

There is a great challenge in elucidating the global importance of interactions, modifications, temporal activation, subcellular localization and tissue contexts that elicit these different responses through the activation of p53.

1.4 ENERGY METABOLISM IN THE CANCER CELL

Normal cells produce more than 90% of their ATP via oxidative phosphorylation in the mitochondria. Already in the 1920s Otto Warburg found that tumor cells differ in their metabolism compared to normal cells. He published papers describing an increase in tumor cell glucose consumption and lactate production alongside a decrease in oxidative phosphorylation under normoxic conditions. This has later been termed “the Warburg effect”. The increased use of glucose by tumor cells is utilized in the clinic for detection and diagnosis of tumors using the radiolabeled glucose analog 18-fluoro deoxyglucose (FDG) in positron emission tomography imaging.

Metabolizing one molecule of glucose to pyruvate through glycolysis generates only two adenosine 5'-triphosphate (ATP) molecules, whereas complete oxidation of glucose through oxidative phosphorylation renders up to 36 ATP (Vander Heiden, Cantley et al. 2009). Why tumor cells employ this inefficient way to generate ATP and whether it confers any advantage to cancer cells has been quite unclear. However, during recent years, many studies have integrated metabolic features into classical tumor cell phenotypes and several models of how metabolism adds survival advantages have been presented. In this field, the role of glycolysis in tumor cell biology has been the pathway most studied.



Glycolysis with glycolytic enzymes and co-factors. Fructose 1,6-PP leads to the generation of two glyceraldehydes 3-P whereby subsequent ATP generation is doubled.

1.4.1 Cancer cell regulation of glycolytic pathways

There are several suggestions for why tumor cells show increased glycolytic activity. One hypothesis is that the tumor microenvironment directly affects the cell metabolism. As the early tumor expands, it will outgrow the diffusion limits of its local blood supply. This leads to hypoxia and stabilization of the hypoxia inducible factor (HIF). Most glycolytic enzymes are regulated by the transcription factor HIFα. A decreased dependence on aerobic glycolysis would be advantageous. However, tumor cells that are not exposed to hypoxic conditions, such as leukemic cells as well as lung tumors of the airways, have also been shown to be highly glycolytic (Nolop, Rhodes et al. 1987; Gottschalk, Anderson et al. 2004).

In addition to being an adaptation to hypoxia, the appearance of the Warburg effect can also be triggered intracellularly. Several oncogenes have been shown to shift tumor metabolism towards glycolysis. Ras overexpression has been shown to increase glucose consumption and to decrease the use of oxygen (Biaglow, Cerniglia et al. 1997). The downstream target Akt of the phosphatidylinositol 3-kinase (PI3K) pathway can regulate the expression of glucose transporter and affects glycolytic enzymes by e.g. enhancing glucose capture by hexokinase and stimulation of phosphofructokinase (PFK) activity (Vander Heiden, Cantley et al. 2009). Also Myc is seen to affect the expression of metabolic genes (Gordan, Thompson et al. 2007).

Not only oncogenes but also tumor suppressors can affect metabolism and glycolysis. The p53 regulated gene TIGAR (TP53-induced glycolysis and apoptosis regulator) can inhibit PFK, leading to increased redirection of glucose to the pentose phosphate pathway (PPP) shunt, responsible for production of pentoses and NADPH needed for anabolic processes (Bensaad, Tsuruta et al. 2006). Another p53-regulated gene is SCO2 (synthesis of cytochrome c oxidase) which regulates the oxygen consumption and aerobic respiration at the mitochondria as it is required for correct assembly of cytochrome oxidase complexes of the respiratory chain (Corcoran, Huang et al. 2006; Matoba, Kang et al. 2006). p53 thereby regulates two features of the Warburg effect, both increased glycolysis and decreased respiration.

The glycolytic pathway in cancer cells shows some protein isoform preferences. Within the glycolytic sequence, the enzyme responsible for net gain of ATP is pyruvate kinase which converts phosphoenolpyruvate to pyruvate. Pyruvate kinase type M2 (M2-PK) isoform is expressed in rapidly proliferating tissues such as tumors. M2-PK exists in tetrameric or dimeric complexes. The tetrameric complexes are highly active while the dimeric show a decrease in activity. Tumors have been shown to harbor the dimeric complexes and thereby show an attenuated pyruvate production that can lead to lower levels of ATP, but with retained glycolytic flux. The tetramer:dimer ratio is controlled by oncogenic signaling like the MAPK-singaling activator Ras and the E7 oncoprotein of human papilloma virus (Mazurek, Boschek et al. 2005). This allows for shunting of glycolysis intermediates towards anabolic biosynthesis.

An important consideration for the glycolytic switch in cancer cells is the availability of carbons for biosynthesis. The rapidly proliferating tumor cells must produce large amounts of proteins, DNA/RNA and lipids to double their size before each cell division. These anabolic processes also require high levels of nicotine adenine dinucleotide (NAD)H that is formed during glycolysis. Pyruvate, the end product of glycolysis, can be transported into mitochondria where acetyl-CoA is formed. This can then enter the citric acid cycle (TCA cycle) where it leads

to complete oxidization. However, acetyl-CoA can also be used for anabolic reactions through a truncated TCA cycle which is stopped after the formation of citrate. Citrate can then be transported to the cytoplasm where acetyl-CoA is reformed and used for lipid synthesis (Kroemer and Pouyssegur 2008; Vander Heiden, Cantley et al. 2009).

1.4.2 Fatty acid metabolism in cancer

Lipid metabolism has not gained the same attention as glucose metabolism in tumors, although many tumors display alterations in lipid metabolism and especially high rates of *de novo* fatty acid biosynthesis (Young and Anderson 2008). This process requires acetyl-CoA, malonyl-CoA and NADPH as reducing equivalents and leads predominantly to palmitate synthesis. In contrast to cancer cells, most normal cells and tissues, including those with high cellular turnover, seem to prefer the use of circulating lipids for the synthesis of new lipids. By contrast, *de novo* fatty acid synthesis is high during embryogenesis and in fetal lungs, where fatty acids are used for the production of lung surfactant. One of the key enzymes for lipogenesis is the fatty acid synthase (FASN). In adults, FASN is highly expressed in hormone-sensitive cells and is regulated by estrogen and progesterone (Kusakabe, Maeda et al. 2000). Levels of FASN has been shown to be increased in cancers like those of the breast, prostate, colon and ovary (Kuhajda, Pizer et al. 2000) and is in fact identical to the well-known diagnostic tumor marker oncogenic antigen 519 (OA-519) (Kuhajda, Jenner et al. 1994).

Molecular inhibition or knockdown of FASN has been shown to induce apoptosis and inhibit tumor growth *in vivo* (Kuhajda, Pizer et al. 2000; Pizer, Pflug et al. 2001; De Schrijver, Brusselmans et al. 2003). The mechanism behind this cell death is not clear, but it has been suggested that accumulation of malonyl-CoA is cytotoxic (Baron, Migita et al. 2004). Accumulation of toxic levels of fatty acids and/or loss of ATP production of fatty acid catabolism through β-oxidation has also been suggested. Downregulation of FASN causes accumulation of malonyl-CoA by inhibition of carnitine palmitoyltransferase-1 (CPT-1) responsible for transporting fatty acids into mitochondria for breakdown, oxidation and generation of ATP. Involvement of survival signaling proteins has also been implicated in the induction of apoptosis by FASN deregulation, since silencing RNA (siRNA) targeted FASN resulted in decreased Akt phosphorylation (Wang, Altomare et al. 2005).

1.4.3 Energy level sensing and AMPK

In order to adapt metabolism according to energy demands and availability the cell needs energy-sensing systems. AMP-activated protein kinase (AMPK) is a central metabolic switch found in all eukaryotic cells. AMPK is regulated by the liver kinase B1 (LKB1), a protein that was originally identified as a tumor suppressor gene (Hemminki, Markie et al. 1998). Loss of LKB1 causes Peutz-Jeghers syndrome – an inherited cancer disease for intestinal polyposis, and it was the search for an LKB1 target that could cause the pathology of e.g. Peutz-Jegher syndrome that led to the identification of AMPK. AMPK is activated when intracellular levels of ATP decrease and levels of AMP increase. Binding of AMP to AMPK induces allosteric changes in AMPK and allows it to be phosphorylated by LKB1. This can happen during for example nutrient deprivation or hypoxia.

Activated AMPK is known to stimulate lipid, cholesterol and glucose metabolism. LKB1-AMPK signaling has been shown to act through the mammalian target of rapamycin (mTOR) pathway (Bolster, Crozier et al. 2002). The mTOR pathway signals through two branches of protein

complexes: mTOR complex 1 and 2 (mTORC1 and 2). The mTORC1 complex can activate eukaryotic translation initiation factor 4E-binding protein (4EBP1) and ribosomal S6 kinase (S6K), which contribute to regulation of protein translation (Holz, Ballif et al. 2005). Upstream of mTORC1 is tuberous sclerosis complex (TSC) 1 and 2, regulating mTORC1 by inhibition. AMPK can when activated directly phosphorylate TSC2 leading to alleviation of inhibition of mTORC1 (Corradetti, Inoki et al. 2004). AMPK has also been shown to modulate p53-dependent apoptosis and directly phosphorylate p53 on serine 15 (Imamura, Ogura et al. 2001; Jones, Plas et al. 2005). LKB1/AMPK can also regulate cellular functions by gene availability through actions on histone acetyl transferases (HATs) and histone deacetyl transferases (HDACs) (McGee, van Denderen et al. 2008). An additional response to metabolic alterations by LKB1 and AMPK is the regulation and the biogenesis of mitochondria. This occurs by positive regulation of peroxisome proliferator-activated receptor gamma coactivator-1α (PGC-1α) which in turn controls diverse energy processes like fatty acid oxidation, gluconeogenesis and mitochondrial biogenesis (Reznick and Shulman 2006). In addition to its effects on metabolism, AMPK/LKB1 signaling is of importance for establishment of cell polarity and cell division (Brenman 2007).

1.4.4 BEC-index

FASN is not the only metabolic protein shown to be a prognostic marker of cancer. Cuezva and colleagues have developed a bioenergetic cellular index (BEC-index) that can be used for classification and prognostic purposes in many types of cancers like breast, liver kidney and colon cancer (Cuezva, Krajewska et al. 2002). This index compares a bioenergetic mitochondrial index with the cellular glycolytic potential. The mitochondrial part of the index consists of the ratio of protein levels of the β-catalytic subunit of the H^+-ATP synthase (β-F1-ATPase) to mitochondrial chaperone protein heat shock protein 60 (Hsp60), while glycolytic potential is represented by protein levels of GAPDH.

$$\frac{\textit{Mitochondrial index}}{\textit{Glycolytic potential}} = \frac{\beta\text{-F1-ATPase / Hsp60}}{\text{GAPDH}} = \text{BEC-index}$$

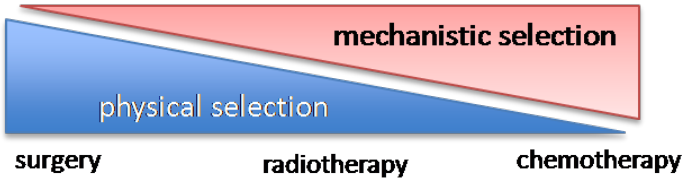
It has been suggested that the levels of β-F1-ATPase display the strongest correlation to the tumor cell phenotype since low levels of this protein alone correlated with more aggressive tumors and worse prognosis in breast cancer (Isidoro, Casado et al. 2005). An inverse correlation between levels of β-F1-ATPase and aerobic glycolysis has been shown (Lopez-Rios, Sanchez-Arago et al. 2007). In some, but not all cancers, the decrease in β-F1-ATPase occurs in parallel with a decrease in mitochondrial mass (Cuezva, Krajewska et al. 2002).

1.5 TREATMENT OF CANCER

If possible, the first strategy to treat cancer is to physically remove the tumor bulk by surgery. Surgical procedures are improving and techniques such as sentinel node biopsies are routinely used to localize lymph node metastases (Rovera, Frattini et al. 2008).

Treatment with radiotherapy is also physically directed against the site of tumor. However, its outcome is still dependent on a mechanistic selection of inflicting damage to cancer cells more than to normal cells.

Chemotherapy is delivered systemically and the success of treatment is dependent on a selective killing of cancer cells. This is made possible by differences in cellular responses between normal cells and cancer cells.



1.5.1 Chemotherapeutic agents

Chemotherapy was first used in clinical cancer treatment after observations of very low white blood cell counts in persons exposed to the chemical warfare agent mustard gas. In the 1940s, patients suffering from lymphoma were hence treated with an intra-venous dose of mustard gas derivatives resulting in patient rapid, albeit temporary, improvement.

Standard chemotherapeutic agents are classified according to their mechanisms of action, but are classically all targeted at cells that proliferate rapidly. Nitrogen mustards belong to the group of *alkylating agents*, together with e g nitrosoureas, alkyl sulfonates and triazines. This group of drugs consists of electrophilic compounds that can directly bind to and damage deoxyribonucleic acid (DNA) of the cell. Platinum compounds are often included in this group because of their ability to bind to DNA; these will be described more closely below.

Antimetabolites are compounds that mimic the building blocks for anabolic reactions in the cell and thereby inhibit this process. The most common antimetabolites mimic bases used for DNA replication and hinder the cell from dividing. A complete genome is an absolute requirement to bring to a daughter cell.

Taxanes are all developed after discovery of taxol, purified from the bark of the Pacific yew tree in the 1960s. These compounds stabilize microtubules and inhibit the dynamics of cytoskeletal function. Taxane treated cells will not succeed in separating chromosomes during cell cycle anaphase and cell division is thereby inhibited.

Vinca alkaloids, such as vincristine and vinblastine, were also originally found in plant extracts, and like taxanes they also affect the microtubules. These drugs prevent tubulin assembly into microtubules and thereby mitosis.

Topoisomerases are enzymes that help to unwind and wind DNA, in order to enable gene transcription from DNA and DNA replication. *Topoisomerase inhibitors* affect cell growth by inhibiting these enzymes and are exemplified by irinotecan (topoisomerase type I inhibition) and etoposide (topoisomerase type II inhibition).

The above mentioned drugs and groups of drugs are used clinically based mainly on empirical observations, and the drug mechanism of action is studied after its clinical use. Contemporary development of anti-cancer drugs is by contrast hypothesis driven, based on the knowledge of

mechanisms involved in cancer progression and cell death. These new drugs are called targeted therapies and are often small-molecule inhibitors or antibodies directed towards key survival factors for the cancer cells. High hopes have been put into the potential of targeted drugs. However, the new compounds have in general not dramatically improved therapeutic outcome and life expectancy.

1.5.2 Platinum chemotherapeutic agents

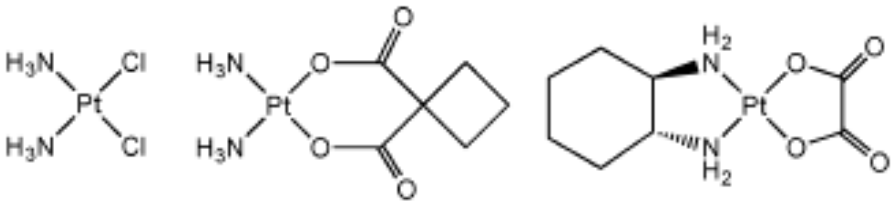
The first platinum compound to be used in treatment of cancer was 3,3,-*cis*-diamminedichloroplatinum(II). Its biological effect was discovered by accident in the 1960s, after the use of platinum electrodes during studies of the effect of electric fields on bacteria. At the electrodes, thought to be inert, platinum salts were formed and the E. coli bacteria studied were after this accidental exposure to cisplatin prevented to divide and kept on growing in long filaments(Rosenberg, Vancamp et al. 1965). The step from discovery to first patient study was rapid, and a US Food and Drug Administration (FDA) approval was granted in 1978. It is now commonly used to treat a variety of cancers, including head- and neck cancer, small cell lung carcinoma, testicular cancer, ovarian cancer and small cell lung carcinoma.

Cisplatin consists of a platinum atom core bound to two ammine groups and two chlorides in *cis* position. When used *in vivo*, the lower intracellular concentration of chloride will exchange one or two of the chlorides for water, rendering an active form of cisplatin. The main mechanism for cisplatin-induced toxicity is thought to be through the covalent bonds formed between aquated cisplatin and purine bases of the DNA, preferably guanines. Cisplatin can form both intra- and interstrand DNA adducts, interstrand adducts being more common.

Even though cisplatin has shown to be very efficient for some cancers – even solely curative as with some testicular cancers – much effort has been put into developing new platinum derivatives. This is not mainly an effort to increase anti-tumor effect, but to decrease the sometimes very severe side effects that accompany cisplatin treatment. Side effects are most prominently nephrotoxicity, ototoxicity and peripheral neurotoxicity (Piel and Perlia 1975).

A great improvement in toxic profile, while retaining anti-tumor effect, came with the development of the second-generation platinum compound carboplatin. Dose-limiting side effects were no longer nephro- and neurotoxicity but myelosuppression. FDA approved carboplatin for treatment of ovarian cancer in 1989. Interestingly, carboplatin was shown to react with DNA much slower than did cisplatin, and much higher doses were required to form the same number of DNA adducts (Knox, Friedlos et al. 1986). Also, elimination of cisplatin is much faster than that of carboplatin (van der Vijgh 1991).

In a continued search for a platinum analog that would be more efficient and affect a broader range of tumors, thousands of compounds were synthesized and screened for anticancer activity. Only one has since then been approved for clinical use, namely oxaliplatin. This third generation platinum drug is since 2002 used to treat colorectal cancer (Kelland 2007).



Cisplatin Carboplatin Oxaliplatin

The major reasons for cancer cell tolerance to platinum drug treatment are decreased uptake of drug, increased scavenging and detoxification of drug (most commonly by increased glutathione (GSH) levels), increased DNA damage repair and avoidance of apoptosis (Kelland 2000; Stewart 2007).

1.5.3 Platinum damage to DNA – repair and recognition

Platinum drugs cisplatin, carboplatin and oxaliplatin form more inter-strand lesions than intra-strand lesions. These are recognized by different DNA repair systems. The most important of these for the repair and removal of platinum adducts is the nucleotide excision repair (NER) process. It is suggested that the exceptional sensitivity of testicular tumors to cisplatin is due to lower levels of proteins of the NER pathway, like the *xeroderma pigmentosum* group A (XPA), excision repair cross-complementation group 1 (ERCC1) and *xeroderma pigmentosum* group F proteins (Welsh, Day et al. 2004). NER can be divided into two subpathways (global genomic NER (GGR) and transcription coupled NER (TCR)) that differ only in their recognition of helix-distorting DNA damage. GGR can as the name implies deal with all reparable lesions throughout the genome while TCR is selective for the transcribed DNA strand in expressed genes (Hanawalt, Ford et al. 2003). Stalled RNA polymerase II acts as damage recognition for TCR, while the GGR is recognized by XPC-human homolog of Rad 23 B (HR23B) binding to the site of damage (Sugasawa, Ng et al. 1998). After recognition, additional proteins are recruited to the site of damage. These can contain helicase activity to unwind the DNA – a process that requires ATP – and endonuclease activity. XPG is responsible for the first incision 3' of the damaged DNA, and XPF-ERCC1 is then assembled for the final cut at the 5' side (Jung and Lippard 2007). A single strand DNA piece of 24-32 nucleotides in length is removed and the gap can be filled by DNA polymerase δ or ε. Another repair system activated after DNA damage is the mismatch repair (MMR) system. This system normally eliminates base-base mismatches as well as insertion and deletion mutations. It is selective for repair of the newly synthesized strand of DNA. Proteins of the Mut family recognize lesions on DNA and initiate the assembly of proteins for excision of bases and following resynthesis of DNA (Martin, Hamilton et al. 2008). In contrast to the NER system, dysregulation of MMR gives protection to cisplatin induced damage to cancer cells (Vaisman, Varchenko et al. 1998). The MMR system can induce cell death through the activation of stress kinases c-Abl and c-Jun N-terminal kinase (JNK) (Nehme, Baskaran et al. 1999) and also by phosphorylation of tumor suppressor protein p53 (Luo, Lin et al. 2004). However, MMR is not important for development of resistance to third generation platinum compound oxaliplatin (Vaisman, Varchenko et al. 1998). Also, recombinational repair has been implicated to have some importance in sensitivity to cisplatin. Loss of homologous recombination repair (HR) increases cisplatin sensitivity whereas knockout of the non-homologous end joining (NHEJ) does not affect sensitivity to the drug (Raaphorst, Leblanc et al. 2005).

The efficiency of drug treatment is dependent on to which extent the cancer cells die, and cell death modes have been widely studied.

1.6 METHODS AND STRATEGIES TO STUDY CELL DEATH

There are several approaches to studying cell death. Different assays have their own specific range of questions that can be answered. For the study of short-term induced cell death, there is a range of established methods for different modes of cell death.

The perhaps most straightforward method to study apoptosis is by microscopy based morphological assessments, e.g. nuclear fragmentation. Apoptosis should then be confirmed by other apoptotic hallmarks like caspase activation and DNA laddering on a separation gel. These different methods assess cell death either as a fraction of apoptotic cells at a given time, or as the difference in levels of apoptotic signaling between two populations (e.g. treated and control samples). Furthermore, after induction of apoptosis, the apoptotic cells are *in vitro* phagocytosed by surrounding cells in response to exposure of phosphatidyl serine on the dead cell surface. Hence, these cells will not be detected by cell death assays.

Cells which have lost their membrane integrity will take up dyes such as trypan blue, a classical method for determining cell death. There are several fluorescent DNA-binding dyes which can pass through only disintegrated cell membranes and are therefore useful for determining cell viability. To distinguish between apoptosis and necrosis, such dyes are conveniently used in combination with fluorescent Annexin V to distinguish between apoptosis and necrosis. Fluorescent-activated cell sorting (FACS) can be used to quantify both types of cell death. Loss of membrane integrity also leads to release of assessable soluble cytosolic macromolecules to the cell medium. The activity of some cellular enzymes such as lactate dehydrogenase are simple to measure using chromogenic substrates and are convenient markers for cell death.

It is also possible to measure a decrease in cell viability instead of actual cell death. The methods measuring viability can be used to study accumulated effects over a longer time, as they are independent of timing of onset of e.g. caspase activation in the study of apoptosis. The viability assays are usually based on cell metabolism. Commonly used are water-soluble tetrazolium salts like 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-5[(phenylamino)carbonyl]-2H-tetrazolium hydroxide (XTT) and 3-(4,5-dimethylthiazol-2-yl)-2-(4-sulfophenyl)-2-tetrazolium, inner salt (MTS). These salts are reduced by intracellular reducing equivalents to a water-insoluble formazan product, the levels of which are quantified spectrophotometrically. The intracellular reducer of tetrazolium was initially thought to be a succinate dehydrogenase (another name for complex II) of the mitochondrial respiratory chain (Mosmann 1983), and the MTT assay was used as a marker for mitochondrial activity and integrity. Later, it has been shown that reduction mainly occurs in the cytoplasm and that reducing equivalents such as NADH and NADPH are the most effective for formazan formation. These cytoplasmic reducing factors are mostly available from an active glycolytic process. Use of these methods can bring misleading results from investigation of cells treated with compounds that affect cellular metabolism.

Also the sodium 5-(2,4-disulfophenyl)-2-(4-iodophenyl)-3-(4-iodophenyl)-3-(4-nitrophenyl)-2H-tetrazolium inner salt (WST-1) assay is based on tetrazolium salt. The formazan product is formed extracellularly depending on the rate of ROS traveling over the plasma membrane.

Long-term total antiproliferative effects of different compounds have in this thesis mainly been assessed using a method to quantitate the total amount of trichloro acetic acid (TCA)-precipitated protein in a micro-well plate. Sulforhodamine B is a spectrophotometrically quantifiable dye that is incorporated into basic side-chains of proteins and polypeptides. The assay does not discriminate between cell arrest and actual cell death.

Acute apoptosis has here been studied by activation of caspases based either on antibodies specific for active caspase-3 or on quantification of caspase-cleaved cytokeratin fragments in total lysates or supernatants using the Apoptosense® ELISA-type 96-well format assay. One important advantage of this indirect assay is that the cytokeratin fragment is stable and thus reflects accumulated apoptosis irrespective of the timing of onset or peaking of caspase activation.

2 AIMS OF THE THESIS

- To better understand the complex apoptosis-related signaling induced by platinum chemotherapeutic agents at the level of mitochondria.

- To investigate a new strategy for increasing antitumor effects of chemotherapeutic drugs by combining them with inhibitors of cell metabolism.

- To investigate the efficiency of thereby identified drug combinations in primary tumor cells and possibly identify a predictive marker of sensitivity.

3 RESULTS

3.1 PAPER I

This paper deals with proapoptotic consequences of MAPK signaling after cisplatin treatment. Earlier work in the group had shown that MEKK1 is important for the apoptotic response after cisplatin treatment. Inhibition of MEKK1 by adenovirus transfection of kinase dead MEKK1 led to decreased levels of Bak in active conformation, as analyzed by conformation specific antibody (Mandic, Viktorsson et al. 2001). The work in Paper I further characterizes MAPK regulation of the proapoptotic functions of Bak.

Human metastatic melanoma cell line AA was treated with cisplatin. Co-incubation with SP600125, a chemical inhibitor of JNK, decreased levels of apoptosis when cells were analyzed for number of fragmented nuclei and cytochrome c release. Also, MEFwt and $MEF^{JNK-/-}$ cells were analysed by FACS for nuclear propodium iodide (PI) incorporation and Annexin V binding to phosphatidyl serine and results showed that $MEF^{JNK-/-}$ more resistant to apoptosis compared to the parental cell line. Since cisplatin induced apoptosis is mediated through intrinsic pathway signaling, and the Bcl-2 family is important for this execution, BH3-only proteins Bid and Bim were investigated for their importance. This was done with isogenic MEF cell lines lacking the gene of interest. Results showed that only Bim had some involvement in the apoptotic response based on morphologic evaluation of fragmented nuclei. Further, JNK ablation did not seem to affect the induction of Bak active conformation. This was analyzed by conformation specific antibodies as well as with cross-linking experiments. 1,6-bis-(maleimido)hexane (BMH) can cross-link proteins between sulfhydryl groups. It can only bind to the neutral inactive form of Bak, and these two can be separated on a gel. Bak activation can thereby be studied as loss of crosslinkability. The finding that JNK had no impact on Bak activation was intriguing, since mitochondrial release of cytochrome c is a point of no return in intrinsic pathway apoptosis and active Bak has been linked to the depolarization of mitochondria (Griffiths, Dubrez et al. 1999; Fadeel and Orrenius 2005; Chipuk and Green 2008). To examine why MEKK-dependent Bak activation did not lead to release of cytochrome c from the mitochondria when JNK was inhibited, Bak complex formation was studied using gel filtration. For this method, heavy membrane (HM) fractions were gently lysed and separated on a sephadex column. Results showed that Bak complexes in fractions corresponding to 80-170 kDa (as determined by reference proteins) corresponded to apoptosis induction. Different patterns were seen in the samples, with some manifestations of Bak-containing complexes larger than 170 kDa. These did not, however, correspond to apoptosis. Adenovirus mediated expression of constitutively active dominant-positive MEKK1, hereafter referred to as dpMEKK1, showed no formation of Bak containing complexes of 80-170 kDa, despite activation of Bak. This was in accordance with no manifestation of apoptosis during this time. Analyses of the cisplatin treated MEF cells showed that parental but not $MEF^{JNK-/-}$ cells harbored 80-170 kDa Bak-containing complexes. dpMEKK1 expression together with treatment with JNK activator sorbitol showed apoptosis similar to the levels induced by cisplatin. It can be concluded that signaling through both members of MAPK signaling family MEKK1 and JNK1 is needed for apoptotic response to cisplatin. They affect Bak at different levels and are both necessary for mitochondrial release of cytochrome c and subsequent apoptosis.

3.2 PAPER II

In this paper, a novel regulation for mitochondrial translocation p53 after short term incubation with cisplatin and oxaliplatin was studied.

Treatment of human colon carcinoma cell line HCT116 showed that at equimolar doses, cisplatin was more efficient than oxaliplatin to induce apoptosis within 24 h. Also, oxaliplatin-induced apoptosis was more dependent on p53 signaling compared to cisplatin. This was examined through comparison of apoptotic responses in HCT116wt cells and in HCT116 cells deficient of p53 and p53 induced proapoptotic gene Bax. The p53-/- and Bax-/- cells were resistant to oxaliplatin while some apoptosis was induced by cisplatin. Oxaliplatin also induced a stronger p53 response as analyzed by FACS and western blot. Cisplatin treatment at 30 μM elicited p53 levels similar to that of oxaliplatin at 20 μM.

Due to reports on DNA-damage-induced mitochondrial translocation of p53, the presence of non-nuclear p53 was investigated upon treatment with the two drugs. Based on western blotting of subcellular fractions, oxaliplatin was found to induce translocation of p53 to the mitochondria within 6h, in contrast to treatment with cisplatin when this was not seen, or only marginally. Since mitochondrial p53 had been shown to involve p53 binding to Bcl-xL, this was investigated in here using immunoprecipitation (IP). Cells treated with oxaliplatin showed co-precipitation of p53 and Bcl-xL while cisplatin did not.

Cisplatin and oxaliplatin have different toxicity profiles, e.g. cisplatin, but not oxaliplatin, can cause ototoxicity. One proposed mechanism for ototoxicity caused by cisplatin has been the induction of inducible nitric oxide synthase (iNOS). iNOS catalyzes the formation of nitric oxide (NO) that rapidly can react with ROS to form reactive nitrogen species (RNS). These, in turn, can modify specific amino acid residues. Analyses by western blot showed an increase in protein levels of iNOS within 6h, i.e. within the time frame of the observed mitochondrial localization of p53. To examine the possibility that RNS modifications of p53 could inhibit this translocation to mitochondria, cells were co-treated with cisplatin and iNOS inhibitor 1400W. This greatly increased translocation of p53 to mitochondria, while 1400W had no effect on the oxaliplatin response, as expected from the lack of iNOS induction by this drug. To further confirm that increase of nitric oxide actually can affect p53 subcellular localization, cells were co-treated with NO-donor S-nitroso-N-acetyl-DL-penicillamine (SNAP). This efficiently prevented p53 from translocating to mitochondria in oxaliplatin treated cells.

Nitrotyrosylation modifications of p53 in the mitochondria and nucleus were investigated by immunoprecipitation of p53 and subsequent blotting with nitrotyrosine-specific antibodies. Results showed that cisplatin-induced p53 was nuclear and nitrotyrosinylated. In the presence of 1400W, the levels of nitrotyrosinylated p53 in the nucleus decreased and non-modified p53 appeared in the mitochondria. Phosphorylation-site specific antibodies were also used to blot after IP of p53 in nuclear and mitochondrial fractions. These results showed that co-treatment with 1400W did not affect the cisplatin-induced phosphorylation pattern of p53. Also, co-treatment with 1400W did not alter the presence of all investigated p53 phosphorylations in either the mitochondrial or nuclear fraction.

3.3 PAPER III

This study explores the effects of combining treatment with anti-tumor drugs with modulators

of cell metabolism. The two metabolism modulators used were one inhibitor of glycolysis, 2-deoxyglucose (DG) and inhibitor of β-oxidation etomoxir. DG inhibits glycolysis by competing with glucose for binding to hexokinase, the enzyme catalyzing the first and often rate-limiting step of glycolysis. In contrast to glucose, DG cannot be further metabolized through glycolysis. Etomoxir inhibits β-oxidation by inhibition of the CPT-1 enzyme that imports fatty acids to mitochondria for catabolic processing. ATP levels after 5h treatment with both inhibitors were assessed, and both DG and etomoxir were shown to decrease intracellular ATP levels in a dose-responsive fashion. Combination of the two inhibitors had additive effects. Also the energy-sensor AMPK was investigated. DG had induced AMPK activation by phosphorylation by 16 h but not by 8 h. Etomoxir treatment did not lead to AMPK phosphorylation at either time point.

Eighteen different anti-cancer drugs, both clinical and experimental, were screened for apoptosis potentiating effects upon addition of either inhibitor. Apoptosis was assessed using M30 Apoptosense® assay in human colon carcinoma cell line HCT116 after 24 h treatment. The analysis of a stable cytokeratin fragment makes this method independent of onset of caspase activation within the 24 h of interest and allows quantification of accumulated apoptosis.

Drug	Function
Cisplatin	DNA and protein adducts
Oxaliplatin	DNA adducts
Etoposide	Topo II inhibitor
Ellipticine	Topo II inhibitor, NCI mechanistic set
U0126	MEK1 inhibitor
MG132	Proteasome inhibitor
Curcumin	Antioxidant, gene regulator
Chloro(triethylphosphine)gold(I) (TEPAu)	Alkylator, oxidative stress, NCI mechanistic set
Betulinic acid	Apoptosis via mitochondria
Arsenic trioxide	Oxidative stress?
5-fluorouracil	Nucleoside analogue
Methotrexate	Antimetabolite
Quercetin	Redox effects, gene regulation
Piroxicam	COX-2 inhibitor
Camptothecin	Topo I inhibitor, NCI mechanistic set
LiCl	GSK3β inhibitor
Echinomycin	DNA intercalation, binds to HIF1α promoter

The eighteen drugs were used at doses which by themselves elicited a low apoptotic response. Potentiation was defined as a greater than 2.4-fold increase in apoptosis after co-treatment

with either DG (10 mM) or etomoxir (200 μM). DG elicited potentiation of apoptotic response to genotoxic drugs cisplatin and ellipticine, MEK1-inhibitor U0126 and proteasome inhibitor MG132. At half the concentration (5 mM), DG potentiated only cisplatin and ellipticine. Etomoxir potentiated cisplatin and lithium chloride induced apoptosis. Since DG was the most potent inhibitor, its potentiating effects were further examined in a screen in HCT116 cells lacking p53. Potentiation by DG at the higher dose was seen only with cisplatin and piroxicam in these cells.

The drugs that did not elicit potentiation in HCT116 parental cells were further examined for the induction of necrosis based on release of cytosolic LDH to extracellular medium. Apoptosis was measured by cleaved caspase cleaved cytokeratin in the same samples. Five of these drugs did not show any increase in necrosis either, while the combination of DG and oxaliplatin, lonidamine or lithium chloride led to a necrotic response.

In addition to tests on cell lines, a 3D-model was also investigated. MDA-MB-231 cells were grown in mice and excised. The tumors were sliced into 200μm thick circular sections that can be kept in culture for several days. These tumor slices are thick enough to be affected by hypoxia signaling and diffusion through the cell layers. Since potentially infiltrating cells in these tumors are of mouse origin, they are not detected in the human specific Apoptosense assay. Slices were kept in culture medium and treated with anticancer drugs alone or in combination with metabolism inhibitors. Accumulated apoptosis was measured at 24, 48 and 72 h and showed that addition of DG or etomoxir can increase apoptotic responses also in this setting.

After studying specific cell death pathways, there was also an interest to study total antiproliferative effects over slightly longer time. This was done by precipitation of total proteins after 48 h treatment and staining of these with sulforhodamine B. The lower dose of DG was used in this assay to avoid too large effects by DG alone and to use a clinically relevant dose. Results showed that DG potentiated a larger number of drugs, while etomoxir potentiated fewer drugs but to a larger extent. Another viability assay was also used, but in order to study short-term effects on red-ox regulation status. AlamarBlue is a dye that can act as an intermediate electron acceptor in the electron transport chain between the final reduction of O_2 and cytochrome oxidase by substituting for molecular oxygen as an electron acceptor (Shappell 2003). ROS-inducing disturbances in respiration are reflected by this method. Drug treatments in this assay were studied over shorter times to exclude differences in cell number and to study early cellular events. DG had no effect in this assay either by itself or in any combination. Etomoxir on the other hand showed increased reduction of AlamarBlue when combined with quercetin and betulinic acid, two of the drugs potentiated in the apoptosis screen. No such effect was observed with other hits like cisplatin and etoposide.

Since cisplatin was well potentiated by both DG and etomoxir in above studies, it was studied more closely. Cisplatin treatment led to a decrease in ATP when examined after 5 h treatment, and combination of DG or etomoxir had additive effects on decreasing ATP levels. Furthermore, low doses of cisplatin that were previously shown to induce senescence and not apoptosis (Berndtsson, Hagg et al. 2007) were used. Cell counting after 48 and 72h revealed that the combination of low dose cisplatin and DG had strong growth inhibitory effect. Interestingly, this could also be seen in cells lacking p53. Clonogenicity experiments showed that etomoxir had strong anti-proliferative effects together with low-dose cisplatin.

3.4 PAPER IV

This paper focuses on *in vitro* studies of platinum-based drugs in combination with DG in ovarian carcinoma. Epithelial ovarian carcinoma cell lines CaOv-4 and SKOV-3 were investigated for sensitivity to cisplatin and carboplatin, both drugs used clinically for treatment of ovarian carcinoma. Platinum drug concentrations needed for reduction of total protein levels over 48 h were studied using the sulforhodamine B assay for antiproliferative effects. This calculated concentration was referred to as an IC_{50} value. SKOV-3 cells were found to be more sensitive than CaOv-4 to cisplatin and carboplatin while CaOv-4 were more sensitive to the glycolysis inhibitor. The cells were then treated with combinations of DG (5 mM) and cisplatin or carboplatin at different doses. Results were presented as DG-mediated decreases in cisplatin IC_{50}, and showed that CaOv-4 showed the greatest decrease. Cells treated with low-dose cisplatin and carboplatin together with DG were studied for ability to regrow after removal of drugs. Cells were first treated for 48 h before addition of drug free fresh medium. During the additional 72 h for recovery, mono-treatments allowed regrowth to approximately control levels while the combination treatment had a sustained effect on proliferation.

The BEC index (see above section 1.4.4) has been shown to be a clinical prognostic marker for several types of carcinomas. It couples clinical outcome to protein levels of metabolic enzymes. Based on densitometric analysis of western blots, ratios of oxidative phosphorylation protein β-F1-ATPase over mitochondrial chaperone Hsp60 are calculated and divided by levels of the glycolytic enzyme GAPDH. CaOv-4 displayed a lower BEC-index than did SKOV-3 – theoretically suggesting higher glycolytic dependence for the CaOv-4 cells. To verify this experimentally, FDG uptake during 1 h was measured. CaOv-4 cells were found to take up more radioactive glucose per µg protein. This was confirmed by a glucose consumption assay over 24 h and by a parallel lactate formation assay.

The study was extended to include primary ascitic tumor cells. Seventeen patient samples that were viable in-culture were treated with cisplatin or carboplatin at different doses, alone or in combination with DG at 5 mM. Similar to the cell lines, IC_{50} values were calculated for the drugs alone and also the decrease in IC_{50} to either platinum drug by the addition of cisplatin. Not surprisingly, these values varied quite much. Potentiation was described as DG-mediated % decrease in platinum drug IC_{50}. The average decrease in cisplatin IC_{50} was 48% (median 68%). Lower potentiation responses were seen with carboplatin treatment. Only 7/17 showed potentiation by DG. Also, induction of acute apoptosis over 24 h was measured in the ascitic samples using M30 Apoptosense®-assay. DG *per se* induced dose-responsive apoptosis in some samples. DG affected cisplatin-induced apoptosis by increasing levels with factors ranging from 1 to about 5. Carboplatin did generally not induce apoptosis, neither was apoptosis potentiated by DG.

BEC-indeces were studied in fifteen out of the seventeen samples using the same western blot approach as with the cell lines. Correlation of BEC-indices with decreases in cisplatin IC_{50} was studied by grouping cisplatin potentiability into high and low potentiated groups (decrease in cisplatin IC_{50} by more or less than 50%, respectively). BEC-indices in the highly potentiated group were homogenously low in contrast to those with lower potentiation, although these differences were not significant. However, the mitochondrial component of the BEC-index, i.e. β-F1-ATPase over mitochondrial chaperon Hsp60, was found to differ significantly between the two groups.

4 DISCUSSION

Papers I and II deal with the complex signaling of proapoptotic proteins upon treatment with platinum chemotherapeutic agents with focus on mitochondrial events.

4.1 PAPER I

The results of this paper show that although they are in the same MAPK pathway, MEKK1 and JNK1 can have separate proapoptotic effects. The target molecule studied is Bak in HM-fractions. Bak in this system has been shown to be a relevant inducer of mitochondrial depolarization (Mandic, Viktorsson et al. 2001). Compared to the other executive Bcl-2 family proapoptotic protein Bax, which showed activation only at time points when caspases were already active, Bak showed earlier conformational activation and was regarded as the most important inducer of cytochrome c release.

The mechanism by which Bak permeabilizes the mitochondria is thought to be through oligomerization and formation of pore complexes in the outer membrane. Exactly how this happens or in which order is not well understood. This study suggests that the activation and pore formation are separate events, and that active conformation of Bak is not enough to induce MOMP in an in otherwise Bak-dependent pathway to apoptosis. The present results show that Bak activation is needed prior to complex.

The Bak complexes correlating with apoptosis are in this study of sizes 80-170 kDa. Unaccountable complexes over 170 kDa were observed in some samples which did not show onset of apoptosis. We do not understand the relevance or consequence of these. There are similar reports, however, of non-apoptotic large protein complexes containing inactive Bak in mitochondria of viable cells (Antonsson, Montessuit et al. 2001; Chandra, Choy et al. 2005).

JNK has been shown to have effects on proteins inducing mitochondrial permeabilization, e.g. phosphorylation and thereby inhibition of Bcl-2 (Yamamoto, Ichijo et al. 1999) and cleavage of BH3-only protein Bid (Deng, Ren et al. 2003). Other Bcl-2 family proteins shown to be phosphorylated by JNK are Bad (at apoptosis-stimulating Ser128) (Donovan, Becker et al. 2002) as well as Bim (Ley, Ewings et al. 2005). However, Bak has not been reported to be modified by phosphorylation upon JNK activation or as a consequence of other kinase activities. The effect on Bak complex formation by JNK in this study is therefore not attributable to direct effects of JNK. There is probably intermediate signaling that exerts the effects of MEKK1 and JNK, but this study does not provide a direct mechanism for the observed effects on Bak proapoptotic function. One can speculate that MAPK signaling effects on Bim could have such a role, since mouse embryonic fibroblasts (MEF) lacking Bim were protected against cisplatin-induced apoptosis.

The adenoviral system used in these experiments expresses the constitutively active kinase domain of MEKK1 and not the full length protein. This fragment does not act in the scaffold protein complexes in which JNK may be activated and this might explain the results that dominant positive transfection of MEKK1 did not induce JNK activation. One may also speculate that additional by other MAP3Ks is needed for the activation of JNK.

4.2 PAPER II

Although it is well accepted that p53 can translocate to mitochondria after genotoxic stress, the mechanism behind mitochondrial translocation is still under debate (see section 1.3.4.1). Furthermore, it has not been possible to attribute subcellular localization of p53 to any specific phosphorylation or acetylation modification (Nemajerova, Erster et al. 2005). Here, we show that nitrosative modification of p53 plays a role. In line with this, we do not find any specific differences in p53 phosphorylation between mitochondrial and total p53. Nitrosative modifications of p53 induced by endogenous levels of NO after treatment with cisplatin were found to be of relevance for mitochondrial localization of p53. This regulation is of negative character. By contrast, positive regulation of mitochondrial p53 translocation is shown to be attributed at least in part to monoubiquitinylation of p53 (Marchenko, Wolff et al. 2007).

iNOS has in other contexts been shown to be upregulated following treatment with cisplatin both *in vivo* and *in vitro*. It has been most well studied as a mechanism for the ototoxicity induced by cisplatin (Watanabe, Hess et al. 2000; Li, Liu et al. 2006). In ovarian carcinoma cell lines, the induction of iNOS has also been coupled to apoptotic response and drug resistance (Leung, Fraser et al. 2008). The role of NO in apoptosis is complex and it has been reported to promote both cell survival and apoptosis (Lala and Chakraborty 2001; Li and Wogan 2005), with the outcome depending on stimuli, cell type, tissue and dose. Proapoptotic actions of NO are e.g. induction of p53 (Schneiderhan, Budde et al. 2003), while NO can function in the antiapoptotic way by inhibiting caspase-3 (Kim, Kim et al. 1998).

The observation that NO modifications are involved in the regulation of the subcellular localization of p53 could not be coupled to levels of apoptosis. Caspase activation at the early time point studied (6h) is very low. We also tried to evaluate the effects on apoptosis by inhibiting translocation of p53 through co-treatment of oxaliplatin and an exogenous NO donor (SNAP). Although this prevented p53 translocation, the onset of apoptosis by SNAP *per se* masked the potential effects of this inhibition. Neither were any major effects on cisplatin-induced apoptosis seen using the iNOS inhibitor 1400W.

Also, the necessity of direct or indirect transcriptional activity of p53 for translocation is not clarified. In this study, we examined translocation after induction of p53 had started. The requirement for transcriptional activity is therefore not known in our setting and the mechanism of translocation can to our understanding fit either into a transcription or non-transcription model.

This paper does not prove that the nitrotyrosinylation of p53 is the final determining factor for inhibition of mitochondrial translocation. In theory, there is also a possibility that this is a secondary sign of iNOS activity so that the presence of nitrotyrosinylation is a confounding finding. Non-modifiable mutants of p53 would be necessary to show this. Nevertheless, nitrosative modification of p53 adds another factor to the already complex regulation of p53.

The work in papers I and II deals with the complex signaling in response to platinum drug treatment. In continuation, increased knowledge about the nature of signaling induced by chemotherapy can ultimately lead to the development of new drugs and identification of

tumors sensitive to certain treatments.

Papers III and IV deal with a metabolism-based strategy for potentiating the antiproliferative effects of chemotherapy, in particular platinum chemotherapeutic agents.

4.3 PAPER III

Tumor cells have higher energy demands, both in terms of ATP and of material for anabolic processes. Inhibition of metabolism alone might not be sufficient to kill cancer cells since effects can range from growth arrest to apoptosis and cell death can be delayed by autophagy. Also, many compounds inhibiting metabolism act in a reversible way that might not give rise to permanent cell damage. In this paper we wanted to explore the strategy of combining anti-cancer agents with inhibitors of energy metabolism. The selected inhibitors are already used in humans and are well tolerated. Etomoxir was developed for the treatment of diabetes mellitus, and radiolabeled DG is used in the clinic for detection of tumors in PET scan. DG has also been investigated as potentiator of radiotherapy treatment of cancer, where oral administration of 200 mg/kg was well tolerated by the patients (Mohanti, Rath et al. 1996). This corresponds to blood concentrations of 5-6 mM, which makes the 5 mM dose used in this study clinically relevant.

In screening experiments, we chose the Apoptosense® method to quantitate levels of apoptosis. Its benefits are that it is accumulative and will therefore not be very dependent on timing of caspase activation and is easy to perform in the microplate format. However, it will over time also show accumulated background apoptosis which makes it less suitable for studies over longer time. We chose to use the sulforhodamine B assay for time points over 24h, an assay that displays the total antiproliferative effects by measuring total protein levels. These two types of assay revealed differences in modes of antiproliferative action of DG and etomoxir, in that DG was more potent in potentiating apoptosis while etomoxir showed potentiation in the reduction of protein content, i.e. cell mass, over 48 h. One should notice, however, that the lower dose of 5 mM DG was used in the 48 h experiments instead of the 10 mM dose used in the 24 h experiments.

In the apoptosis screen, DG and etomoxir potentiated the effect of drugs representing different mechanisms of action. The drug best potentiated by both inhibitors was cisplatin. Interestingly, third generation platinum drug oxaliplatin was not potentiated in the same manner but did show a necrotic response. The results reflect the fact that cytotoxic effects by these two related DNA–damaging platinum drugs differ. Cisplatin is a highly reactive molecule that has been shown to induce various stress responses such as ER stress (Mandic, Hansson et al. 2003), and induction of ROS (Berndtsson, Hagg et al. 2007). In addition, cisplatin has also been shown to have direct effects on mitochondria, e.g. binding to mtDNA and mitochondrial proteins (Yang, Schumaker et al. 2006). These direct effects might be of importance for the decrease in ATP levels seen after short time incubation with cisplatin. Stress signaling, such as ER stress, is also reported components of the cytotoxic response seen with other drugs potentiated in apoptotic response by the addition of DG, e.g. ellipticine and MG132.

The set of drugs screened in this work revealed no clear factor that was relevant for potentiation by DG or etomoxir, probably depending on the relative small set of drugs.

Potentiation responses varied from apoptotic to necrotic and growth arrest. The screen identified cisplatin as a chemotherapeutic drug whose effect was well potentiated, even in cells lacking p53. If an autophagic response is elicited by drug combination treatments, this could be affected by the p53 status, since p53 has been shown to important for the induction of autophagy (Jin 2005).

4.4 PAPER IV

After the identification of cisplatin as a drug that showed substantial potentiation on apoptotic effects after cotreatment with glycolysis inhibitor DG, paper IV is a next step in trying to explore *in vitro* efficiency of the combination.

Ovarian cancer is treated with platinum drugs cisplatin and carboplatin, carboplatin having to a large extent replaced cisplatin due to its better toxicity profile. Here, in ovarian cancer cells, carboplatin was less potentiated by DG compared to cisplatin. One can hypothesize that this is coupled to the higher reactivity of cisplatin towards cellular nucleophiles (such as proteins, DNA and RNA) (Oguri, Sakakibara et al. 1988; Goodisman, Hagrman et al. 2006), possibly explaining ROS and RNS induction which might in turn interact with DG effects for higher potentiation of apoptotic response. A similar model might be applied to the reactive drugs being potentiated by cisplatin in paper III. Furthermore, the multiple cellular effects of cisplatin make it difficult to compare doses of cisplatin and carboplatin. This may also partially explain why they are administered in very different doses in the clinic. Differences in cytotoxic responses were especially obvious in the apoptosis experiments, where true synergistic effects were seen with cisplatin and not carboplatin.

Experiments over longer time with low-dose platinum treatment and subsequent regrowth in fresh medium did however show similar results for both platinum compounds. This is in line with previous work showing that induction of growth inhibition is different from the acute apoptotic response seen upon cisplatin treatment (Berndtsson, Hagg et al. 2007). Here, results suggest that addition of DG to cisplatin treatment can increase both short term and long term cytotoxicity in ovarian carcinoma cells.

The BEC index as a prognostic factor in several different cancers was developed by the group of Prof José Cuezva at the Universidad Autonoma of Madrid. Here, we have investigated if the BEC index could be a predictive marker for potentiating treatment using DG. This was in turn also based on the assumption that tumors are increasingly glycolytic with progression and also show higher resistance to chemotherapeutic drugs. That the sensitive samples did not show significant differences in BEC index might be due to an insufficiently large sample size. By contrast, we find that the mitochondrial part of the BEC index (β-F1-ATPase:Hsp60) is better than the whole BEC index for identification of samples that are differently sensitive to potentiation with DG.

As to the mechanisms for potentiation in both Paper III and IV, one might speculate on several mechanisms on how decreased levels of glycolysis and ATP can affect drug toxicity. First, DNA damage repair is ATP-consuming and NAD^+ dependent. Decreased levels of these two factors through decreased glycolysis may thereby inhibit the DNA repair. This could have a major effect with highly DNA-damage dependent drugs like oxaliplatin. Decreased ATP/NAD may

also lead to the inhibition of macromolecule synthesis, likely leading to an autophagic response, which may be prosurvival or death-inducing depending on the time frame. Autophagic responses are therefore interesting as explanations for the long term effects of DG in combination with low dose platinum drugs. Long term effects may also include cisplatin damage to mitochondrial DNA and subsequent effects on respiration. Since both platinum drug treatment and inhibition of glycolysis induce a broad range of responses, the mechanism for increased toxicity after addition of DG most likely includes various factors and is cell dependent.

The combination of two drugs that each has a tumor cell-selective feature is an attractive idea. Combining DG with cisplatin led in all samples studied to increased antiproliferative effects compared to treatment with cisplatin alone, although in some cases the difference between combination and monotreatment was small. As a point for concern, organs or tissues that are commonly affected by platinum side effects might also suffer from potentiated toxicity. This will be investigated in the guinea pig ototoxicity model used in one of the additional papers. However, there is a theoretical benefit of the combination of cisplatin and DG as the concentration of DG is higher in tumor cells compared to many other organs.

There is a growing interest in using tumor cell energy metabolism as a target for cancer treatment (Pelicano, Martin et al. 2006; Chen, Lu et al. 2007; Kroemer and Pouyssegur 2008). This strategy adds a bioenergetic basis of preferential killing of tumor cells to existing criteria such as proliferation rate, hormone dependence and tyrosine kinase activation.

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