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TRANSLATIONAL STUDIES OF DRUG-INDUCED TUMOR CELL DEATH

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ABSTRACT

It is essential to understand the mechanisms of action of anticancer drugs in order to obtain good treatment results. I have in the present thesis studied clinically used anticancer agents as well as other agents that induce tumor cell death. Cisplatin, an important agent used to treat various forms of malignancies, was found to have a more complex mode of action than previously appreciated. Different doses of cisplatin were found to induce two distinct cellular outcomes: low doses induce senescence and high doses apoptosis via a mechanism involving reactive oxygen species and calcium. Interestingly, these outcomes appeared to be triggered by different signaling mechanisms: apoptosis by a cytoplasmic mechanism and senescence by DNA damage. The plant alkaloid ellipticine was identified as an agent that synergizes with cisplatin. Detailed studies showed that ellipticine induces an endoplasmic reticulum stress response characterized by induction of GRP78/BiP expression and splicing of the XBP1 transcription factor. Our studies of cisplatin and ellipticine emphasize the complex modes of action of many anticancer drugs.

Calcium signaling has been implicated in apoptosis induced by many agents, including cisplatin. In order to characterize the role of calcium signaling in apoptosis, we examined apoptosis by 40 different compounds that were identified by screening a chemical library. Our study suggested that calpain and calmodulin kinase II are important mediators of calcium signaling in apoptosis. The requirement for calcium was generally late during the apoptotic process, and calcium signaling was implied in activation of Jun N-terminal kinase.

Another aim of the work was to develop a method to monitor the activity of anticancer drugs *in vivo.* We measured caspase-cleaved cytokeratin 18 fragments in patient serum during cancer therapy. The method generated interesting clinical information. Docetaxel was found to be more effective in inducing tumor apoptosis in prostate cancer patients compared to two other agents. Of particular interest was that apoptosis was induced during a number of treatment cycles. By measuring the fraction of caspase-cleaved to total cytokeratin 18 in patient serum we obtained evidence suggesting that the CEF combination used to treat breast cancer induces a predominantly necrotic response. Improved methods to assess drug effects *in vivo* are expected to aid clinical development of new drugs and to be quite useful in clinical work.

LIST OF PUBLICATIONS AND MANUSCRIPTS

I. **Maria Hägg**, Kenneth Bive´n, Takayuki Ueno, Lars Rydlander, Peter Björklund, Klas G. Wiman, Maria C. Shoshan ans Stig Linder
A novel high-through-put assay for screening of pro-apoptotic drug
Inv New Drugs; 20:253-259

II. **Maria Hägg**, Maria Berndtsson, Aleksandra Mandic, Rong Zhou, Maria C. Shoshan and Stig Linder
Induction of endoplasmic reticulum stress by ellipticine plant alkaloids
Mol Cancer Ther; 3:489-497

III. Maria Berndtsson, **Maria Hägg**, Theocharis Panaretakis, Aleksandra Mandic Havelka, Maria C. Shoshan and Stig Linder
Acute apoptosis by cisplatin requires induction of reactive oxygen species but is not associated with damage to nuclear DNA
Int J Cancer, in press

IV. **Maria Hägg Olofsson**, Yun-Chun Li, Maria C Shoshan, Stig Linder and Aleksandra Mandic Havelka
Identification of calmodulin kinase II as an important mediator of calcium stimulated apoptosis pathways using chemical library
Manuscript

V. Gero Kramer, Stephan Schwarz, **Maria Hägg**, Aleksandra Mandic Havelka and Stig Linder (2006)
Docetaxel induces apoptosis in hormone refractory prostate carcinomas during multiple treatment cycles
Br J Cancer 94:1592-8

VI. **Maria Hägg Olofsson**1*, Takayuki Ueno1, 2*, Yang Pan3, Ren Xu3, Heiko van der Kuip4, Thomas Muerdter4, Maike Sonnenberg4, Walter Aulitzky4, Maria C. Shoshan1, Stephan Schwarz1, Yun-Chun Li1, Aleksandra Mandic Havelka1, Masakazu Toi2 and Stig Linder1
Cytokeratin-18 as a serum biomarker for response to chemotherapy
Manuscript

Additional papers:

K. Bivén, H. Erdal, **M. Hägg**, T. Ueno, R. Zhou, M. Lynch, B. Rowley, J. Wood, C. Zhang, M. Toi, M. C. Shoshan and S. Linder (2003)
A novel assay for discovery and characterization of pro-apoptotic drugs and for monitoring apoptosis in patient sera
Apoptosis; 3: 263 – 268

Berndtsson M, Konishi Y, Bonni A, **Hägg M**, Shoshan M, Linder S, Havelka AM (2005)
Phosphorylation of BAD at Ser-128 during mitosis and paclitaxel-induced apoptosis.
FEBS Lett; 579:3090-4.

LIST OF ABBREVIATIONS

AIF	apoptosis inducing factor
Apaf	apoptosis protease activating factor
ASK	apoptosis stimulating kinase
ATF	activating transcription factor
ATM	ataxia-telangiectasia mutated
ATP	adenosine three phosphate
ATR	ATM- and Rad3-related
Bak	Bcl-2 homologous antagonist killer
Bax	Bcl-2 associated X protein
Bcl-2	B cell lymphoma-2
Bid	BH3-interacting-domain death agonist
$[Ca^{2+}]_i$	intracellular calcium
CaM	calmodulin
CaMK	calmodulin kinase
caspase	cysteinyl aspartate proteinase
CBP	CREB binding protein
CEF	cyclophosphamide/epirubicin/5-fluorouracil
CK	cytokeratins
CREB	cAMP-response element binding protein
CsA	cyclosporin A
cyt c	cytochrome *c*
DAP-kinase	death-associated protein-kinase
DSB	double strand breaks
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
ERSE	ER stress response element
FACS	fluorescence-activated cell sorter
GRP	glucose-regulated protein
HRPC	hormone refractory prostate cancer
HTP	high-throughput screening
$InsP_3$	inositol-1,4,5-triphosphate
JNK	c-jun NH_2-terminal protein kinase
LMP	lysosomal membrane permeabilization
MMP	mitochondrial membrane permeabilization
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NCI	National Cancer Institute
PERK	PKR-like endoplasmic reticulum kinase
PI	propidium iodide
PI3K	phosphatidylinositol 3-kinase
PS	phosphatidyl serine
PSA	prostate-specific antigen
ROS	reactive oxygen species
RyR	ryanodine receptor

SERCA	sarcoplasmic/endoplasmic Ca^{2+}-ATPase
SRF	serum response factor
TNF	tumor necrosis factor
TNFR	TNF receptor
UPR	unfolded protein response

CONTENTS

INTRODUCTION

GENERAL INTRODUCTION TO CANCER

Every third person will develop cancer during their lifetime. In Sweden more than 50 000 persons will receive a cancer diagnosis every year. After cardiovascular disease, cancer is the most common cause of death. Cancer is not a single disease: there are more than 200 different types of cancer. The most common forms are the carcinomas, tumors that are derived from epithelial cells (breast, prostate, lung, colon and ovarian carcinomas).

Tumors originate from single progenitor cells that proliferate and generate many sublines that further branch or may become extinct (Nowell 1976). Already in the 1950s, Foulds suggested that cancer progression is a process of Darwinistic selection of malignant clones of tumor cells (Foulds 1957). Tumors tend to have more aggressive characteristics as they develop, and he suggested that tumor progression occurred stepwise, each step determined by the mutation or loss of specific genes. In addition, the process is driven by the development of aneuploidy (Duesberg and Rasnick 2000) and epigenetic changes.

Hanahan and Weinberg suggested that cancer is an expression of six changes that collectively determine malignant growth: self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of apoptosis, limitless replicative potential, sustained angiogenesis and tissue invasion and metastasis (Hanahan and Weinberg 2000). Each of these changes is shared by most if not all cancers during their development. The genes that contribute to tumorigenesis can be divided into two groups: oncogenes and tumor suppressor genes. An oncogene is a gene whose activation expresses a gain of function, whereas a tumor suppressor is a gene whose activation represents a loss of function mutation. About 200 different cellular proto-oncogenes have been identified (Futreal, Kasprzyk et al. 2001), which when activated convert the gene into an oncogene. In the normal cell, the expression of these proto-oncogenes is tightly controlled. Proteins encoded by proto-oncogenes include growth factors, growth factor receptors, signal transducers and transcription factors. There are three main mechanisms, structural gene alterations, gene amplification and deregulated expression, that lead to activation of proto-oncogenes. The second group of genes that plays an important role in tumorigenesis, the tumor suppressor genes, acts by restricting cell growth. In most instances both copies of the gene have to be mutated before tumors develop.

Tumor progression generates heterogeneous tumors that are genetically unstable. The heterogeneity and instability are major reasons for the rather limited progress in therapy of solid tumors. The presence of multiple unstable sublines generates a large potential for selection of clones that are resistant to therapy.

CANCER TREATMENT

Cancers are treated with surgery, radiation therapy, chemotherapy, hormone therapy or immunotherapy. A combination of therapies is often used. For solid tumors, surgery is used to treat local disease. Ionizing radiation of various types and energies are used to destroy localized populations of cancer cells. As with surgery, results are best with relatively small tumors detected before they are locally or systemically widespread.

Tumors arising from the breast, prostate and uterine lining may respond to hormonal therapy. Certain hormones (e.g. estrogens) act by binding to specific receptors in the tumor cell. Breast and prostate cancers may be treated by removing sources of circulating hormones that could stimulate or support tumor growth or by the administration of estrogens, androgens, progesterones, glucocorticoids to suppress tumor growth.

Chemotherapy is used at various stages of cancer therapy. Approximately 50 drugs, which may be used as single agents or in various combinations, are used for chemical management of cancer. Chemotherapy is administered with different objectives. Adjuvant therapy is given in order to kill micrometastases after surgical removal of a primary tumor. The objective of neo-adjuvant therapy is both to shrink tumors prior to surgery and to combat micrometastases. Anti-cancer agents are also used to treat patients with advanced cancer, usually with the objective to retard the progress of disease.

Although cytostatic treatment is less important than surgery for the complete curing of patients, it still is of major importance for adjuvant therapy. If there is a possibility of curing the patient, it could be reasonable to give intensive treatment with the risk of severe side-effects. If there are no chances of cure, the patient is treated only to decrease or slow down tumor growth and to relieve symptoms and prolong survival. Examples of tumor diseases that can be completely cured by cytostatic treatment are acute leukemia in children, testicular cancer and Hodgkins lymphoma. However, substantial advances in molecular oncology have yet to effect on mortality statistics. It is unfortunate that some of the most common cancers, such as breast- ovarian- and lungcancer, are relatively insensitive to cytostatic treatment. In the type of cancer

most common in men, prostate cancer, as well as in malignant melanoma, cytostatics have limited effects.

ANTICANCER DRUGS

Chemotherapy has been used in anti-cancer treatment for about seventy years. For a long time, it was not known how or why treatment actually killed tumor cells. It is only with recent knowledge about DNA transcriptional regulation, cell cycle regulation and the concept of programmed cell death – apoptosis – that we have begun to understand the effects of chemotherapy on a molecular level. This understanding is in turn important for understanding why some tumors are resistant to therapy, and why some drugs are more efficient than others. Chemotherapeutic drugs may be classified according to the type of cellular damage they cause. However, little is known about how this damage induces the molecular signals which lead to cell death. Some of these signals appear to be common to most drugs, while others are likely to be drug-specific. Some anticancer drugs and novel agents have been reported to affect several cellular targets.

Anticancer drugs are grouped based on their cellular mechanisms of action

- Alkylating agents and platinum agents
- Antimetabolites
- Topoisomerase inhibitors
- Microtubular-targeting agents
- Other

Alkylating agents and Platinum agents

Alkylating agents are a chemically diverse group that acts through covalent binding of alkyl groups to intracellular macromolecules. They contain either one or two reactive groups and are classified as monofunctional or bifunctional. Monofunctional agents can only modify one base in the DNA which in turn led to DNA single-strand breaks. Bifunctional alkylating agents form crosslinks between biological molecules and interstrand crosslinking of DNA seems to be one of the major mechanisms of cytotoxity. Most alkylating agents can be divided into two main groups, nitrogen mustards and nitrosoureas.

The platinum compounds consist of complexes of platinum with ligands that can be substituted by electron-rich atoms. The platinum agents form strong covalent bonds with proteins and nucleic acids. Cisplatin was the first platinum antitumor compound discovered (Rosenberg, Vancamp et al. 1965; Rosenberg, VanCamp et al. 1969) and entered into clinical trials in the early 70s (Lippman, Helson et al. 1973; Higby, Wallace et al. 1974). Cisplatin was found to have antitumor activity against testicular cancer, lymphoma, ovarian cancer, bladder cancer and a number of other solid tumors and has become one of the most frequently used antitumor agents. The dose-limiting toxicity of cisplatin is renal toxicity (Gottlieb and Drewinko 1975; Piel and Perlia 1975), ototoxicity and neurotoxicity (Von Hoff, Schilsky et al. 1979). In contrast to many other anticancer drugs, cisplatin has moderate immunosuppressive effects which make the compound suitable for combination therapy.

Topoisomerase inhibitors

Many topoisomerase inhibitors are planar hydrophobic compounds that intercalate DNA with high affinity. Topoisomerase I and III are classified as type I enzymes, since they alter DNA supercoiling by cleaving a single strand of the DNA duplex, whereas the topoisomerase II enzymes are classified as type II and cleave both strands. The anthracyclines are fermentation products of *Streptomyces peucetius*. Daunomycin and doxorubicin were the first anthracyclines shown to have antitumor activity in the 60s (Arcamone, Cassinelli et al. 1969; Zunino, Gambetta et al. 1976). Anthracyclines are positively charged, favoring intercalation with DNA. Although daunomycin and doxorubicin differ only by a single hydroxyl group, they have distinct spectra of antitumor activity. Epirubicin is a semi-synthetic derivative of doxorubicin produced for the purpose to decrease cardiac toxicity. Anthracyclines generate reactive oxygen species that damage DNA, mRNA, proteins and lipids and their peroxidation of lipids may account for much of the cardiac toxicity typical of these drugs (Doroshow 1986; Sinha, Katki et al. 1987). Doxorubicin has the broadest spectrum of activity of all anthracyclines and is involved in the treatment of many solid tumors and in Hodgkin´s and non-Hodgkin´s lymphoma.

The topoisomerase I and topoisomerase IIα and IIβ are also known to be targets of naturally occurring plant alkaloids. Ellipticine [5,11-dimethyl-6*H*-pyrido(4,3-*b*)carbazole] is a plant alkaloid with antitumor activity (Dalton 1967). It was first isolated 1959 from the leaves of the Australian tree *Ochrosia elliptica* and 10 years later anticancer activity by this substance was reported. The main mechanism of cytotoxicity is believed to be inhibition of topoisomerase IIβ (Jurayj, Haugwitz et al. 1994)

Microtubular-targeting agents

The vinca-alkaloids are naturally occuring or semi-synthetic derivatives of alkaloids from the periwinkle plant. They bind to tubulin and inhibit its polymerization to microtubules (Kaufmann, Peereboom et al. 1996). The taxanes paclitaxel and docetaxel are two well studied plant alkaloids. Paclitaxel was isolated in 1971 from the bark of *Taxus brevifolia* and was found to have antitumor activity (Fuchs and Johnson 1978). Paclitaxel binds to microtubules and inhibits their depolymerization (molecular disassembly) into tubulin, leading to a block in cell cycle progression in the G_2–M phase. Paclitaxel is most effective against ovarian carcinomas and advanced breast carcinomas.

Docetaxel is a semi-synthetic taxane with higher aqueous solubility. Docetaxel is synthesized from the naturally occurring compound, 10-deacetyl baccatin III, which is extracted from the needles of yew plants. The mechanisms of docetaxel activity is similar to that of paclitaxel (Brown, Shalli et al. 2004). The dose-limiting toxicity of taxanes is myelosuppression with increased risk of infection and both docetaxel and paclitaxel can cause hypersensitivity.

NEW GENERATION OF CANCER DRUGS

Despite intensive research with the aim to develop new anticancer drugs, only a few new drugs with good clinical activity have been identified. Imatinib (Gleevec) is a tyrosine kinase inhibitor (TKI) with good activity in chronic myelogenous leukemia (CML). Almost all CML cases have a chromosome translocation which results in the expression of a fusion protein. This protein, BCR-ABL, has a constitutively active tyrosine kinase activity. Proliferation of CML cells is dependent on this kinase for survival, and inhibition of BCR-ABL by imatinib results in apoptosis (Jacquel, Herrant et al. 2003). Imatinib produces milder side effects and results in a better response than conventional therapy in patients with previously untreated CML (O'Brien, Guilhot et al. 2003).

Chromosome translocations leading to activation of tyrosine kinases are not observed in carcinomas, the major group of malignancies in human. In distinction to CML cells, carcinomas are therefore generally not “addicted” to the activity of a specific kinase and kinase inhibitors do not show as dramatic effects as in CML. An interesting exception to this rule is the activity of inhibitors of the epidermal growth factor receptor (EGFR) such as gefitinib and erlotinib in some patients with non-small-cell lung cancer (NSCLC). Gefitinib induces responses in approximately 10% of Caucasian patients with advanced NSCLC and higher responses in Asians (Giaccone, Debruyne et al. 2005) Responses are observed in patients with tumors

expressing activating EGFR mutations, which are more common in Asians compared to Caucasians. The prescence of these mutations correlates with a positive clinical response to gefitinib an erlotinib (Lynch, Bell et al. 2004; Paez, Janne et al. 2004).

The anti-EGFR TKIs show different degrees of antitumor activity when administered as single agents, but no additional survival benefit was evident when they were combined with conventional therapy (Baselga 2006). However, in a phase III study comparing erlotinib with supportive care in NSCLC patients with advanced disease, patients who received erlotinib showed a statistically significant improvement in response rate and overall survival (6.7 months versus 4.7 months) (Shepherd, Rodrigues Pereira et al. 2005). Herceptin (Trastuzumab) is a monoclonal antibody to the HER2 tyrosine kinase receptor which is overexpressed in many cases of metastatic breast cancer (Nahta and Esteva 2006). For patients with breast cancers that overexpress HER2, Herceptin combined with chemotherapy increases time to disease progression and survival. However, most cancers that initially respond to Herceptin begin to progress within one year.

The new TKI sorafenib (Nexavar) is a "multi kinase inhibitor" that blocks several different kinases, including Raf, VEGFR-2, Flt-3 and c-Kit. Sorafenib has both antiproliferative and antiangiogenic properties and has been shown to be effective in the treatment of kidney cancer (Ratain, Eisen et al. 2006). However, similar to conventional chemotherapy, sorafenib produces toxicity, including neutropenia and diarrhea (Richly, Henning et al. 2006) .

In summary, most of the advanced solid tumors have complex genetic alterations and are generally not dependent on one abnormally activated kinase or signaling pathway for its malignant behavior. There is also an important level of cross talk between distinct signaling pathways. As with conventional chemotherapeutic agents, which are often most effective when administered as combination therapies, rationally developed combinations of molecularly targeted agents are likely to be more potent than single-agent therapies (Baselga 2006).

ANTI-PROLIFERATIVE RESPONSES AND CELL DEATH

For several decades, apoptosis has been discussed as the principal mechanism of programmed cell death. Accumulating evidence suggests that tumor cell response to chemotherapy is not confined to apoptosis but also includes other modes of cell death (Brown and Attardi 2005). The best-described modes of anti-proliferative response are apoptosis and necrosis, followed by senescence, mitotic catastrophe and autophagy.

Apoptosis

In 1972, Kerr and colleagues noted that apoptosis was responsible for maintaining tissue homeostasis by connecting the equilibrium between cell proliferation and death (Kerr, Wyllie et al. 1972). Apoptotic cells show a characteristic morphology characterized by cell membrane blebbing, cell shrinkage, chromatin condensation and fragmentation. One of the surface changes on apoptotic cells is the loss of phospholipids asymmetry in the plasma membrane and the translocation of phosphatidyl serine (PS) from the inner lipid bilayer to the surface (Martin, Reutelingsperger et al. 1995). The exposure of PS on the outer membrane occurs early during the apoptotic process where they function as "eat-me" signals. Cells at late stages of apoptosis become fragmented into apoptotic bodies. These apoptotic bodies are phagocytosed by neighboring cells or macrophages and removed before they disintegrate. Apoptosis does therefore not generate an inflammatory response.

Necrosis

While apoptosis is well defined at the molecular level, necrosis is referred to as a form of cell death that is uncontrolled. The characteristics of necrotic cells are loss of membrane integrity, swelling of cytoplasm and mitochondria and finally with total cell lysis. It is nowadays believed that necrosis represents an alternative pathway for tumor cells to be eliminated. Necrosis triggers an inflammatory response, resulting in stimulation of an immune response that could lead to additional tumor cell death. Necrosis is induced by inhibition of cellular energy production, unequal intracellular calcium flux, generation of ROS and activation of non apoptotic proteases. Despite the idea that necrosis is an uncontrolled or default form of cell death, recent studies have suggested that this may not be true. Alkylating DNA damage was reported to stimulate a regulated form of necrotic cell death, independent of the major apoptotic effectors p53, Bax, Bak and caspases (Zong, Ditsworth et al. 2004). These authors suggested that there exists an intrinsic cellular control point that determines cell fate in response to PARP activation. Programmed cell necrosis may be just as an important cell fate as apoptosis (Zong and Thompson 2006).

Senescence

Replicative senescence was first described in normal human cells explanted in culture that failed to divide beyond a finite number of population doublings (Hayflick and Moorhead 1961).

Telomeres are repetitive DNA sequences that protect the ends of chromosomes. The uncapping of telomeres can be defined as the "loss of the end protection". Telomere uncapping can cause a degeneration of the cells ability to regulate its own telomere length. This results in drastic lengthening or shortening of telomeres, an increase in recombination in telomere regions, and a loss of the mean length regulation (Smith and Blackburn 1999). Premature senescence is thought to be related to replicative senescence which has been a DNA damage response (d'Adda di Fagagna, Reaper et al. 2003). During replicative senescence, signaling pathways involving ATM (Ataxia telangiectasia mutated) and p53 are activated by telomere uncapping (d'Adda di Fagagna, Reaper et al. 2003; von Zglinicki, Saretzki et al. 2005). Several classes of chemotherapeutic agents induce senescence *in vitro* and in mouse tumor xenografts. In addition to induction of apoptosis cisplatin also induces premature senescence (Wang, Wong et al. 1998), but the factors that decide whether cisplatin will trigger apoptosis or senescence are not well understood.

Mitotic catastrophe

Mitotic catastrophe is a process occurring during or shortly after a failed mitosis and can be followed by morphological alterations such as formation of micronuclei. Generally, mitotic catastrophe is not considered as form of death, it is rather an irreversible trigger of death (Weaver and Cleveland 2005). Large nonviable cells with multiple micronuclei are morphologically distinguishable from apoptotic cells since the micronuclei do not contain condensed chromatin. One definition of mitotic catastrophe is "death that occurs during mitosis, resulting from a combination of deficient cell-cycle checkpoints and cellular damage" (Castedo, Perfettini et al. 2004). Mitotic catastrophe can be the primary mode of cell death following treatment with ionizing radiation and occurs in response to several anticancer agents (Chang, Broude et al. 1999; Morse, Gray et al. 2005).

THE MAIN APOPTOTIC PATHWAYS

It is clear that hematological malignancies are sensitive to chemotherapy induced apoptosis. However, whether clinically used doses of ionizing radiation or anticancer drugs induce apoptosis of carcinomas is more controversial (Brown and Attardi 2005). Irrespective of how important apoptosis is for the anti-proliferative responses to conventional chemotherapy, apoptosis is an attractive mechanism of action of potential anticancer drugs. Apoptosis has therefore attracted a lot of interest in the field of cancer research (Reed 1999). In recent years,

the molecular machinery responsible for apoptosis has been elucidated, revealing a family of intracellular proteases, the caspases, which are responsible directly or indirectly for the morphological and biochemical changes that characterize the phenomenon of apoptosis (Reed 2000).

Caspases

Caspases are a family of proteases that are important effectors of apoptosis. About 14 mammalian caspase have been identified. Caspases are synthesized as inactive zymogens containing prodomains and two subunits. In order to become active, the pro-caspases must be proteolytically cleaved at specific aspartate residues. Caspase substrates are widely distributed in the nucleus, cytoplasm and cytoskeleton. Based on their function, the caspases can be classified into two groups. Caspases-2,-8, -9 and -10 are the major initiator caspases and their function is to cleave and activate the downstream caspases. Caspases-3, -6 and -7, the effector pro-caspases, are normally cleaved and activated by various death substrates. Caspase-3 has been recognizes as the crucial executioner caspase because caspase-3 knockout mice display an apoptotic defect in response to both intrinsic and extrinsic pathway stimuli (Kuida, Zheng et al. 1996; Woo, Hakem et al. 1998).

There are two major signaling pathways that lead to apoptotic cell death: the intrinsic, or mitochondria-mediated pathway, and the extrinsic, or receptor-mediated pathway. Both pathways involve activation of caspases. The extrinsic pathway is activated by the tumor necrosis factor (TNF) family and the receptors for these ligands (TNFR) (Locksley, Killeen et al. 2001). After extracellular ligand binding, the TNF receptor (TNFR) recruits initiating caspases.

The intrinsic pathway is usually activated in response to intracellular stress signals like DNA damage, high levels of reactive oxygen species (ROS) and activation of oncogenes. The mitochondria are the central regulators and mitochondrial outer membrane permeabilization (MOMP) is mainly mediated by Bcl-2 family members.

THE ROLE OF MITOCHONDRIA IN APOPTOSIS

In response to apoptotic stimuli, cytochrome c is released from the mitochondria to the cytosol. Cytochrome c in turn binds to Apaf-1 and the binding of cytochrome c induces a

conformational change in Apaf-1, allowing it to bind the nucleotide dATP or ATP. The nucleotide binding to the Apaf-1-cytochrome c complex triggers its oligomerization to form the apoptosome, which recruits procaspase-9. The binding of procaspase-9 to the apoptosome cleaves and activates the downstream caspases, such as caspase-3.

The human Bcl-2 family members can be divided into three groups. They all contain at least one of four Bcl-2 homology (BH) domains. Anti-apoptotic members, including Bcl-2 and Bcl-X_L promote cell survival. Bcl-2-deficient mice display apoptosis of lymphocytes, developmental renal cell death and loss of melanocytes (Veis, Sorenson et al. 1993). The pro-apoptotic "multi-BH domain"Bcl-2 family members Bak and Bax have been shown to control the apoptotic process at the level of the mitochondria and instead elicit cell death (Wei, Zong et al. 2001). The BH3-only family of proteins share sequence homology with Bcl-2 only in the BH3 domain, an amphipathic helix required to interact with other Bcl-2 family members (Huang and Strasser 2000). These proteins are normally located in other cellular compartments and translocate to the mitochondria in response to apoptotic stimuli. Once translocated to the mitochondria, they cause mitochondrial damage and release of apoptogenic proteins by interacting with other members of the Bcl-2 family.The signal from the BH3-only protein can be either neutralized by the antiapoptotic protein, such as Bcl-2 or Bcl-x_L, or further transduced to mitochondria by the proapoptotic protein such as Bax or Bak (Wang 2001). In comparison to the execution phase of the mitochondrial apoptotic pathway, we know relatively little about how the upstream signaling pathways to the mitochondria are regulated.

CALCIUM SIGNALING AND APOPTOSIS

Cellular Ca^{2+} import into the cell occurs mainly by receptor-operated, voltage sensitive and store-operated channels. Ca^{2+} can either interact with Ca^{2+}-binding proteins or become sequestered into the endoplasmic reticulum (ER) or mitochondria (Orrenius, Zhivotovsky et al. 2003). Both Ca^{2+} overload and depletion of the ER Ca^{2+} pool can lead to changes in protein folding which in turn can lead to ER stress. The mitochondria take up Ca^{2+} from the cytosol through an energy dependent uniport transporter and release it through different pathways.

Calmodulin (CaM) is a multifunctional protein and one of the major intracellular Ca^{2+}-binding proteins and has been implicated in apoptosis. Several calmodulin-dependent kinases have been identified. CaMKII is a mediator of cellular Ca^{2+} effects and has been reported to be activated in apoptosis induced by UV light and TNF (Wright, Schellenberger et al. 1997).

Calcineurin (protein phosphatase 2B [PP2B]) is a calcium-calmodulin-activated, serine/threonine PP that is activated by sustained elevations in intracellular calcium (Crabtree 1999; Crabtree and Olson 2002; Hogan, Chen et al. 2003). Calcineurin consists of a catalytic subunit (CnA), and a calcium binding subunit (CnB), and calmodulin (Crabtree 1999). Calcineurin is also a critical effector of cell death, where it has been shown to either agonize or antagonize apoptosis. Calcineurin enzymatic activity is inhibited by the drugs cyclosporine (CsA) and FK506 (Crabtree 1999).

The mammalian calpain family includes at least 12 members with ubiquitous and tissue specific patterns. The most well-known members are the m-and μ-calpains which have similar substrate specificities but differ in calcium concentrations required for their activation *in vitro*. m-calpain requires mM concentrations of Ca^{2+} while μ-calpain requires μM Ca^{2+} concentration. Both enzymes are heterodimers composed of a homologous 80 kDa catalytic subunit and a 30 kDa regulatory subunit.

Calpains are involved in several cellular functions including cytoskeletal remodeling, differentiation, proliferation and apoptosis. Mice lacking the calpain regulatory subunit and thereby calpain activity die during embryonic development. Calpain activity is regulated by different mechanisms including autoproteolytic cleavage, phosphorylation, calcium requirement and inhibition by an endogenous inhibitor, calpastatin. Cleavage of calpastatin is essential for activation of the Ca^{2+}-dependent calpain (Porn-Ares, Samali et al. 1998; Wang, Posmantur et al. 1998). Calpain has been demonstrated to cleave Bax (Wood and Newcomb 1999) and Bid (Mandic, Viktorsson et al. 2002) during apoptosis.

INVOLVEMENT OF CELL ORGANELLES

Various forms of endoplasmic reticulum stress lead to apoptosis. The endoplasmic reticulum apoptosis response is likely to be a defense mechanism to limit the replication of viruses which synthesize high amounts of envelope proteins in this compartment (He 2006). The ER initiates apoptosis by the unfolded protein response (UPR) and Ca^{2+} signaling (Ferri and Kroemer 2001). Accumulation of unfolded or misfolded proteins leads to activation of Ire1-α and β, PKR-like endoplasmic reticulum kinase (PERK) and cleavage of the ER membrane protein ATF-6. The cytosolic domain of ATF-6 translocates to the nucleus which lead to an activation of genes having an ER stress element in the promoter region. The chaperones Grp78 and Grp94 are markers for ER stress. The transmembrane protein ATF-6 is a transcription factor which

binds Grp78 in unstressed cells, but translocates and is cleaved in the Golgi following ER stress induction (Shen, Chen et al. 2002).

The ER is the major organelle involved in calcium storage. Ca^{2+} is taken up from the cytosol by sarcoplasmic/endoplasmic Ca^{2+}-ATPase (SERCA) and released through the inositol-1,4,5-triphosphate ($InsP_3$) receptor/Ca^{2+} channels ($Insp_3R$) or ryanodine receptor/Ca2+ channels (RyR) (Ferri and Kroemer 2001). Classical agents related to ER are tunicamycin (inhibits N-linked glycosylation), brefeldin A (inhibits ER-Golgi transport) and thapsigargin (inhibits SERCA).

Lysosomes have recently been identified as important apoptotic signal integrators in response to various stimuli. Recent studies have shown that lysosomal membrane permeabilization (LMP) is an early event in apoptosis triggered by ligation of death receptors, lysosomotropic agents, oxidative stresses, or serum withdrawal (Isahara, Ohsawa et al. 1999; Foghsgaard, Wissing et al. 2001; Kagedal, Johansson et al. 2001; Boya, Andreau et al. 2003). The specific release of cathepsins from lysosomes into the cytosol is critical for lysosome-mediated cell death and is responsible for activation of downstream signal pathways. Cathepsins can indirectly induce MMP by modifying cytosolic proteins, including Bax or Bid, conferring upon them the ability to translocate to the outer mitochondrial membrane (Boya, Andreau et al. 2003; Cirman, Oresic et al. 2004).

Nuclear DNA damage is one initiator of apoptosis. Cells respond to DNA damage by activating a complex DNA-damage-response pathway that includes cell-cycle arrest, the transcriptional and post-transcriptional activation of a number of genes including those associated with DNA repair. It is widely believed that these DNA damage responses are required for the maintenance of genomic stability and prevention of tumor development (Elledge 1996). The ataxia telangiectasia mutated (ATM) gene plays an important role in sensing DNA double strand breaks (DSBs). ATM is a kinase involved in activating the appropriate damage response pathway, leading to either cell cycle arrest or apoptosis, and is a key checkpoint molecule in regulating cell cycle responses to DNA damage (Meyn 1995; Shiloh 1995). The ATM and ATR (ataxia telangiectasia and Rad3 related) kinases play central roles in the cellular response to several types of DNA damage occurring in S and G_2 phases of the cell cycle, including aberrant replication intermediates and DNA DSBs (Abraham 2001). The p53 tumor suppressor protein plays a central role in a cells decision to induce either cell cycle arrest or apoptosis. Activated ATM and ATR phosphorylate p53, resulting in the stabilization of p53 and subsequently amplifying the downstream p53 cascade, which modulates both cell cycle and apoptosis (Vogelstein, Lane et al. 2000).

METHODS FOR ASSESSMENT OF CELL DEATH

Apoptosis has been considered a major mechanism of chemotherapy-induced cell death, and pathways regulating apoptosis are the focus of many preclinical drug discovery investigations. During apoptosis caspases are activated and cleave different cellular substrates. In epithelial cells, one such substrate is cytokeratin-18 (CK18). A problem in drug-screening projects is that normal and tumor cells are both sensitive to anticancer drugs when grown on plastic. Under standard *in vitro* conditions, normal cells and tumor cells both proliferate, partially explaining their similar drug sensitivities. Developing drug-screening systems that better distinguish between normal and tumor cells is likely to speed up the drug-screening process.

Morphological criteria for apoptosis include chromatin condensation, DNA fragmentation, cell shrinkage and cytoplasmic blebbing (Kerr, Wyllie et al. 1972). Various molecular indicators of apoptosis are also available. Cleavage of DNA can be detected in cultured cells and tissue sections by using labelled deoxynucleotide triphosphates and terminal dUTP nick end labelling. The appearance on the surface of apoptotic cells of phosphatidyl serine is exploited using the Annexin V assay. Multiple flow cytometric assays that employ DNA binding fluorochromes such as Hoechst 33342 and propidium iodide are also used to identify apoptotic subpopulations (Darzynkiewicz, Bedner et al. 2001). A variety of caspase activation assays can also be applied and the use of antibodies to activated forms of caspases, e.g. to active caspase 3.

BIOMARKERS OF RESPONSE IN CLINICAL STUDIES

Phase I studies are performed to identify the maximum tolerated dose (MTD) but also to identify the maximum biologically active dose (MBD). The definition of MBD is the dose at which maximum inhibition of the target molecule occurs. For a drug to be clinically effective, the MBD should occur below the MTD. Accelerated drug approval by the Food and Drug Agency (FDA) is now possible based on surrogate response biomarkers (Bross, Kane et al. 2004). Thus, the identification and incorporation of robust biomarkers early in the drug development process is becoming increasingly attractive.

The ability to track the effect of anticancer drugs during patient treatment usually requires serial sampling. Serial collection of biopsies from solid tumors is connected with practical and ethical problems. An alternative approach is to use biomarkers which are detectable in

peripheral blood, allowing serial sampling throughout the course of treatment. Typical products of cell death are nucleosomes, complexes that are formed from a core particle of several histone components and DNA. In cases of enhanced cell death, as during chemotherapy, they are released into the circulation and can be detected in elevated amounts in serum or plasma (Stroun, Maurice et al. 2000; Lichtenstein, Melkonyan et al. 2001; Holdenrieder, Stieber et al. 2004). A main objective behind the development of the M30-Apoptosense and M65 biomarker assays described in the present thesis was to use these assays as response biomarkers in clinical studies. The assays allow assessment of the levels of cytokeratins release from carcinoma cells, as well as qualitative assessments on the types of fragments released (Bivén et al., 2003)(Kramer, Erdal et al. 2004) (paper V, paper VI).

AIMS OF THE THESIS

The aim of this work was to achieve a better understanding of drug-induced cell death. Anticancer drugs have complex modes of action, and we were interested in studying signaling events induced by different drugs. Understanding the mechanisms of action of anticancer drugs is important in order to understand how drugs should be used and why cells become resistant.

Another aim of the work was to develop a method to monitor the activity of anticancer drugs *in vivo*. Improved abilities to assess drug effects *in vivo* are expected to aid clinical development of new drugs and to be quite useful in clinical work.

RESULTS AND DISCUSSION

PAPER I: DEVELOPMENT OF A METHOD TO DETECT APOPTOSIS

Cell based screening is traditionally performed using proliferation-assays such as the MTT-assay. This type of assay is based on the ability of viable cells to metabolize various substrates (Twentyman and Luscombe 1987; Larsson and Nygren 1990). Toxic compounds, irrespective of mechanisms of action, will reduce cell numbers and score positive in viability assays.

Many anti-cancer drugs can induce apoptosis (Costantini, Jacotot et al. 2000; Kaufmann and Earnshaw 2000). Most apoptosis assays can not be adapted for high-throughput screening. An assay ideal for screening of pro-apoptotic drugs should have high-throughput-capability and also measure the accumulation of a product produced during apoptosis to avoid the measuring of samples at many time-points. During the execution phase of apoptosis, caspases are activated and cleave different substrates. One such substrate in epithelial cells is cytokeratin-18 (CK18) (Caulin, Salvesen et al. 1997; Ku, Liao et al. 1997) which is cleaved by caspases at 2 different sites during apoptosis. We developed an ELISA assay based on the M30 monoclonal antibody (M30) which recognizes a neo-epitope on cytokeratin-18 exposed after cleavage at residue Asp-396. M30 was conjugated to horseradish peroxidase and was used as tracer. A monoclonal antibody directed against an epitope on the 284-396 fragments was used as a catcher in the ELISA assay.

Only drugs that induce apoptosis drugs will show activity in the M30-ELISA assay, not compounds that are growth inhibitory or induce necrosis. By adding non-ionic detergent to the medium it is possible to detect apoptosis both in cell extracts and from floating and dead cells in a single time-point. This is a clear advantage since it is possible to measure the accumulation of cleavage products both in medium and cell extracts. We demonstrated that paclitaxel induces increased activity in the M30-ELISA assay, and that the activity was blocked by co-treatment with a caspase inhibitor (zVAD). To demonstrate that the assay could be used for drug-screening in the 96-well format, a chemical library from the National Cancer Institute was used for screening. Of 500 compounds screened, 16 drugs showed a strong pro-apoptotic activity. One of the pro-apoptotic drugs identified was a derivative of ellipticine which was studied further (paper II). Ellipticine is an anti-tumor alkaloid which intercalates with DNA and has been reported to induce apoptosis.

The M30-ELISA assay has been used for drug-screening in this study, and also in other studies (Erdal, Berndtsson et al. 2005)(paper IV). The sensitivity is sufficient for adapting the assay to the 384 well format. The assay may also be used for screening using tumor spheroids (paper VI). This is of considerable interest since spheroids are considered to be better models than monolayer cultures.

PAPER II-IV: STUDIES OF MECHANISMS OF ACTION OF PRO-APOPTOTIC DRUGS

Chemotherapeutic drugs may be classified according to the type of cellular damage they cause. However, little is known about how this damage induces the molecular signals which lead to cell death. Some of these signals appear to be common to most drugs, while others are likely drug-specific. Cis-diamminedichloroplatinum(II) (cisplatin) is a potent anticancer agent with a high cure rate in testicular cancer and one of few curative drugs. Cisplatin is classified as a DNA-damaging agent that generates intra- and inter-strand breaks and also reacts with RNA and protein. Ellipticine is a plant alkaloid with anti-tumor activity. Ellipticine has been shown to be a topoisomerase-II inhibitor and has also been reported to activate the transcriptional function of mutant p53 (Peng, Li et al. 2003). Anticancer drug are used at rather high concentrations (micromolar). Since these agents often are reactive (alkylating agents, platinum agents), it is very likely that they have several cellular targets. Although it is convenient to classify anticancer agent as "DNA damaging", "microtubule interacting" etc., is important to keep in mind that these agents are likely to interact with a number of targets and that the classification is partly misleading.

PAPER II: Induction of ER stress by ellipticine alkaloids

In paper II we continue to examine apoptosis induction by ellipticines, and especially the derivative 6-propanamine ellipticine (6-PA-ELL), one of the 16 apoptosis-inducing drugs that were identified in the first study. One of the reasons for our interest in this particular compound was that we found it to have a synergistic effect in combination with cisplatin (Fig. 1).

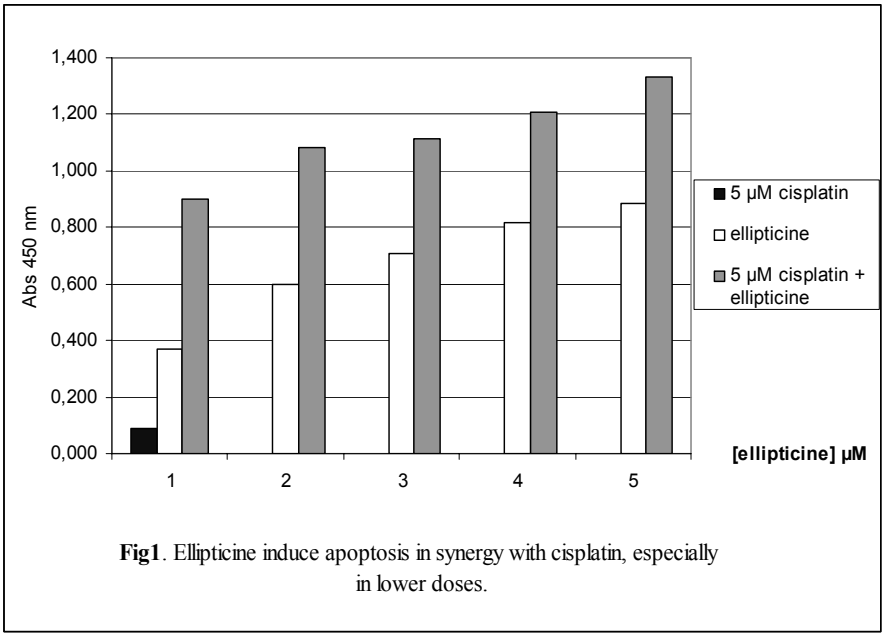

Fig1. Ellipticine induce apoptosis in synergy with cisplatin, especially in lower doses.

Ellipticine is a plant alkaloid known to inhibit topoisomerase II (see above). We found that 6-PA-ELL induced a more rapid apoptotic response (within a few hours) than DNA damaging agents such as doxorubicin and cisplatin. This observation made us hypothesize that 6-PA-ELL induced apoptosis by other mechanisms than DNA damage. Apoptosis was preceded by an early conformational change in Bak followed by cytochrome c release. We found that 6-PA-ELL rapidly increased the expression of the endoplasmic reticulum chaperones GRP78/BiP and GRP94, markers for ER-stress (Shiu, Pouyssegur et al. 1977; Li, Alexandre et al. 1993; Lee 2001). This response was faster than that observed with cisplatin, and was speculated to be due to direct induction of ER-stress. We were able to directly demonstrate induction of the endoplasmic reticulum stress response element (ERSE) using a reporter construct. Furthermore, 6-PA-ELL induced splicing of the mRNA encoding the XBP1 transcription factor. The induction of the ERSE, which requires the ATF6 transcription factor, and induction of XBP1 splicing, indicates that 6-PA-ELL induces both a protein refolding response (ATF6 mediated) and a refolding plus degradation response (XBP1-mediated) (Yoshida, Matsui et al. 2003).

6-PA-ELL induced cleavage of caspase-12, a caspase reported to be activated during ER stress (Nakagawa, Zhu et al. 2000). The role of caspase-12 in apoptosis of human cells is, however, unclear since the human caspase-12 gene does not encode a functional protein (Fischer, Koenig

et al. 2002). Human caspase-4 may be the equivalent of mouse caspase-12 since reduction of caspase-4 expression by siRNA decreases ER stress-induced apoptosis (Hitomi, Katayama et al. 2004). Whether the antibody to mouse caspase-12 reacts to human caspase-4 is unclear. As a further complication of this issue, it was recently reported that neither caspase-12 or caspase-4 are required for ER stress-induced apoptosis (Obeng and Boise 2005).

Our results show that ellipticine derivatives can induce ER-stress. We found an association between the ability of different ellipticines to induce GRP78/BiP expression and their ability to induce caspase-cleavage activity. This suggests that ER-stress is an important factor for the cytotoxic activities of ellipticines. Consistent with a mechanism of apoptosis induction distinct from the previously described topoisomerase II inhibition was that 6-PA-ELL induced apoptotic signaling in enucleated cells.

PAPER III: Cisplatin induces apoptosis and senescence by different mechanisms

Cisplatin is generally believed to induce DNA-damage-induced apoptosis (Wang and Lippard 2005). The p53 protein has an important role in many cellular processes. p53 inhibits cell proliferation by inducing cell-cycle arrest or induces apoptosis in response to cellular stress. The relationship between p53 and cisplatin cytotoxicity depends on several factors such as cell type, activation of specific signaling pathways and the presence of genetic alterations (Wang and Lippard 2005). Following DNA damage, p53 is activated and trans-activates different target genes, leading to various cellular responses. Part of the DNA damage response involves the activation of protein kinases of the PI3-K family, including ATM and ATR that can specifically target p53 for phosphorylation. Opposing the common view that cisplatin induces DNA-damage dependent apoptosis, we previously reported that cisplatin induces apoptotic signaling in enucleated cells (Mandic, Hansson et al. 2003). Apoptosis was associated with induction of endoplasmic-reticulum stress. Another group reported similar findings for oxaliplatin (Gourdier, Crabbe et al. 2004).

It is important to clarify whether cisplatin induces apoptosis by mechanisms that are unrelated to DNA damage. First, cisplatin is a commonly used cancer therapeutic and it is important to understand its mode of action. Secondly, cisplatin is used as a model compound for induction of DNA-damage induced apoptosis in many studies. In paper III we show that cisplatin concentrations $\geq$ 20 μM are required to induce apoptosis of HCT116 colon cancer cells. These concentrations are one magnitude higher than the IC50 in clonogenic assays. We observed

induction of cellular superoxide formation by these concentrations of cisplatin and found that the superoxide scavenger Tiron inhibited cisplatin-induced apoptosis. Caspase activation was also observed in enucleated cells, and was inhibited by Tiron. These observations demonstrate that cisplatin induces apoptosis by a superoxide dependent mechanisms which occurs independently of nuclear DNA damage.

Lower concentrations of cisplatin, which do not induce apoptosis, were sufficient for induction of DNA damage signaling. In this lower concentration range, cisplatin inhibits clonogenic outgrowth of cells and induces premature senescence. We also observed secondary, non-stress induced apoptosis (occuring at 2 – 3 days after exposure) after exposure to low cisplatin concentrations.

These findings explain many inconsistencies in the literature. Cell lines with deficiencies in DNA repair are sensitive to cisplatin in clonogenic assays (Bhattacharyya, Ear et al. 2000). We believe that this loss of clonogenicity is due to induction of senescence by a p53-dependent mechanism. It has not been clear why cisplatin has to be used at doses much higher than the IC50 to induce apoptosis. Our data explain this paradox. Acute apoptosis over 24 – 48 hours is not caused by DNA damage, but by induction of reactive oxygen species. Our previous observation of endoplasmic reticulum stress by cisplatin (Mandic, Hansson et al. 2003) is likely due to release of Ca^{2+} from intracellular stores caused by ROS.

PAPER VI: Mapping calcium signaling pathways

The aim was to investigate the importance of different cellular Ca^{2+} stimulated pathways for apoptosis. From the NCI Mechanistic Drug Set we identified 40 agents with strong caspase-cleavage activity. Since the Mechanistic Drug Set is assembled from drugs with different patterns on growth inhibition in the NCI 60 cell line panel, the 40 agents are likely to induce apoptosis by different mechanisms. In order to examine the role of Ca^{2+} for apoptosis, cells were treated with these 40 compounds in the presence or absence of the intracellular Ca^{2+} chelator BAPTA-AM and caspase-cleaved CK18 was measured using the M30-ELISA. We found that apoptosis by a majority of the compounds (26 of 40) was inhibited by 30% or more. Preliminary studies using the Fluo-4 calcium indicator showed that these compounds induce increases in cytosolic calcium at late stages of apoptosis (unpublished observations). We then attempted to investigate which calcium signaling pathways that were stimulated by our set of compounds.

Calpain is a Ca^{2+}-activated protease that has previously been reported as an important mediator of apoptotic signals (Wang 2000; Mandic, Viktorsson et al. 2002; Harwood, Yaqoob et al. 2005). We examined the effects of the calpain inhibitor calpeptin on drug-induced apoptosis. Not unexpected, 19 of 40 compounds were inhibited by the calpain inhibitor calpeptin. The degree of inhibition (median level of inhibition of apoptosis by all compounds) was lower compared to that achieved with BAPTA-AM, suggesting that calpain induction could not alone account for the effects on apoptosis observed by chelating calcium.

Calmodulin (CaM) is the major intracellular Ca^{2+}-binding protein and has been shown to either agonize (Devireddy and Green 2003) or antagonize (Ahn, Pan et al. 2003; Mishra, Mishra et al. 2005) apoptosis. We examined the effect of the calmodulin inhibitor W7 on apoptosis and found that apoptosis was inhibited by 17 of 40 compounds. A correlation was observed between W7 and BAPTA-AM, suggesting that calmodulin was an important mediator of Ca^{2+}-induced apoptosis.

Calcineurin (protein phosphatase 2B [PP2B]) is a calcium-calmodulin-activated, serine/threonine PP that is activated by intracellular calcium (Crabtree 1999; Crabtree and Olson 2002; Hogan, Chen et al. 2003). Calcineurin dephosphorylates kinase apoptosis signaling kinase (ASK-1) which decreases the association of ASK-1 with 14-3-3, leading to increased ASK1 activity. ASK1 then activates JNK and p38 leading to apoptosis. Calcineurin has been implicated as a modulator of cell death in many studies, the majority of which relied on the inhibitory agents CsA and/or FK506 (Liu, Wilkins et al. 2006). Calcineurin is also sensitive to oxidative stress, although in this case oxidation results in inactivation of the enzyme (Wang, Culotta et al. 1996). Somewhat surprisingly, we did not find any strong inhibition of apoptosis by FK506.

Several calmodulin-dependent proteins have been identified, and one of these, calmodulin kinase II (CaMKII) is a mediator of cellular Ca^{2+} and calmodulin-mediated apoptosis (Fladmark, Brustugun et al. 2002). CaMKII has been reported to phosphorylate ASK-1. However, compared to calcineurin, CaMKII has not widely been studied in relation to apoptosis except for a few studies (Wright, Schellenberger et al. 1997; Fladmark, Brustugun et al. 2002). We found that the CaMKII inhibitor KN-93 was able to block apoptosis by 21/40 compounds which was similar to the calmodulin inhibitor W7.

Several downstream mediators of JNK apoptotic signaling have been described, including p53 (Davis 2000), Bak (Ihrlund, Hernlund et al. 2006) and Bax (Papadakis, Finegan et al. 2006). We found that apoptosis by a number of the compounds studied were inhibited by the JNK inhibitor SP600125. Calcium signaling has been proposed to activate JNK in some studies (Ko, Park et al. 1998; Sreedhar and Srinivas 2002; Shen and Liu 2006). Since a strong correlation between inhibition of apoptosis by KN-93 and SP600125 was observed we suggested that some of the compounds may induce a calmodulin-CaMKII-JNK pathway. Further studies are in progress to examine whether JNK activation precedes via CaMKII.

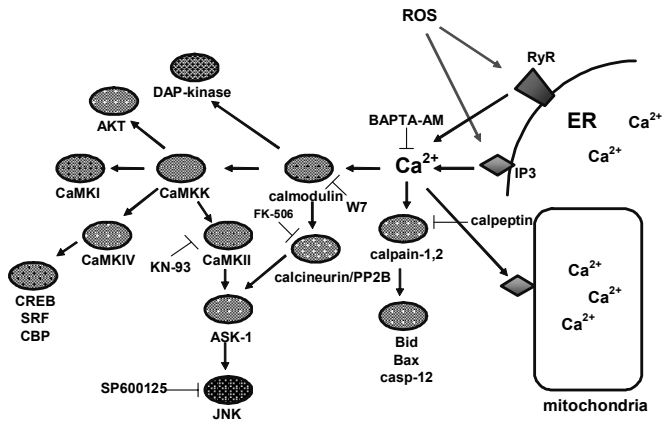

Fig. 2 Different cellular calcium stimulated pathways. We suggested that calpain and calmodulin kinase II are important mediators of calcium signaling in apoptosis. The requirement for calcium was generally late during the apoptotic process, and calcium signaling was implied in activation of JNK.

PAPER V AND VI: USING CYTOKERATIN MARKERS TO ASSESS TUMOR CELL DEATH IN BLOOD

A major problem in the process of evaluating drug activity is related to the poor performance of cancer animal models (Kamb 2005). These models are often week indicators of clinical activities of investigational drugs, particularly on determining toxicity.

A major aim of developing the M30-ELISA assay was to investigate whether it would be possible to measure tumor apoptosis in blood samples. Initial studies showed that the levels of

caspase-cleaved CK18 were elevated in serum from cancer patients (Bivén, Erdal et al. 2003; Ueno, Toi et al. 2003). It was subsequently shown that the M30-ELISA could be used together with an ELISA that measures total CK18 (the M65-ELISA)(Fig.3) to assess the fraction of cleaved CK18 molecules (Kramer, Erdal et al. 2004). Induction of apoptosis in cultured cells will result in release of caspase cleaved CK18 and in relatively high M30:M65 ratios, whereas induction of necrosis will almost exclusively result in release of CK18 molecules that are not caspase cleaved and in a low M30:M65 ratio. The M30:M65 ratio will therefore reflect the mode of cell death of epithelial cells.

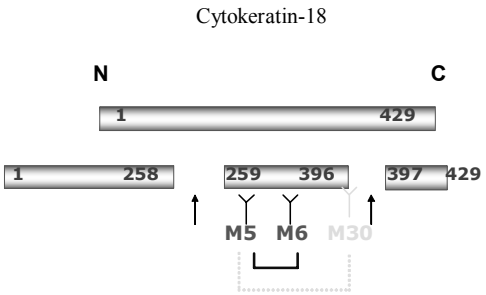

Fig.3 A method to to assess the relative amounts of apoptosis and necrosis (total), M65 ELISA

PAPER V: Examining treatment response in prostate cancer

In this study we monitored CK18 levels during treatment of patients with hormone refractory prostate cancer. Prostate cancer is stimulated to proliferate by male hormones, particularly testosterone. Therefore, patients are treated with hormone therapy to reduce levels of male hormones available to cancer cells. This removes the growth stimulus produced by male hormones and causes the cancer to shrink. Unfortunately, patients ultimately stop responding to hormone therapy. Such patients are referred to as having hormone-refractory or androgen independent prostate cancer. The relationship between prostate-specific antigen (PSA)–defined recurrence and prostate cancer–specific mortality remains unclear and PSA level should be used with caution. When hormone therapy is no longer successful, chemotherapy is a treatment

option. However, current single-agent treatment has shown to have limited benefit. Similar to other malignant diseases, prostate cancer is treated with a combination of different agents.

Estramustine phosphate (EMP) is a nornitrogen mustard-estradiol conjugate that interferes with microtubule dynamics (Smith 1999). It has been used in the treatment of prostate cancer for many years. Vinorelbine, a microtubule interacting agent, has shown activity in prostate cancer. Docetaxel is a semi synthetic taxane, a class of chemotherapeutic agents that bind to beta tubulin, thereby stabilizing microtubules and inducing cell cycle arrest and apoptosis (Hennequin, Giocanti et al. 1995; Pienta 2001). Treatment with docetaxel every three weeks led to superior survival and improved rates of response as compared with mitoxantrone (Tannock, de Wit et al. 2004).

We chose to analyze the data using non-parametric statistics. Median CK18 levels were determined before therapy, and after 48 and 96 hours of estramustine treatment. The 96 hour time point was used as a basal level for subsequent vinorelbine or docetaxel treatment. One potential problem of this design was that CK18 increases induced by docetaxel/vinorelbine may not be detected against a background of activity induced by the estramustine treatment. This was not observed: on the contrary, estramustine induced small increases in serum CK18 and no detectable increase in caspase-cleaved CK18. Treatment with vinorelbine and in docetaxel in particular, induced more pronounced increases.

These findings suggest that estramustine and vinorelbine are not as effective as docetaxel in inducing apoptosis (or cell death). This interpretation is consistent with clinical data: docetaxel is the first agent shown to improve survival in HRPC (Petrylak, Tangen et al. 2004; Tannock, de Wit et al. 2004). We also demonstrate that higher CK18-Asp396 increases were observed during third-line docetaxel treatment compared to second-line vinorelbine treatment in the same group of patients, providing direct evidence that docetaxel is more effective than vinorelbine.

These results are encouraging as they suggest that the CK18 markers are useful for assessing the efficiency of anticancer drugs. The results suggest that docetaxel may be as effective as monotherapy as a combination with estramustine. Estramustine shows toxic side effects, and the possibility that it is not of major benefit for the patients raises the question of whether this drug should be clinically used.

Increases of CK18-Asp396 and CK18 levels during therapy due to release from dead tumor cells, normal epithelial cells or both. If CK18 molecules are released from tumor cells during

treatment, higher increases are expected in patients with a large tumor load. We were very encouraged to find that there was a significant observation between docetaxel-induced increases of CK18-Asp396 and baseline levels of PSA, CK18 and CK18-Asp396. This finding strongly suggests that CK18-Asp396 release induced by docetaxel is due to tumor apoptosis.

PAPER VI: Response to therapy in breast cancer patients

In this study we determine CK18 levels in the serum of breast cancer patients during therapy. We examined patients treated with docetaxel and patients treated with CEF (cyclophosphamide/ epirubicin/5-FU). The response to these drugs was also examined on cultured cells, both in monolayer and spheroid culture.

A problem in anticancer drug research is that cell cultures are poor models for assessment of drug toxicity to tumor cells *in vivo*. Tumor cells are usually grown on plastic support in the presence of rich nutrition in a well oxygenated environment. The properties of normal cells and tissues are also poorly reflected in tissue culture and this makes it difficult to use *in vitro* cultures to assess whether anticancer drugs have a therapeutic window. Spheroid cultures represent a type of technology developed in order to mimic malignant tumor growth in tissue culture experiments (Sutherland and Durand 1976; Mueller-Klieser 1987). Spheroids are 3-D cell colonies, allowed to form in agarose coated culture wells. At the surface of the colonies, cells are in contact with oxygen and nutrients and proliferate quickly. Further inward, cell viability depends on diffusion of nutrients in and waste out. Proliferation assays such as MTT assay require addition of exogenous substrate. Such substrates do not freely enter multicellular aggregates. The M30 ELISA system does not rely on the use of substrates and is ideal for high through put (HTP) screening of apoptosis in 3-D spheroid cultures. van der Kuip et al. have described an organ culture system where 200 µM tumor slices are cultivated for 4-5 days (van der Kuip, Murdter et al. 2006). The cultured tumor slices can be treated with anticancer drugs and apoptosis checked by microscopy. Such 3-D cultures are ideal for secondary screening with M30 ELISA system.

We found that docetaxel and CEF therapy induce apoptosis of MDA-MB-231 cells *in vitro*. The apoptotic response was characterized by an increased release of caspase-cleaved CK18 both in monolayer and in spheroids. The *in vivo* responses to docetaxel and CEF were found to differ. Docetaxel induced increased serum levels of caspase-cleaved CK18 molecules. In contrast, CEF therapy was found to lead to a preferential increase in the serum levels of

uncleaved CK18 fragments in patient serum. The pattern of release of preferentially uncleaved CK18 molecules, consistent with induction of a mainly necrotic response *in vivo*, could not be reproduced *in vitro* using 2-D or 3-D cultures.

One reason for the inconsistency between the *in vitro* and the *in vivo* results could be that the necrotic response by DNA damage agents may require high drug concentrations (Zong, Ditsworth et al. 2004) and it is known that bolus injection of 150 mg/m^2 epirubicin will result in high plasma concentrations (Tjuljandin, Doig et al. 1990).

The application of cyclophosphamide is often accompanied by a number of potential toxicities. Pharmacokinetics of cyclophosphamide has been studied extensively, but there is still an incomplete understanding on the role of cyclophosphamide metabolites in the efficacy and toxicity cyclophosphamide therapy. Studies are ongoing aiming to reproduce the *in vivo* results using cyclophosphamide metabolites, hypoxic culture conditions and tumor spheroids.

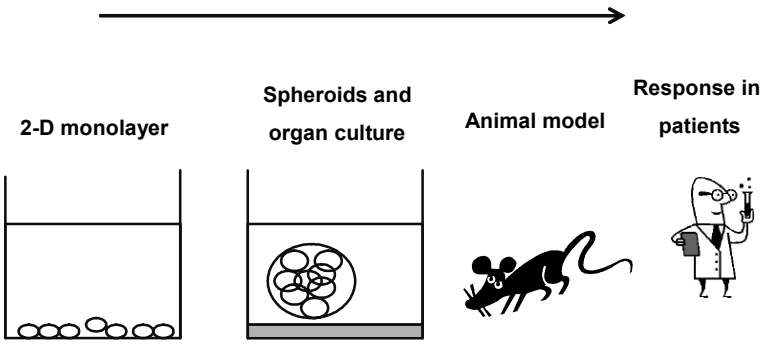

Fig. 4 The M30 ELISA: from screening in cell culture to response in patients.

Conclusion paper V – VI

There is a need to develop surrogate markers that can be used to rapidly assess treatment efficiency. Methods which can determine if apoptosis and/or necrosis have been induced by treatment may assist in monitoring the effectiveness of therapy. Monitoring the increase of caspase cleaved (M30 reactive) or total cytokeratin 18 (M65 reactive) levels in human serum

samples appear to be a promising candidate for a surrogate marker to demonstrate treatment efficiency. We conclude that assessment of release of cytokeratins from dying cells provided unique opportunities for translational research. The possibility to use the same cell death assay system for *in vitro* cultures, *ex vivo* cultures, blood from xenografted mice and finally from patients is an advantage and make it easier to characterize new cancer therapeutics.

♥ – tack till alla som på något sätt funnits med

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