Cisplatin-resistance and cell death in malignant pleural mesothelioma cells

Veronica Janson

Faculty of Medicine
Department of Medical Biosciences, Clinical Chemistry

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Cover: Cisplatin-induced, early-phase membrane blebbing in a P31 human malignant pleural mesothelioma cell (Phase-contrast microscopy image, photo by Veronica Janson)
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Abstract

Malignant pleural mesothelioma (MPM) is an aggressive, treatment-resistant tumour. Cisplatin (cis-diaminedichloroplatinum (II)) is the best single-agent chemotherapy for MPM, but platinum-based combination therapies give the best overall response rates. Cisplatin use is limited by resistance and severe side effects. This thesis has increased the knowledge concerning cisplatin-induced cell death in MPM by describing a novel potential therapeutic target, and three novel phenotypes of cisplatin-resistance in a human MPM cell line (P31) and its cisplatin-resistant subline (P31res1.2).

The novel potential therapeutic target, and one of the novel phenotypes, was cisplatin-resistant pro-apoptotic BH3-only proteins. In the P31 cells, cisplatin transiently increased pro-apoptotic BH3-only proteins during 6 h of exposure. This was almost completely abrogated in the P31res1.2 cells. De-regulated caspase activity and activation was the second novel phenotype identified. The P31res1.2 cells had earlier, possibly mitochondria-independent, caspase-3 activation, increased basal caspase-3 activity and increased basal cleavage of caspase-8 and -9. Despite these differences, 6-h equitoxic cisplatin exposures rendered 50-60% of the cells apoptotic in both cell lines. The third novel phenotype was abrogated Na⁺K⁺2Cl⁻-cotransporter (NKCC1) activity. Although NKCC1 activity was dispensable for cisplatin-induced apoptosis, balanced potassium transport activity was essential for P31 cell survival. Finally, the survival signalling protein Protein Kinase B (PKB or Akt) isoforms α and γ were constitutively activated in a PI3K-independent manner in P31 cells. In the P31res1.2 cells, PKBα and γ activities were increased, and there was PI3K-dependent activation of PKBβ. However, this increase in PKB isoform activity was not strongly associated to the cisplatin-resistance of the P31res1.2 cells.

Keywords: apoptosis, BH3-only proteins, caspase, cell morphology, potassium (K⁺) transport, protein kinase B (PKB/Akt), signalling pathways, time

Author: Veronica Janson, VMD, Department of Medical Biosciences, Clinical Chemistry, Building 6 M 2nd floor, Umeå University, S-901 85 Umeå, Sweden. E-mail: veronica.janson@medbio.umu.se; phone: +46 (0) 90 785 2740; fax: +46 (0) 90 785 2829
In memory of

my father, Jens-Erik
my aunt, Anita

Cancer strikes without remorse,
always painful, always unfair.
I miss you.
I love you.
“Writing a book is like washing an elephant: there is no good place to begin or to end, and it is hard to keep track of what you already covered”

Anonymous
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## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
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<tbody>
<tr>
<td>AFC</td>
<td>7-amino-4-trifluoromethyl coumarin</td>
</tr>
<tr>
<td>AIF</td>
<td>Apoptosis inducing factor</td>
</tr>
<tr>
<td>Apaf-1</td>
<td>Apoptotic protease activating factor 1</td>
</tr>
<tr>
<td>Bad</td>
<td>Bcl-2 antagonist of cell death</td>
</tr>
<tr>
<td>Bak</td>
<td>Bcl-2 antagonist/killer</td>
</tr>
<tr>
<td>Bax</td>
<td>Bcl-2 associated protein X</td>
</tr>
<tr>
<td>Bcl-X</td>
<td>Bcl-2 related protein X (Xl = long form)</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>B-cell lymphoma 2</td>
</tr>
<tr>
<td>BH</td>
<td>Bcl-2 homology domain</td>
</tr>
<tr>
<td>Bid</td>
<td>Bcl-2 interacting domain death agonist</td>
</tr>
<tr>
<td>Bik</td>
<td>Bcl-2 interacting killer-like</td>
</tr>
<tr>
<td>Bim</td>
<td>Bcl-2 interacting mediator of cell death</td>
</tr>
<tr>
<td>Blk</td>
<td>Bik-like</td>
</tr>
<tr>
<td>Bmf</td>
<td>Bcl-2 modifying factor</td>
</tr>
<tr>
<td>Caspase</td>
<td>Cysteinyl aspartate proteases</td>
</tr>
<tr>
<td>CD&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Cytotoxic dose for 50% effect</td>
</tr>
<tr>
<td>DISC</td>
<td>Death-inducing signalling complex</td>
</tr>
<tr>
<td>EDTA</td>
<td></td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor (receptor)</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular-signal regulated kinase, also known as p44/42</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence-activated cell sorting</td>
</tr>
<tr>
<td>FLIP</td>
<td>FLICE-like inhibitory protein (FLICE is old name for caspase-8)</td>
</tr>
<tr>
<td>FMCA</td>
<td>Fluorometric microculture cytotoxicity assay</td>
</tr>
<tr>
<td>FSC</td>
<td>Forward scatter, determined in flow cytometry</td>
</tr>
<tr>
<td>GSK3</td>
<td>Glycogen synthase kinase-3</td>
</tr>
<tr>
<td>Hrk/DP5</td>
<td>Harakiri/death protein 5</td>
</tr>
<tr>
<td>Hsp</td>
<td>Heat-shock proteins e.g. Hsp27, Hsp70, Hsp90</td>
</tr>
<tr>
<td>IAP</td>
<td>Inhibitor of apoptosis</td>
</tr>
<tr>
<td>(I)CAD</td>
<td>(Inhibitor of) caspase-activated DNase</td>
</tr>
<tr>
<td>IC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Inhibitory concentration for 50% effect</td>
</tr>
<tr>
<td>JNK</td>
<td>Jun-N-terminal kinase, also known as SAPK - stress-activated protein kinase</td>
</tr>
</tbody>
</table>
K+ Potassium ions
LDH Lactate dehydrogenase
MAPK Mitogen-activated protein kinase
MPM Malignant pleural mesothelioma
mTOR Mammalian target of rapamycin
NKCC1 The secretory Na⁺-K⁺-2Cl⁻-cotransporter
Omi/HtrA2 Omi/High temperature requirement protein A2
PARP Poly(ADP-ribose) polymerase
PBFI(-AM) Cell membrane permeable fluorescent probe benzofuran isophtalate (acetomethyl ester)
PBS Phosphate-buffered saline
PCA Projected cell area, calculated in image analysis of phase-contrast microscopy
PCM Phase-contrast microscopy
PDK Phosphoinositide-dependent kinase 1
(PH)LPP (Pleckstrin homology domain) leucine-rich repeat protein phosphatase
PI Propidium iodide
PI3K Phosphoinositide 3-kinase
PIP₃ Phosphatidylinositol-3,4,5 phosphate
PKB Protein kinase B, also known as Akt
PKC Protein kinase C
Puma p53-upregulated modulator of apoptosis
SEM Scanning electron microscopy
SF Shape factor, geometrically calculated in image analysis of phase-contrast and scanning electron microscopy
Smac/DIABLO Second mitochondria-derived activator of caspases/direct inhibitor of apoptosis-binding protein with low pI
SSC Side scatter, determined in flow cytometry
TBS Tris-buffered saline
TMR Tetra-methyl-rhodamine-dUTP
TNF(R) Tumour-necrosis factor (receptor)
TUNEL Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling
XIAP X-linked inhibitor of apoptosis
1 Introduction

Virtually all cancers must acquire six hallmark capabilities: self-sufficiency in growth signals, insensitivity to anti-growth signals, evasion of apoptosis, limitless replicative potential, sustained angiogenesis and tissue invasion and metastasis (1). The therapeutic goal of cancer treatment is to kill the tumour cells without affecting the normal tissue. The main method to achieve this is by triggering tumour-selective cell death (2). An important limiting factor for therapeutic success is thus the cancer cells’ capacity to evade apoptosis. To increase the chance of therapeutic success it is necessary to understand the effect of the apoptosis-inducing therapy on the tumour cells, as well as the efficiency of the apoptotic machinery in the tumour cells.

The work presented in this thesis has focused on the apoptosis-inducing effect of the chemotherapeutic drug cisplatin and the efficiency of the apoptotic machinery in human malignant pleural mesothelioma cells.

1.1 Cisplatin

Cisplatin is a chemotherapeutic drug that is used to treat solid tumours, but its use is limited by intrinsic or acquired resistance and severe side effects subsequent to increased dose (3-5). Much effort has been put into understanding the mechanisms of cisplatin-induced cell death and the resistance mechanisms since the discovery of the drug in the 1960-1970s [reviewed by the discoverer in (6)] (3-19). It was long considered that the cytotoxic effect was solely due to cisplatin intercalations into DNA and consequent activation of apoptotic signalling pathways that executed cell death programs. Cisplatin-induced cell death can be executed through apoptosis or necrosis, depending on the cellular context (9, 10). However,
cisplatin chemotherapy activates several signalling pathways (Figure 1), which can lead to cell cycle arrest, DNA repair or cell survival, in addition to execution of cell death (17, 20). The cellular outcome of cisplatin exposure is determined by the relative intensity and duration of these signalling pathways (17).

**Figure 1.** An overview of pathways and proteins that can mediate cisplatin-induced cellular effects. Cisplatin intercalation in DNA will activate DNA damage recognition proteins that transduce the DNA damage signals to downstream effectors. The outcome of cisplatin exposure depends on the relative intensity of the signals generated and the crosstalk between the pathways. The pathways shown are simplified and incomplete. The figure is based on (9, 17, 18, 20) and references therein.

Subsequent to the many potential effects of cisplatin on cellular signalling there is a plethora of potential mechanisms of resistance (5, 12, 14, 16, 19). These are summarised in Figure 2. With the increased insight into cisplatin effects on cellular signalling, and the plethora of resistance mechanisms, it has become clear that cisplatin also has effects on cellular mechanisms distinct from the effects on DNA integrity. Recent evidence shows that
cisplatin-induced apoptotic signalling can occur independently of DNA damage (21-23). It has even been suggested that the acute apoptosis-inducing effect of cisplatin is an off-target effect i.e. independent of DNA damage (24).

**Figure 2.** An overview of cisplatin-resistance mechanisms. There has been much work on determining the role of cisplatin uptake (1), sequestration in the cytosol (2), cisplatin efflux (3) and response to cisplatin intercalation in DNA (4) in cisplatin-resistance. Subsequently, the role of decreased apoptosis (5) as a consequence of 1-4 has been well studied. However, the role of decreased apoptosis (5) subsequent to defects in the apoptotic machinery has only recently received increased attention. Figure based on (5, 12, 14, 16, 19) and references therein.

The time-dependent uptake and cellular distribution of cisplatin has been studied with the aid of fluorescence-labelled cisplatin (25-28) and electron microscopy analysis (29). As illustrated in Figure 3, fluorescence-labelled cisplatin binds to the cell membrane within a few min of exposure, and after 15-30 min it is distributed throughout the cytoplasm and nucleus (25-
This has also been shown with electron microscopy analysis (29). Accumulation of cisplatin in the Golgi apparatus precedes accumulation in the nucleus (26). After 1-2 h, cisplatin accumulates in the nucleus (25, 26, 28), thereafter it is gradually removed from the cell, probably via the Golgi apparatus (25, 28). Although there is no time-resolved study of cisplatin-DNA adducts published, separate studies have shown presence of cisplatin-DNA adducts after 1 h (30), 2 h (31) and 4 h (32) of cisplatin exposure.

Figure 3. The time-dependent uptake and cellular distribution of cisplatin determined with the aid of fluorescence-labelled cisplatin and electron microscopy. Cisplatin concentration is indicated by the shade of gray: white = no cisplatin, dark gray = maximal concentration of cisplatin. Figure based on (25-29).

1.2 Cell death

There are several forms of cell death, mainly defined by morphological criteria disregarding biochemical mechanisms (33, 34). Most often, researchers focus on differentiating between necrosis and apoptosis. Apoptosis was originally defined by morphological criteria (35). Classically, necrosis and apoptosis were considered each other’s opposites – necrosis representing uncontrolled cell disintegration and apoptosis representing controlled cellular break-down (34, 36). However, there is increasing evidence that necrosis also is executed in a controlled manner i.e. through activation or inactivation of specific proteins and signalling pathways. In addition, there are forms of cell death that do not fit into either apoptosis or necrosis (34). The Nomenclature Committee on Cell Death published recommendations for classification of cell death in 2005 (33), and some of these are summarised in Table 1.

In cancer therapy, the aim is to kill the cells in an orderly manner i.e. through apoptosis, since in vivo necrosis results in unwanted inflammatory
responses and tissue damage. In this thesis I have focused on apoptosis as the main form of cell death, although I appreciate that other forms of cell deaths may have occurred which have not been investigated.

**Table 1. Summary of the morphology of some cell death types, with comments on the use of their nomenclature (33).**

<table>
<thead>
<tr>
<th>Name</th>
<th>Morphology</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apoptosis</td>
<td>Cells round up, retract pseudopods, shrink, detach from substrate and separates into apoptotic bodies. Chromatin condensation and nuclear fragmentation.</td>
<td>Biochemical changes (e.g. caspase activation, DNA fragmentation) are used to diagnose the form of cell death.</td>
</tr>
<tr>
<td>Autophagy</td>
<td>Vacuolisation of the cytoplasm: two-membraned vacuoles contain degenerating cytoplasmic organelles or cytosol. No chromatin condensation.</td>
<td>Cell death does not occur through autophagy, it occurs with autophagy.</td>
</tr>
<tr>
<td>Necrosis</td>
<td>Often that of oncosis.</td>
<td>I.e. cell death without signs of apoptosis or autophagy.</td>
</tr>
<tr>
<td>Oncosis</td>
<td>Swelling/dilation of cytoplasm and cytoplasmic organelles, moderate chromatin condensation, mechanical rupture of plasma membrane.</td>
<td>The use of the term oncrosis should be limited for the preference of necrosis, for historical reasons.</td>
</tr>
<tr>
<td>Anoikis</td>
<td>Apoptotic.</td>
<td>I.e. apoptosis induced by loss of attachment to substrate or surrounding cells.</td>
</tr>
<tr>
<td>Programmed cell death</td>
<td>Apoptotic.</td>
<td>An imprecise term that describes the genetically programmed cell death that occurs during development and aging. Should be replaced by e.g. developmental cell death. Not synonymous to apoptosis.</td>
</tr>
</tbody>
</table>

1.2.1 The morphology of apoptosis

The term apoptosis was introduced by Kerr et al (1972) to describe a particular morphological aspect of cell death: attached cells retract pseudopods, round up and shrink, there is chromatin condensation and fragmentation of the nucleus, and plasma membrane blebbing precedes the separation of the cell into membrane-bound apoptotic bodies (35) (Figure
More recently, an early phase of membrane blebbing was described in adherent cells, which does not occur in non-adherent cells (Figure 4). The early-phase blebs are dynamic with rapid protrusions and retractions, they do not contain any organelles or chromatin and they infrequently detach from the main body of the cell (37, 38). The late-phase blebs are more static, contain chromatin and cytoplasmic organelles (37) and ultimately detach from the main cell body, forming apoptotic bodies (37, 38).

Figure 4. The sequence of apoptotic morphological changes: 1) normal adherent cell, 2) retraction of pseudopods and rounding up, 2b) early-phase blebbing, 3) rounding up and shrinkage, 4) chromatin condensation, 5) nuclear fragmentation and late-phase blebbing, 6) separation into membrane-bound apoptotic bodies. Figure based on (35, 37, 38).

The early-phase membrane blebbing is similar to stress-induced membrane blebbing, which occurs concurrent with regulation of the MAP kinase proteins p38 and ERK1/2 (39, 40). It is suggested that the early-phase apoptotic membrane blebbing is a stress-response that is neither necessary nor sufficient for apoptosis (37, 38). The late-phase blebbing occurs concurrently with caspase activation (37, 38) and involves activation of the Rho effector proteins (41-43). Although apoptosis is a strictly morphologically defined term, there are numerous biochemical changes that are associated with the execution of apoptosis, including externalization of phosphatidylserine, activation of apoptotic caspases and oligonucleosomal fragmentation of DNA (33). However, it is important to recognise that the execution of cell death by apoptosis can occur in the absence of caspase activation and DNA fragmentation, although recent
evidence indicate that caspase-3 activation may be required for the apoptotic morphological changes to occur (33, 44).

1.2.2 Apoptotic signalling pathways

The process of apoptosis can be divided into three phases – initiation, effector and degradation (45, 46). The initiation phase depends on the cell or tissue type, the cellular context and the apoptotic stimulus. During the initiation phase, the scene is set for the effector phase, and the efficacy of the effector and the degradation phase can be influenced by the initiation phase (46). In the effector phase the proteases, nucleases and other participants in the degradation phase are activated. It is during the effector and degradation phases that the classical morphological features of apoptosis are detected (46). As yet, there is no single “point-of-no-return” identified for the execution of apoptosis (33). Instead a number of critical control points are identified (47). In Figure 5, a brief overview of some of the apoptotic signalling pathways is presented. Often when apoptotic signalling pathways are described, only the mitochondrial and the receptor-mediated pathways are mentioned. For increased understanding of the complexity of apoptotic signalling I include some of the other regulatory proteins and pathways, in addition to the two classical pathways.

The mitochondrial signalling pathway

The classical description of the mitochondrial signalling pathway (© in Figure 5), also known as the intrinsic signalling pathway, starts with the activation of pro-apoptotic BH3-only proteins and inhibition of pro-survival Bcl-2 proteins (48-53). This results in activation of pro-apoptotic Bax or Bak, which cause mitochondrial membrane permeabilisation and release of pro-apoptotic proteins to the cytosol, e.g. cytochrome C, Smac/DIABLO and Omi/HtrA2. Cytochrome c activates the apoptosome and subsequently caspase-9 (54-57). Smac/DIABLO and Omi/HtrA2
Figure 5. Brief overview of apoptotic signalling. For details and abbreviations, see the text. 

1. The mitochondrial, or intrinsic, signalling pathway.
2. Caspase-2 signalling.
3. The receptor-mediated, or extrinsic, signalling pathway.

↓ indicates activation, ⊥ indicates inhibition. See text for references.
competitively bind to caspase inhibitors and cause the release of inhibitor-bound caspase-3 and -9 (58-60). Caspase-9 is the initiator caspase of the mitochondrial signalling pathway and its substrate is pro-caspase-3 and -7 (54-57, 61-64). Cleavage of these pro-caspases yields active caspase-3 and -7, executioner caspases common to both the mitochondrial and the receptor-mediated signalling pathway (33, 46, 61).

Recently, it was suggested that another caspase is necessary for the activation of the mitochondrial signalling pathway – caspase-2 (\(\alpha\) in Figure 5) (65-68). Caspase-2 is unique among the caspases in that it has characteristics and functions of both initiator and executor caspases. In common with the initiator caspases it has a long pro-domain that is structurally related to that of caspase-9 (65). The executioner role is defined by the ability of caspase-2 to cleave \(\alpha\II\)-spectrin at the same site as caspase-3 and -7, the two established execution caspases of apoptosis (69). Caspase-2 activity is also important for Bax translocation to the mitochondrial membrane (66). In addition, caspase-2 can permeabilise the outer mitochondrial membrane and stimulate the release of cytochrome c and Smac/DIABLO, but not apoptosis-inducing factor (AIF) (68). There are additional functions for caspase-2 not mentioned here, and I have not discussed the relevance of the subcellular distribution of caspase-2 (65). Furthermore, there is increasing evidence that caspase-2 is necessary for the onset of apoptosis subsequent to many different insults, probably in a cell- and trigger-specific manner (65).

The receptor-mediated signalling pathway

In the receptor-mediated signalling pathway (\(\beta\) in Figure 5), also known as the extrinsic signalling pathway, activation of death receptors of the tumour necrosis factor receptor (TNFR) superfamily is the first step. The multimeric receptor recruits adaptor proteins that form a death-inducing signalling complex (DISC) (46, 53, 55, 70). The details differ slightly for different receptors, but the concept is the same. Caspase-8, the initiator caspase of the receptor-mediated signalling pathway, is activated within the
DISC. Caspase-10 is similar to caspase-8 and is also activated within the DISC, but it is not yet established how important caspase-10 is for apoptosis signalling (62). There are two consequences of caspase-8 activation – activation of caspase-3 and cleavage of Bid to truncated Bid (tBid) (46, 53, 55, 61, 70). Cleavage of Bid confers cross-talk to the mitochondrial signalling, as tBid is a potent pro-apoptotic BH3-only protein that can inhibit all five pro-survival Bcl-2 proteins (49-52, 71).

**Caspase-independent signalling**

Apoptosis can also be executed in a caspase-independent manner, and some examples of this are shown in Figure 5 (8). Apoptosis-inducing factor (AIF) can be released from the mitochondrion after mitochondrial membrane permeabilisation. It translocates to the nucleus and triggers chromatin condensation and DNA fragmentation (72, 73). Endonuclease G is also released from the mitochondrion and translocated to the nuclease where it acts as a DNase (74, 75). The cysteine protease cathepsins B and L and the aspartatic protease cathepsin D are the most abundant proteases in lysosomes, and can trigger apoptosis via several different pathways. For instance, cathepsin B can take the role of primary executor protease in death-receptor induced apoptosis (76). Cathepsin D can activate Bax and cause selective translocation of AIF to the cytosol (77). The lysosomal proteases can also cleave Bid, and thereby initiate the mitochondrial signalling pathway (78).

**Negative regulators of apoptotic signalling**

Apoptotic signalling is kept under strict control to prevent accidental cell death. The negative regulators of apoptotic signalling include inhibitor-of-apoptosis proteins (IAPs, e.g. IAP-1, IAP-2, X-linked IAP or XIAP) (79), heat-shock proteins (e.g. Hsp27, Hsp70, Hsp90) and the FLICE-like inhibitory protein (FLIP) (80). XIAP binds to active caspase-3 and -9 (79) and FLIP inhibits caspase-8 (80) (Figure 5). The Hsp have several targets,
and some are shown in Figure 5. In addition to these regulatory proteins, other signalling pathways also influence apoptotic signalling. One such signalling pathway is the phosphoinositide 3-kinase (PI3K)/protein kinase B (PKB, also known as Akt) pathway. This pathway and its effects on apoptotic signalling are discussed in detail in section 1.4.

1.2.3 The Bcl-2 family of proteins

Members of the Bcl-2 protein family are critical regulators of the mitochondrial signalling pathway of apoptosis (49-52, 71, 81). The family includes both pro-survival and pro-apoptotic proteins that interact to maintain or disrupt mitochondrial membrane permeabilisation (48, 49, 51, 82, 83). The family members share one or more Bcl-2 homology domains (BH), and can be classified by the presence of these (51, 82):

- pro-survival Bcl-2 proteins have four BH domains (BH1-4): Bcl-2, Bcl-XL, Bcl-w, Mcl-1, A1, Boo/Diva
- pro-apoptotic Bax-like proteins (or multidomain proteins) have three BH domains (BH1-3): Bax, Bak, Bok/Mtd
- pro-apoptotic BH3-only proteins have only the BH3 domain: Bid, Bad, Bim, Bmf, Bik, Hrk/DP5, Blk, Nip3, BNip3/nix, Puma, Noxa

The status of the Bax-like proteins determines the mitochondrial membrane permeabilisation (42, 43). If they are inactive, or inhibited by the Bcl-2 proteins, mitochondrial membrane integrity is maintained (Figure 6 A). Apoptotic stimuli can activate one or several of the BH3-only proteins and they can bind to and neutralise the Bcl-2 proteins (Figure 6 B). The Bcl-2 family of proteins are differently regulated, both transcriptionally and post-transcriptionally (81, 84). Some examples of known modes of regulation are shown in Table 2.
Table 2 Examples of transcriptional activation, post-transcriptional activation and inhibition of the Bcl-2 family of proteins. N.I. = not included due to not known or not well described.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Transcription factor</th>
<th>Post-transcriptional Activation</th>
<th>Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Puma p53 (85) FOXO3A (86)</td>
<td>N.I.</td>
<td>N.I.</td>
<td></td>
</tr>
<tr>
<td>BimEL BimL FOXO3A (87)</td>
<td>Release from dynein motor complex (88) Dephosphorylation (81, 84)</td>
<td>Phosphorylation (81, 84, 89)</td>
<td></td>
</tr>
<tr>
<td>BimS N.I. N.I.</td>
<td>N.I.</td>
<td>N.I.</td>
<td></td>
</tr>
<tr>
<td>Bid N.P.</td>
<td>Proteolytic cleavage to tBid</td>
<td>N.I.</td>
<td></td>
</tr>
<tr>
<td>Bad p53 (90)</td>
<td>Dephosphorylation (91, 92)</td>
<td>Phosphorylation (81, 84, 93, 94)</td>
<td></td>
</tr>
<tr>
<td>Bmf N.I.</td>
<td>Release from actin-myosin motor complex</td>
<td>N.I.</td>
<td></td>
</tr>
<tr>
<td>Bik E2F (95)</td>
<td>N.I.</td>
<td>Proteasome degradation (96)</td>
<td></td>
</tr>
<tr>
<td>Bcl-Xl STAT1 (97) CREB (98)</td>
<td>N.I.</td>
<td>N.I.</td>
<td></td>
</tr>
<tr>
<td>Bcl-2 CREB (98)</td>
<td>Phosphorylation (99-101)</td>
<td>Dephosphorylation (91)</td>
<td></td>
</tr>
<tr>
<td>Mcl-1 CREB (98)</td>
<td>N.I.</td>
<td>Ubiquitin-proteasome degradation</td>
<td></td>
</tr>
<tr>
<td>Bax N.P.</td>
<td>Translocation (102) and conformational change (103)</td>
<td>Phosphorylation (104)</td>
<td></td>
</tr>
<tr>
<td>Bak N.P.</td>
<td>Conformational change (103)</td>
<td>N.I.</td>
<td></td>
</tr>
</tbody>
</table>
Figure 6. Interactions between the Bcl-2 family of proteins to regulate mitochondrial membrane permeabilisation. ⊥ = inhibition. **A.** Preservation of mitochondrial membrane integrity by inhibition of the Bax-like proteins. **B.** Inhibition of the Bcl-2 proteins by BH3-only proteins. Figure based on (42, 43) and references therein.

1.2.4 The apoptotic caspases

A brief description of the activation of the well-studied apoptotic caspases is found in the previous section on apoptotic signalling pathways (Figure 5).

The cysteine-aspartate protease (caspase) family of proteins includes inflammatory and apoptotic caspases (105). The apoptotic caspases are caspase-2, -3, -6, -7, -8, -9 and -10, and will hereafter be referred to as caspases. There are two sub-families of the apoptotic caspases: initiator and executor caspases. Caspase-8, -9 and -10 are initiators, -3, -6 and -7 are executors (57, 62, 105) and caspase-2 can take the role of either initiator or executor (65), as discussed in the previous section on apoptotic signalling pathways. All caspases are produced as inactivated zymogens, or pro-caspases, and must be activated (105, 106).

The initiator caspases, and caspase-2, have long N-terminal regions with adaptor domains important for their recruitment to the multimeric protein complexes that mediate their activation (105, 106). This pro-
domain is much shorter in the effector caspases, and they are activated through proteolytic cleavage by the initiator caspases. The catalytic domain of all caspases is comprised of a smaller and a larger subunit or chain (105, 106). Caspase-9 and caspase-2 can exhibit activity without proteolytic cleavage (54, 55, 107). Although cleavage of the pro-caspase is not always obligatory for caspase activity, all activated caspases can be detected as cleaved fragments in apoptotic cells (62, 105, 108). The caspases regulate each other's activity in a more complex manner than shown in Figure 5 (Figure 7) (61, 64, 109, 110).

![Figure 7](image)

**Figure 7.** Regulation of caspases by caspases. Top part: The activation of executioner caspases (dark gray shapes) by initiator caspases (light gray shapes). Lower part: additional activations are shown. The dashed line indicates a weaker role in the processing of the relevant caspase (64), full lines indicates a strong role. Figure based on (61, 64, 109, 110).

### 1.3 The role of K⁺ in apoptosis

Volume regulatory processes are integral parts of a variety of cellular functions including apoptosis, and much effort has been put into understanding regulatory volume increase and regulatory volume decrease and the consequences when they go wrong (111-123).

Potassium ions (K⁺) have important regulatory roles in apoptosis. Efflux of K⁺ is necessary for cell shrinkage (124-128), and excessive K⁺ efflux induces apoptosis (125, 129-131). Prevention of K⁺ efflux by elevating extra-cellular K⁺ or blockage of K⁺-efflux channels inhibits apoptosis (116, 126). Cell shrinkage appears to be a prerequisite for apoptotic events leading to cell death (116, 119, 132). Early cell shrinkage occurs within two h of apoptosis induction, prior to cytochrome c release and caspase activation.
A later cell shrinkage then occurs, associated with increases in cytoplasmic cytochrome c, caspase activity, and DNA fragmentation (116, 132). Membrane ion channels and ion transporters appear to be important in both phases (118, 119, 132). A reduced intracellular K⁺ concentration is necessary for caspase activation and apoptotic nuclease activity (125). However, caspase activity is independent of the intracellular K⁺ concentration (126). The transition of an apoptotic cell from a state of high to low K⁺ content permits both the loss in cell volume and the activation of enzymes that mediate apoptosis (118, 126, 132). The role of ion regulation in volume regulation, apoptotic and non-apoptotic, is summarised in Figure 8.

![Figure 8](image)

**Figure 8.** A. The normal ion transport of the active Na⁺K⁺ATPase and Na⁺K⁺2Cl⁻cotransporter (NKCC1). B. Summary of the ionic control of volume regulation during apoptotic stimulus (the left part of the figure) or in a hypertonic environment (the right part of the figure). The roles of the K⁺ transporters are indicated. Figure based on (119, 123, 125, 134, 135) and references therein.

1.3.1 Membrane transport of K⁺

There is a plethora of K⁺ channels and transporters that mediate the transport of K⁺ across cellular membranes (134-141). Many different types of K⁺ channels contribute to the ionic fluxes during apoptosis (141, 142).
However, it appears as if distinct channel involvement depends on the cellular and apoptotic context (125, 141). In contrast, there are some transporters that are suggested to be important for the K⁺-mediated volume regulation - the Na⁺K⁺ATPase (135, 140) and the secretory Na⁺K⁺2Cl⁻ cotransporter (NKCC1) (111, 115, 134). The Na⁺K⁺ATPase in particular is suggested to be important for apoptotic volume regulation (135). The roles of Na⁺K⁺ATPase and NKCC1 in volume regulation, apoptotic and non-apoptotic, are summarised in Figure 8.

1.4 Protein kinase B (PKB) signalling

Protein kinase B (PKB/Akt) is a serine/threonine protein kinase with critical functions in the regulation of cell survival and proliferation (98). There are three PKB isoform; α, β and γ, with a high degree of structural similarity and sequence homology. Despite this, they can be differentially regulated (143, 144) and have, to an extent, different roles in cell proliferation, tumour development and cell migration (145-147).

PKB is activated by phosphorylation at two residues (148), serine 473 (ser473) and threonine 308(thr308) (98, 149). Both sites need to be phosphorylated for maximal activation of PKB (98, 149). Phosphorylation of thr308 alone partially activates PKB, but phosphorylation of ser473 is not sufficient for PKB activity (149). However, phosphorylation of ser473 facilitates thr308 phosphorylation (150). Thr308 phosphorylation is mediated by phosphoinositide-dependent kinase 1 (PDK1) (149, 151, 152). Phosphoinositide 3-kinase (PI3K) generates phosphatidylinositil-3,4,5 phosphate (PIP₃). In turn, PIP₃ recruits PDK1 and PKB to the plasma membrane by binding their pleckstrin homology (PH) domains. The two proteins can then interact, and PDK1 phosphorylates PKB at thr308. For complete activation of PKB, the mammalian target of rapamycin (mTOR):rictor complex phosphorylates ser473 (150).
**PKB targets in the apoptosis signalling pathways**

Constitutive activation of PKB promotes both cellular survival and resistance to e.g. chemotherapy (153), and elevated PKB activity is a common finding in cancers (153-156). PKB can affect its targets directly through phosphorylation, or indirectly via effects on transcription factors, cell death is inhibited through inhibition of pro-apoptotic proteins and activation of pro-survival proteins (86, 87, 89, 94, 98, 104, 157, 158). Some of the effects of PKB activity on the mitochondrial signalling pathway of apoptosis are summarised in Figure 9.

**Figure 9.** Protein kinase B (PKB) regulation of pro-apoptotic and pro-survival proteins in the mitochondrial signalling pathway of apoptosis. ⊙ Direct inhibition of pro-apoptotic proteins by phosphorylation. ⊙ Indirect inhibition of pro-apoptotic proteins by inhibition of the forkhead transcription factor FOXO3α. ⊙ Indirect activation of pro-survival proteins by activation of the cyclic AMP response element binding protein (CREB). Figure based on (86, 87, 89, 94, 98, 104, 157, 158) and references therein.
1.5 The mesothelium and malignant mesothelioma

Mesothelial cells compose the mesothelium - the monolayer of specialised cells that line the serosal cavities and internal organs of mammals (159, 160). Squamous-like cells of about 25 µm diameter dominate the mesothelium, but cuboidal cells can be found in various areas. The two cell types differ in ultrastructure; the cuboidal cells appear to be in a more metabolically active state. The luminal surface of the cells has a well-developed microvilli border. Adjacent cells are often overlapping, and they have well-developed cell-cell junctional complexes.

The mesothelial cells are derived from the mesoderm and express the mesenchymal intermediate filaments vimentin and desmin, as well as cytokeratins which are intermediate filaments characteristic of epithelial cells (159, 161). However, mesothelial cells can change their phenotype. After several passages in culture, mesothelial cells lose their cytokeratin expression and adopt a fibroblast-like phenotype, but on re-establishment of an intact mesothelium the epithelial-like phenotype returns (162, 163).

In addition to the two classical functions - protective barrier and frictionless interface for organ and tissue movement, the mesothelium has many important roles (159, 160). It is involved in fluid transport across the serosal cavities, tissue repair and secretion of various lubricants and immunomodulatory mediators. The mechanism of tissue repair is unusual in that it involves both stimulation of cell proliferation in the borders of the wound and detachment of cells that become free floating (159, 160, 162). The floating cells are attracted to exposed extracellular matrix and attach to it, proliferate and reconstitute the mesothelial monolayer (162). This mechanism suggests that mesothelial cells have an intrinsic resistance to release-induced apoptosis.

Malignant mesothelioma

Malignant mesothelioma is an aggressive, treatment-resistant tumour, often presenting as pleural in origin (159, 164). The pathogenesis of malignant mesothelioma has remained obscure due to the long latency time
until development of disease, up to 40 years (165). Asbestos is linked to malignant mesothelioma pathogenesis, and the mechanisms of asbestos carcinogenesis are still under investigation (166). There are also other factors relevant for the development of malignant mesothelioma, including other mineral fibers, genetic predisposition and presence of SV40 (a DNA tumour virus) (166).

In Western Europe, the incidence of malignant mesothelioma appears to be leveling, probably due to the actions taken to restrict asbestos exposures (167-169). Worldwide, however, the incidence of malignant mesothelioma is expected to continue to increase for some time (168). The median survival after diagnosis is less than 12 months, and during the progression of the disease the patient suffers increased dyspnea and pain that can become chronic (170, 171). In a literature review with meta-analysis, single-agent chemotherapy had little effect; the most effective single-agent was cisplatin (172). The best overall objective response rate (28%) was achieved by combining cisplatin and doxorubicin (172). Cisplatin and perametrexed is also an effective combination (171). The common treatment strategies usually include surgery of resectable tumours, chemotherapy and immunotherapy, often used in combination (171). To delay symptom progression, chemotherapy should be started as soon after diagnosis as the performance status of the patient allows (170).

Most mesotheliomas have abnormal karyotypes, aneuploidy and structural rearrangements (165, 171, 173). Chromosomal losses are more common than gains (173). One of the most common deletions is at the 9p21 locus encoding two critical inhibitors of cyclin dependent kinases, p16\textsuperscript{INK4a} and p15\textsuperscript{INK4b}, and the p53 regulator p14\textsuperscript{ARF} (173, 174). The loss of these proteins results in loss of cell cycle control and neoplastic transformation (173, 174). There are also numerous defects in the apoptotic machinery of malignant mesothelioma cells (174, 175). Elevated levels of pro-survival Bcl-X\textsubscript{L} is found in most malignant mesothelioma cell lines and tumour tissues investigated (176, 177), and down-regulation of Bcl-X\textsubscript{L} expression increases apoptosis \textit{per se} (178, 179). Elevated levels of pro-survival Bcl-2 are less
common (176, 177). There is less known about the role of the pro-apoptotic Bcl-2-family proteins in malignant mesothelioma resistance to apoptosis (180-184). It has been suggested that Bax is either dysfunctional or antagonized downstream of its interaction with the outer mitochondrial membrane in malignant mesothelioma cells (175). Inhibitor of apoptosis proteins (IAPs) are also suggested to have an important role in the apoptosis-resistance of malignant mesotheliomas (175). EGFR has been held as an attractive therapeutic target in malignant mesotheliomas, however, it is not clear to what extent EGFR expression contributes to the resistance phenotype (175, 185). A more investigated target is PKB signaling, which is frequently up-regulated in malignant mesotheliomas, and can be targeted for inhibition to increase apoptosis (186-188).

1.6 Putting it all together

In this thesis, the roles of three “signalling systems” were investigated - apoptosis signalling pathways, K⁺ transport and the PI3K/PKB signalling pathway. The proteins involved in, or affected by, these three systems partially overlap (Figure 10). During apoptosis, it is necessary to either override the PKB survival signals or abrogate them. For instance, PKB-mediated inhibitory phosphorylation of Bad can be abrogated through dephosphorylation (91, 92) and PKB-mediated inhibition of Puma expression can be counteracted by p53 activation (85). However, this can fail if PKB signalling is over-activated. The importance of K⁺ regulation during apoptosis has already been outlined in section 1.3. Much less is known about interactions between PKB activity and regulation of K⁺ in cancer cells. However, K⁺ deprivation caused dephosphorylation of PKB in cerebellar granule cells (189), and activation of PKB inhibited K⁺ deprivation-induced apoptosis in cerebellar granule neurons (190).

Many of the proteins that are targeted by cisplatin are involved in at least two of the three “signalling systems” investigated in this thesis (Figure
Furthermore, many of the resistance mechanisms involve proteins affected by at least two of the “signalling systems”.

**Figure 10.** Examples of overlap of proteins involved in, or affected by, the three “signalling systems” investigated in this thesis: apoptosis signalling, PKB signalling and K⁺ transport. Proteins targeted by cisplatin are enhanced in **bold**, and proteins involved in cisplatin-resistance are enhanced with *italics*. The proteins are grouped roughly according to function, as indicated by the gray shading. For reference suggestions see text and Figures 1, 2, 5 and 9.
2 Objectives

The overall objective of this thesis project was to determine the effect of in vitro acquired cisplatin-resistance on apoptotic signalling pathways. This was to be achieved through time-resolved comparative studies of a human malignant pleural mesothelioma cell line (P31) and its cisplatin-resistant sub-line (P31res1.2).

The specific objectives for each paper were to:

Paper I:
- Develop a new 96-well application for measuring changes in intracellular K+ content with PBFI-AM
- Determine, with the aid of the new application, if K+ depletion per se could induce apoptosis in P31 and U1690 (small-cell lung cancer) cells

Paper II:
- Determine if very early (< 30 min of exposure) cisplatin-induced changes of P31 morphology can be used to determine cisplatin cytotoxicity and induction of apoptosis

Paper III:
- Determine the role of membrane K+ transporters, particularly the Na+K+2Cl--cotransporter (NKCC1), in early-phase cisplatin-induced morphological changes of P31 and P31res1.2 cells
- Determine if NKCC1 has a role in the cisplatin-resistance of P31 and P31res1.2 cells
Paper IV:

- Investigate, in a time-resolved manner, the effect of acquired cisplatin-resistance on the cellular contents of pro-survival and pro-apoptotic Bcl-2 proteins in P31 and P31res1.2 cells
- Determine, in a time-resolved manner, the effects of cisplatin exposure on the cellular contents of pro-survival and pro-apoptotic Bcl-2 proteins in P31 and P31res1.2 cells

Paper V:

- Determine, in a time-resolved manner, the effect of acquisition of cisplatin resistance on the expression and activity of apoptotic caspases by comparing P31 and P31res1.2 cells

Paper VI:

- Determine, in a time-resolved manner, the effect of acquired cisplatin resistance on protein kinase B (PKB) activity by comparing P31 and P31res1.2 cells
- Investigate the effects of cisplatin exposure and LY294002-inhibition of PI3K on PKB activity and P31 and P31res1.2 cell survival
3 Materials and Methods

The materials and methods used are thoroughly described in each paper. Therefore, the descriptions here will be brief. A summary of the methods used is presented in Table 3.

<table>
<thead>
<tr>
<th>Subject</th>
<th>What is detected</th>
<th>Method</th>
<th>Paper</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell morphology</td>
<td>Membrane morphology and cell size</td>
<td>Phase-contrast microscopy</td>
<td>III, VI</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Phase-contrast microscopy &amp; image analysis</td>
<td>II, III</td>
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<tr>
<td></td>
<td></td>
<td>Scanning electron microscopy</td>
<td>II</td>
</tr>
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<td>LDH release</td>
<td>Flow cytometry</td>
<td>II</td>
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<tr>
<td></td>
<td>Cellular hydrolysis of fluorescein diacetate</td>
<td>Colorimetric assay</td>
<td>I</td>
</tr>
<tr>
<td>Biochemical diagnosis of apoptosis</td>
<td>Phosphatidylserine externalisation</td>
<td>FACS analysis of annexin V-FITC &amp; PI-staining</td>
<td>II</td>
</tr>
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<td>Caspase cleavage</td>
<td>Western blot</td>
<td>V</td>
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<tr>
<td></td>
<td>Caspase activity</td>
<td>Fluorometric assay with AFC-substrates</td>
<td>I-III, V</td>
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<tr>
<td></td>
<td></td>
<td>Western blot</td>
<td>V</td>
</tr>
<tr>
<td></td>
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<td>FACS analysis of TUNEL-staining</td>
<td>I- VI</td>
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<td>K+ transport</td>
<td>Changes in intracellular K+</td>
<td>PBFI-AM</td>
<td>I</td>
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<td>Influx and efflux of K+</td>
<td>86Rb+</td>
<td>III</td>
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<td>Proteins</td>
<td>Unmodified, phosphorylated or cleaved proteins</td>
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<td>Phosphorylated serine/threonine kinases</td>
<td>Proteome Profiler Human phosphor-MAPK array - nitrocellulose membrane array</td>
<td>VI</td>
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<tr>
<td>Cell cycle</td>
<td>DNA</td>
<td>FACS analysis of PI-stained DNA content</td>
<td>VI</td>
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</table>
3.1 Cell lines and cell culture

The P31 cell line was established from a male patient with a pleural malignant mesothelioma tumour in the early 1980s (191). To establish the cisplatin-resistant sub-line, P31 cells were maintained in cisplatin-containing medium, step-wise increasing the concentration. The cells’ acquisition of cisplatin-resistance was monitored with a fluorometric cytotoxicity assay (see below). After about four months, the cells had acquired a four-fold increased resistance to cisplatin, and the increase in cisplatin-concentrations was stopped. The resulting cell line was named P31res1.2, because they are maintained in 1.2 mg/L cisplatin.

The U1690 cell line is a small-cell lung cancer cell line (192) that was used in paper I to show that the PBFI-AM method could be applied to other cell lines than P31. However, it was not used in any of the other papers, and the results for the U1690 cell line will only be mentioned very briefly.

All cell lines were maintained at 37 °C in a humidified atmosphere containing 5% CO₂, grown as monolayer cultures in Eagle’s minimal essential culture medium with Earl’s salts supplemented with 10% foetal calf serum, 200 µmol/L L-glutamine, and gentamicin. The cells were propagated 2-3 times per week. For propagation, the medium was removed and the cells were released from the substrate by incubation with 0.1% sterile trypsin for ten min at 37 °C. The released cells were suspended in culture medium and aliquotted to maintenance flasks (75 cm²), and to cell culture petri dishes or multiwell plates (Table 4) for experiments. When P31res1.2 cells were plated for experiments, cisplatin was excluded from the medium.

Cell line stocks were stored at -144 °C. After thawing, the cell lines were propagated five times before being used in experiments to ensure stable cell cultures. After twenty propagations, the cells were discarded and not used for any more experiments. Once or twice yearly, cell line stocks were controlled for Mycoplasma ssp infection by 4’,6-diamidino-2-phenylindole staining. There were no Mycoplasma infections identified in the cell lines during the work with this thesis.
Table 4 Number of cells plated prior to start of experiments. The P31res1.2 had a slower growth rate compared to the P31 cells, therefore more cells were plated to ensure similar cell densities at start of experiments.

<table>
<thead>
<tr>
<th></th>
<th>Petri dish, 15 cm ∅</th>
<th>Petri dish, 10 cm ∅</th>
<th>6-well plate</th>
<th>96-well plate</th>
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</thead>
<tbody>
<tr>
<td>P31</td>
<td>1.2 – 2.0 × 10⁶</td>
<td>0.80 × 10⁶</td>
<td>0.10 × 10⁵</td>
<td>0.010 – 0.020 × 10⁶</td>
</tr>
<tr>
<td>P31res1.2</td>
<td>1.7 – 2.3 × 10⁶</td>
<td>1.20 × 10⁶</td>
<td>0.15 × 10⁵</td>
<td>0.010 – 0.025 × 10⁶</td>
</tr>
<tr>
<td>U1690</td>
<td>~2.0 × 10⁶</td>
<td>0.40 × 10⁶</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Time¹</td>
<td>48 – 72 h</td>
<td>18 – 36 h</td>
<td>18 – 36 h</td>
<td>18 – 48 h</td>
</tr>
<tr>
<td>Method</td>
<td>Proteome</td>
<td>TUNEL</td>
<td>TUNEL</td>
<td>LDH release</td>
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<tr>
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<td>Caspase activity</td>
<td>⁸⁶Rb⁺</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>Cell cycle</td>
<td></td>
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</tbody>
</table>

¹Time necessary for cells to reach ~ 80% confluence

3.2 Cell morphology

3.2.1 Phase-contrast microscopy (II-VI, VI)
Cell cultures were always inspected in an inverted phase-contrast microscope (Olympus CK2 microscope) before starting an experiment as well as at the end of it. This was done to determine cell density and cell health at the start and the end of experiments. Phase-contrast images of cell cultures during or after exposures were collected with a Nikon Coolpix 4300 camera with an ocular adaptor Nikon UR-E4 (III, VI). Images of semi-adherent cells were collected every 5 min during exposure using a CK40 microscope (Olympus) and a DP12 camera (Olympus) (III).

Image analysis (II, III)
For image analysis of phase-contrast microscopy (PCM) images, a method developed by the group was used, for a detailed description see papers II, III and references (193-195). In brief, images of a single, semi-adherent cell were collected every minute during a total of 40 min of exposure. The initial 5-10-min perfusion with drug-free medium was regarded as a base-line to
which the treatment period was compared for each individual cell (expressed as % of basal).

The different refractive indices between cell and medium produce an optical halo around the cell. The images were processed with reference to both the inside and outside border of this optical halo. The two-dimensional projected cell area (PCA) was determined and then used to calculate a shape factor (SF) (193, 196). The SF describes the geometric roundness of the halo outline, and a perfect circle has a SF of 1 and more irregular outlines a SF < 1.

**Scanning electron microscopy (II)**
For scanning electron microscopy (SEM), the cells were initially treated as described for PCM except that the cells were allowed to adhere to a thin glass slide. The cells were fixated with, and stored in, 2.5% glutaraldehyde in PBS. The cells were prepared for SEM by ethanol dehydration, coated with gold and examined in a Cambridge Stereoscan 360 IXP scanning electron microscopy (LEO Electron Optics, UK) at a standard magnification of 6 000 times.

A Leica Quantimet QWin image analysis software (Leica Microsystems Imaging Solutions Ltd) was used for image analysis. The SF again corresponded to the geometric roundness of the cell outline and a perfect circle had a SF of 1, but in contrast to the SF in the PCM image analysis, more irregular outlines had SF > 1. Blinded semi-quantitative estimations (value 1-5) of small blebs, large blebs, and membrane sprouts was performed by three persons. Cellular flatness was evaluated in a similar fashion.

**Flow cytometry (II)**
Flow cytometry was tested as method to study changes in the morphology of adherent cells. It is widely used for cells growing in suspension, but the process of removing adherent cells from their substrate causes profound
changes in cell shape and membrane morphology, reducing the reliability of the method for such cells. A FACSCalibur™ flow cytometer (BD Biosciences, San Jose, CA) was used for analysis of forward scatter (FSC) on a linear scale and side scatter (SSC) on a logarithmic scale.

3.3 Cytotoxicity (I, II, III, VI)

3.3.1 Lactate dehydrogenase (LDH) release (I)
The effects of K⁺ modulators on membrane integrity were determined with a colorimetric cytotoxicity assay measuring the release of LDH (CytoTox 96, Promega Corporation). The assay was performed according to the manufacturers’ instructions with cells plated on 96-well plates (Table 4). Untreated cells were used to set the background release, and untreated, lysed cells were used to determine maximal LDH release. The amount of colour formed was measured in a microplate reader (Molecular Devices). The results were corrected for background release and presented as % of maximal LDH release.

3.3.2 Cellular hydrolysis of fluorescein diacetate (II, III, VI)
Drug cytotoxicity and 50%-cytotoxic concentrations (CD₅₀) were determined with a semi-automated fluorometric microculture cytotoxicity assay (FMCA) with fluorescein diacetate (197). Cellular hydrolysis of the non-fluorescent substrate to a fluorescent product is linearly correlated to cellular density (197). The assay was performed with cells plated on 96-well plates (Table 4). Fluorescence was measured with a FluoroScan II fluorometer (Labsystems) (II), or a LS55 Luminescence spectrometer (PerkinElmer) (III, VI). Changes in cellular density are expressed as % of control i.e. untreated cells cultured on the same 96-well plate. The method does not discern between cell death and growth inhibition as causes of reduced cellular density.
3.4 Biochemical diagnosis of apoptosis (I-VI)

3.4.1 Phosphatidylserine externalisation (II)
Phosphatidylserine externalisation was determined with annexin V staining (TACS™ Annexin V-FITC Apoptosis detection kit, R&D Systems). The staining was performed according to the manufacturer’s instructions and analysed in a FACScalibur™ flow cytometer (BD Biosciences). Cells (Table 4) were exposed, then harvested with cold 0.2% EDTA and stained with annexin-V FITC. Propidium iodide (PI) staining was used to discern cells with membrane damage from cells with intact membrane. The FL1 channel was used for FITC detection and the FL2 channel for detection of PI fluorescence.

3.4.2 Caspase cleavage (V)
Caspase cleavage was determined with Western blotting, see separate section below.

3.4.3 Caspase activity (I-III,V)
To determine activity of apoptotic caspases, fluorometric caspase activity assays (R&D Systems, Minneapolis, MN, USA) were used according to manufacturers’ instructions. After appropriate exposures, cells (Table 4) were lysed with kit-included lysis buffer. The total protein content of the cell lysates was determined with a bichinconinic acid protein assay reagent kit (Pierce Biotechnology, Rockford, IL, USA). Cleavage of 7-amino-4-trifluoromethyl coumarin (AFC) was used to determine caspase activity: synthetic substrates were DEVD-AFC for caspase-3/7, IETD-AFC for caspase-8 and LEHD-AFC for caspase-9. Fluorescence was measured with a LS55 Luminescence spectrometer (PerkinElmer), and the results were presented as fluorescence per 100 µg protein.
Caspase activity was also determined with Western blotting to detect cleavage of endogenous substrates, see separate section below.

3.4.4 Oligonucleosomal DNA fragmentation (I-VI)
TUNEL staining (In Situ Cell Death Detection Kit, Roche) was used for detection of oligonucleosomal DNA fragmentation. After appropriate exposures, cells (Table 4) were collected, fixed and stained according to the manufacturer's instructions. The staining was analysed in a FACSCalibur™ flow cytometer (BD Biosciences) using the FL3 channel for detection of TMR red.

3.5 K⁺ transport (I,III)
3.5.1 Chemical modulators of K⁺ transport (I,III)
Ouabain, a specific inhibitor of the Na⁺K⁺ATPase pump (198), and bumetanide, a specific inhibitor of Na⁺K⁺2Cl⁻-cotransport (138) were used. Amphotericin B is a polyene anti-fungal antibiotic that induces cell membrane pores through which K⁺ can leak, and can potentiate cisplatin cytotoxicity (199). Nigericin is another ionophore that stimulates K⁺ efflux.

3.5.2 Changes in intracellular K⁺ (I)
The 96-well application for measurement of the cell permeable fluorescent probe benzofuran isophtalate acetomethyl ester (PBFI-AM) was developed by Britta Andersson in my lab, and paper I is the first publication describing this application. In brief, cells plated in 96-well plates (Table 4) were exposed to low-serum (1%) medium for 45 min, then loaded with PBFI-AM for 100 min. Excess PBFI-AM was removed by washing with low-serum medium and test substances were added. Exposures were terminated by removing the medium and washing with physiological NaCl solution. Fluorescence was measured with a LS55 Luminescence spectrometer.
(PerkinElmer), and expressed as % of control i.e. untreated cells cultured on the same 96-well plate.

3.5.3 $^{86}$Rb$^+$ influx and efflux (III)

The K$^+$ analog $^{86}$Rb$^+$ was used to study K$^+$ influx and efflux across the cell membrane. In brief, cell and supernatant $^{86}$Rb$^+$ content was determined with Optiphase SuperMix Liquid Scintillation Cocktail (Perkin-Elmer Wallac) and a MikroBeta counter (PerkinElmer Wallac). For determination of influx, cells (Table 4) were exposed to 0.75 µCi/mL $^{86}$Rb$^+$ and drugs simultaneously. For efflux determinations, cells were pre-loaded with 0.75 µCi/mL $^{86}$Rb$^+$ for 24 h, and then exposed to the drugs. $^{86}$Rb$^+$ content was expressed as counts per minute (cpm), $^{86}$Rb$^+$ influx was expressed as % of control and $^{86}$Rb$^+$ efflux was expressed as % of total content (cellular + supernatant content of $^{86}$Rb$^+$).

3.6 Proteins (III-VI)

Cells (Table 4) were lysed with lysis buffer [50 mmol/L Tris 7.5, 1% Triton X-100, 150 mmol/L NaCl, 1 mmol/L NaF, 1 complete protease inhibitor EDTA-free cocktail tablet to 50 mL buffer, Roche] for 30 min at +4 °C. The supernatants were aliquoted and stored at -80 °C. In some cases, lysates prepared for Caspase activity assays (kit-included lysis buffer) or for Proteome Profiler™ assays (kit-included lysis buffer) were used for Western blotting. The total protein content of lysates was determined with a bichinconinic acid protein assay reagent kit (Pierce Biotechnology).

3.6.1 Unmodified, phosphorylated or cleaved proteins (III-V)

Cell lysates and a pre-stained molecular weight marker (Bio-Rad Laboratories, Hercules, CA, USA) were separated with SDS-PAGE on Tris-
HCl gradient gels (Bio-Rad Laboratories) and transferred to PVDF-membranes (Bio-Rad Laboratories). The standard procedure was to block the membranes 60 min at room temperature with 5% non-fat milk in Tris-buffered saline (TBS), primary antibodies were applied over night at +4 °C, and horseradish peroxidase-conjugated secondary antibodies were applied for 60 min at room temperature. Protein bands were detected with ECL Advance Western Blotting Detection Kit (GE Healthcare, UK) visualized and quantified with the Chemidoc XRS system and Quantity One 1-D Analysis software (Bio-Rad Laboratories) and on X-ray film (GE Healthcare). All membranes were stripped with 1 M glycine, pH 2.0 or with Restore™ Western blot stripping buffer (Pierce Biotechnologies) and reprobed with an actin antibody (Millipore) to use actin content as protein loading controls. The antibodies used in the different papers are summarised in Table 5.

3.6.2 Phosphorylated threonine-serine kinases (VI)

A Proteome Profiler™ Human phospho-MAPK array was used to determine the relative levels of phosphorylation of mitogen-activated protein kinases (MAPKs) and some other serine/theronine kinases (R&D Systems).

The assay was performed according to the manufacturer’s instructions after cell lysates with 300 µg protein were applied to each membrane. Protein spots were detected with ECL Advance Western Blotting Detection Kit (GE Healthcare, UK), visualized and quantified with the Chemidoc XRS system and Quantity One 1-D Analysis software (Bio-Rad Laboratories) and on X-ray film (GE Healthcare). The spot volumes were corrected for background using the array-included negative controls, and then expressed as % of array-included positive controls.
<table>
<thead>
<tr>
<th>Protein detected</th>
<th>Paper</th>
<th>Type</th>
<th>Company</th>
<th>Isoforms/cleaved products</th>
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</thead>
<tbody>
<tr>
<td>Actin</td>
<td>III-VI</td>
<td>Mouse, mAb</td>
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<td>IV</td>
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<td>α, β</td>
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</table>

*CST = Cell Signaling Technology*
3.7  Cell cycle analysis (VI)
The distribution of cells in the different phases of the cell cycle was
determined according to Vindelov et al. (200). Cells (Table 4) were
exposed, and after the supernatant and any floating cells was discarded, the
remaining cells were washed with cold PBS and released with 0.2% EDTA.
After centrifugation, the cell pellet was resuspended in PBS with 2% foetal
calf serum. Cellular DNA was stained with PI (200) and analyzed using the
FACScan flow cytometer (Becton Dickinson), the Cellfit software program,
and the RFIT evaluation model from Becton Dickinson.

3.8  Statistical analysis (I-VI)
All statistical analyses were performed with the SPSS 12.0.1 and SPSS 16.0
statistical software for Windows. In paper I, the effect of treatment was
determined with one-way analysis of variance. In papers II-VI, the
following tests were used. If normal distribution could be assumed paired t-
tests or independent t-tests were used. If normal distribution could not be
assumed, the Wilcoxon’s Signed Ranks tests or the Mann-Whitney U-tests
were used. The level of significance for rejecting the null hypothesis of zero
effect was taken to be $p \leq 0.05$.

For the semi-quantitative scoring of SEM images (II), agreement
between the evaluators was determined with Cohen’s kappa determination,
and the effect of cisplatin concentration was determined with the Mann
Whitney U-test. The level of significance for rejecting the null hypothesis of
zero effect was taken to be $p \leq 0.100$. 
4 Results and Discussion

The overall objective of this thesis was to determine the effect of in vitro acquired cisplatin-resistance on apoptotic regulatory mechanisms in malignant pleural mesothelioma cells. This objective can be subdivided into three parts: 1) the role of cell membrane K⁺ transporters in apoptosis and cisplatin-resistance (I-III), 2) the effect of acquired cisplatin-resistance on apoptotic signalling pathways (III-V), and 3) the role of PI3K/PKB signalling in acquired cisplatin-resistance (VI).

4.1 Equitoxic concentrations of cisplatin

In order to compare the effects of cisplatin exposure on the two cell lines, it was necessary to determine the equitoxic cisplatin concentrations.

The cisplatin cytotoxicity was monitored with FMCA performed 72 h after the end of a 2-h exposure to cisplatin. Quadratic curves were fitted to the FMCA data and the final 50%-cytotoxic concentrations (CD₅₀) were calculated to 15 mg/L for P31 cells and 45 mg/L for P31res1.2 cells (III). Because FMCA does not differentiate between cell death and cell cycle arrest as the cause to a reduced no of viable cells compared to control, concentration-response experiments for determining the extent of cisplatin-induced apoptosis by detecting DNA fragmentation with TUNEL staining were performed. These showed that the 50%-apoptosis-inducing concentrations were higher than the CD₅₀ concentrations. Both FMCA and TUNEL concentration-response curves were then determined for different times of cisplatin exposure. These showed that equitoxic concentrations of cisplatin, i.e. concentrations that cause a similar number of cells to die through apoptosis in both cell lines, were about 10 and 40 mg/L cisplatin for a 6-h exposure.
In earlier studies, the cisplatin CD$_{50}$ for P31 cells had been estimated to 10 mg/L (193), and this concentration resulted in cell membrane blebbing, which was absent when 5 mg/L cisplatin was used (II). Furthermore, 5 or 10 mg/L cisplatin was used in previous studies of the effect of K$^+$ modulations on cisplatin cytotoxicity to P31 cells (128, 201). Therefore, I continued to use 10 mg/L cisplatin in the experiments with the P31 cells. Thus, 40 mg/L cisplatin were used for the P31res1.2 cells.

These concentrations of cisplatin are higher than the 50%-inhibitory concentration (IC$_{50}$) reported in most other studies with malignant mesothelioma cell lines (184, 202-204). However, in all these studies, the time of cisplatin exposure was $\geq 24$ h. When the IC$_{50}$ was determined 24 h after a 1-h cisplatin exposure, it ranged from 1.3 to 36.6 mg/L for five different human malignant pleural mesothliomas (205). Thus, when I determined cisplatin CD$_{50}$ for longer exposures, it was within the same magnitude as for previously reported cell lines (not shown).

4.2 The role of cell membrane K$^+$ transporters in apoptosis and cisplatin-resistance (I-III)

The cisplatin-resistant P31res1.2 cells were initially established to investigate the role of K$^+$ fluxes in cisplatin-resistance – in particular related to the manipulation of K$^+$ fluxes to induce or enhance apoptosis. At least 90% of the K$^+$ transport in the P31 cells is carried out by Na$^+$K$^+$ATPase activity and the secretory Na$^+$K$^+$2Cl$^-$-cotransporter (NKCC1) [III] (206, 207), and the effect of K$^+$ transport manipulation on P31 cell survival has been thoroughly investigated [I] (127, 128, 201, 206-209).

4.2.1 Na$^+$K$^+$ATPase and Na$^+$K$^+$2Cl$^-$-cotransporter activities are necessary for P31 cell survival (I)

All of the earlier work on characterising membrane K$^+$ transport in P31 cells has been done using $^{86}$Rb$^+$ as an analogue for K$^+$ (127, 128, 201, 206-209). This method can be used to characterise K$^+$ influx and efflux, but does not
yield information on the intracellular K⁺ concentration. Intracellular K⁺ concentrations can be estimated with the aid of the cell membrane permeable fluorescent probe benzoquinone isophtalate acetomethyl ester (PBFI-AM) (129, 210-212). Previous studies have used PBFI-AM on adherent cells (210, 211) and cells in suspension (129, 212). To increase efficiency, a 96-well PBFI-AM application was developed and tested.

The PBFI-AM fluorescence deteriorates with time, but in the 96-well application the fluorescence remained stable for 3.5 h after a 100-min loading period. It proved difficult to establish a calibration curve to calculate intracellular K⁺ concentration; instead the results were compared to untreated control.

To test the 96-well PBFI-AM application, substances known to affect K⁺ fluxes in the P31 cells were used (127, 128, 201, 206-209). A 3-h exposure to the selected substances was not cytotoxic to the P31 cells as determined with an LDH-release assay. Bumetanide and ouabain were used in combination to inhibit NKCC1 and Na⁺K⁺ATPase activity, respectively. Previous studies had shown that this resulted in almost complete inhibition of K⁺ influx, at least during the first hour of exposure (207). However, there was no change in intracellular K⁺ concentration during the 3-h exposure. Despite this, caspase-3 activity was increased 24 h after end of exposure, and at 48 h after end of exposure about 20% of the cells had died through apoptosis. In contrast, the K⁺ efflux stimulator amphotericin B reduced intracellular K⁺ concentration to about 50% of control after 2 and 3 h of exposure, but did not induce caspase-3 activity or DNA fragmentation. This suggested that amphotericin B-induced K⁺ depletion could be efficiently counteracted by activation of K⁺ influx mechanisms i.e. regulatory volume increase. When amphotericin B was added to bumetanide and ouabain, the K⁺ depletion was enhanced, as was the caspase-3 activity and DNA fragmentation. This supported the suggestion that functional regulatory volume increase was sufficient to rescue cells from K⁺ depletion-induced apoptosis. On the other hand, the K⁺ efflux stimulator nigericin alone did not decrease the intracellular K⁺ concentration, increase caspase-3 activity
or increase DNA fragmentation. However, when nigericin was added to bumetanide and ouabain, the reduction in intracellular K$^+$ concentration over time was enhanced after 3 h, as was the caspase-3 activity and DNA fragmentation, resulting in the highest levels observed in this study.

The results were similar for the U1690 cell line, therefore I concluded that NKCC1 and Na$^+$K$^+$ATPase activity was essential for P31 and U1690 cell survival. Whether this is directly associated to the regulation of intracellular K$^+$ concentrations or not remains to be determined, since a 3-h inhibition of NKCC1 and Na$^+$K$^+$ATPase activity did not reduce the intracellular K$^+$ concentration, but was followed by caspase activation and DNA fragmentation. A concurrent stimulation of K$^+$ efflux enhanced the apoptosis, and decreased the intracellular K$^+$ concentration.

4.2.2 Early-phase cisplatin-induced membrane blebbing is dissociated from apoptosis and depends on a functional Na$^+$K$^+$2Cl$^-$-cotransporter (II, III)

Inhibition of K$^+$ influx can induce apoptosis per se, and enhance cisplatin-induced apoptosis in the P31 cells (128, 201, 209). NKCC1 activity is transient – after about 60 min in fresh medium the P31 cells have almost no bumetanide-sensitive NKCC1 activity, although the ouabain-sensitive Na$^+$K$^+$ATPase activity remains [II] (207). When activated, the NKCC1 carries out about 20-30% of the K$^+$ transport in the P31 cells [II] (207). In contrast to this, the P31res1.2 cells had almost no bumetanide-sensitive NKCC1 activity during the first 60 min in fresh medium [II]. However, there was ample evidence that NKCC1 activity occurred in the P31res1.2 cells. In the $^{86}$Rb$^+$ assays, direct evidence for active NKCC1 was found after bumetanide exposure: bumetanide 1) enhanced the effect of ouabain on K$^+$ influx, 2) transiently inhibited K$^+$ efflux during the first min of exposure and 3) inhibited K$^+$ efflux when combined with cisplatin or ouabain. Furthermore, bumetanide exposure inhibited P31res1.2 proliferation. Inhibition of NKCC1 impairs cell proliferation (213), and over-expression of NKCC1 causes cell proliferation (214). The P31res1.2 cells have a reduced
proliferation rate compared to the P31 cells (unpublished data). Thus, the P31res1.2 cells appeared to express functional NKCC1 that for some reason had abrogated activation. Abrogated NKCC1 activity was also suggested by the decreased phosphorylation of ERK1/2 in the P31res1.2 cells. Both P31 and P31res1.2 cells had similar contents of the glycosylated NKCC1 protein. Glycosylation of NKCC1 is necessary for membrane targeting, thus there was no evidence of decreased NKCC1 expression or aberrant localisation of the protein in the P31res1.2 cell. This suggested that the P31res1.2 cells had impaired activation of NKCC1.

A number of factors can stimulate NKCC1 activity, and both phosphorylation and interaction with other proteins appear to be important. Factors that can stimulate NKCC1 activity include cell shrinkage, growth factors including serum, reduced intracellular Cl⁻ concentration and, in some cases, cell swelling (215). Furthermore, NKCC1 is activated in the early G1 phase of the cell cycle (213).

The early morphological changes observed in P31 cells during cisplatin exposure are membrane blebbing and reduced projected cell area (PCA) (193, 194) [I,II]. This is reminiscent of apoptotic morphology (35). Apoptotic membrane blebbing of adherent cells can be divided into two phases. One early-phase at the onset of the apoptotic release phase, and a late-phase concurrent with phosphatidylserine (PS) externalisation and caspase activation (37). In particular, caspase-3 activity is important for the late-phase apoptotic membrane blebbing (43, 216, 217). The cisplatin-induced membrane blebbing found in the P31 cells was independent of caspase-3 activation [I,II]. The lack of membrane blebbing in the P31res1.2 cells [II] that had increased basal caspase-3 activity [II,III] emphasised this observation. There were also no changes in the caspase-8 or -9 activities in either cell line after 30 min of exposure [III].

Early-phase membrane blebbing appears both morphologically and biochemically similar to stress-induced membrane blebbing (38-40, 218, 219). In both cases the membrane blebs protrude and retract rapidly, as observed in the P31 cells, and they both seem to involve actin, p38 and
ERK1/2 [11-14]. However, there was no evidence that p38 or ERK1/2 was involved in the membrane blebbing of the P31 cells.

4.2.3 Inhibition of Na\(^+\)K\(^+\)2Cl\(^-\)-cotransporter activity does not confer cisplatin-resistance [III]

The observation that abrogation of NKCC1 activity inhibited cisplatin-induced cell membrane blebbing [III], combined with the importance of functional Na\(^+\)K\(^+\)ATPase and NKCC1 for prevention of apoptosis [I] (112) led to the hypothesis that abrogated NKCC1 activity conferred cisplatin-resistance to the P31 cells. This was suggested by the results in a previous study, where P31 cells exposed to both bumetanide and cisplatin had better survival than cells exposed to cisplatin alone, as determined with a clonogenic assay and with FMCA (128). Despite this, there were no changes in the induction of apoptosis, as determined with measurements of free nucleosomes and caspase-3 activity (128). When I examined the hypothesis specifically [III] the previous results were partially confirmed. The previous results pertaining to the lack of bumetanide effect on apoptosis induction (128) were confirmed; analysis of DNA fragmentation showed that although bumetanide exposure per se decreased the basal cell death by apoptosis in P31 cell culture, but it did not affect the extent of cisplatin-induced apoptosis [III]. Furthermore, bumetanide per se did not affect caspase-3 or -9 activities, and when bumetanide was added to cisplatin, the caspase-3 and -9 activities were similar to those induced by cisplatin alone (unpublished data). However, the previous results that bumetanide exposure augmented cisplatin cytotoxicity were not confirmed. Instead, the addition of bumetanide to P31 cells during exposure to cisplatin decreased the number of viable cells compared to cisplatin alone, as determined with FMCA.
4.3 The effect of acquired cisplatin-resistance on apoptotic signalling pathways (IV-V)

As outlined in the introduction, many of the suggested mechanisms of cisplatin-resistance involve deregulation of apoptotic signalling pathways. From previous studies it was known that a 6-h cisplatin-exposure induces apoptosis in the P31 cells (128). Cisplatin exposure caused oligonucleosomal DNA fragmentation, detected as DNA laddering on agarose gel, and caspase-3 activation. However, it was not determined which apoptotic signalling pathway was activated by cisplatin in the P31 cells, and nothing was known about the cisplatin-induced cell death of the P31res1.2 cells.

Cell morphology after cisplatin exposures suggested that most of the cisplatin-induced cell death was executed through apoptosis (III). Equitoxic concentrations of cisplatin caused both P31 and P31res1.2 cells to retract, round up and release from the substrate (III). The rounding up and release was concurrent with extensive membrane blebbing, often resulting in separation of the cells into several apoptotic bodies (Figure 4). When the cells were exposed to cisplatin for 72 h there were large numbers of floating cells or apoptotic bodies in the cell cultures. This suggested that there was little cell lysis, or necrosis, consequent to equitoxic cisplatin exposures.

4.3.1 Cisplatin activates the mitochondrial signalling pathway in P31 and P31res1.2 cells

As described above, cisplatin exposure induced DNA fragmentation in both cell lines in a concentration- and time-dependent manner (III-V). In contrast to the TUNEL assays, which were performed at 72 h after end of cisplatin exposure, the effect of cisplatin on caspase activation was determined immediately after the end of cisplatin exposure. In both cell lines, a 24-h exposure to cisplatin resulted in increased caspase-3 activity (III,V). A 24-h exposure to cisplatin also increased the caspase-8 and -9 activities in both cell lines (V). However, the caspase-8 activity was rather
low, and did not result in cleavage of Bid (V). I therefore concluded that the main effect of cisplatin exposure was activation of the mitochondrial signalling pathway.

4.3.2 Pro-survival Bcl-2-family proteins after acquisition of cisplatin-resistance (IV)

The regulation of mitochondrial membrane permeabilisation and subsequent release of pro-apoptotic factors is dependent on a balance between pro-survival and pro-apoptotic proteins of the Bcl-2 protein family (Figure 6). Much effort has been put into understanding the role of the pro-survival Bcl-2 family proteins in apoptosis-resistance of malignant mesotheliomas (176-179, 184, 204, 220, 221) and other solid tumours (222). Elevated levels of Bcl-XL is found in most malignant mesothelioma cell lines and tumour tissues investigated (176, 177), and down-regulation of Bcl-XL expression increases both apoptosis per se (178, 179) and cisplatin-sensitivity (204, 221). Elevated levels of Bcl-2 are less common (176, 177). However, simultaneous down-regulation of Bcl-XL and Bcl-2 sensitised malignant mesothelioma cells to cisplatin more effectively than down-regulation of Bcl-XL alone, although the Bcl-2 levels prior to down-regulation were very low (220).

In the P31 cells the predominant pro-survival protein was Bcl-XL, and there were very low levels of Bcl-2. After acquisition of cisplatin-resistance, there was no change in the Bcl-XL content, but the Bcl-2 levels increased. There was also increased phosphorylation of Bcl-2 on serine 70 (ser70) in the P31res1.2 cells. Phosphorylation of Bcl-2 at ser70 is necessary for the pro-survival function of Bcl-2 (99-101), and enhances Bcl-2 inhibition of the G1/S transition (99). However, the P31res1.2 cells appeared to be delayed in the G2 phase rather than G1 or S (VI). Phosphorylation of Bcl-2 also occurs during the G2/M transition (223) and appears to arrest the cells in the G2 phase (224), consistent with the G2 delay in the P31res1.2 cells. Up-regulation of Bcl-2 as a consequence of
acquired cisplatin-resistance has also been found in bladder cancer (225), ovarian adenocarcinoma (222) and cervical carcinoma (222) cell lines.

Cisplatin transiently decreased the Bcl-X₅ levels of both cell lines during the first 0.5 h of exposure (IV), and after 24 h of cisplatin exposure the Bcl-X₅ levels were again reduced in both cell lines (not shown; Poster presentation Janson & Grankvist 2006 3rd Focused Meeting on PI3K signalling and disease, Bath, England). Cisplatin decreased the Bcl-2 levels of P₃₁res1.2 cells after 2 and 6 h of exposure, although the levels still remained higher than in the P₃₁ cells. There was also decreased phosphorylation of Bcl-2 after 6 h of cisplatin exposure.

These results show that acquisition of cisplatin-resistance can result in increased levels of Bcl-2, with consequent effects on cell cycle progression and possibly increased cisplatin-resistance. However, cisplatin exposure decreased both Bcl-X₅ and Bcl-2 levels; therefore it seemed unlikely that the increased cisplatin-resistance was due to changed responsiveness of the two proteins to cisplatin exposure.

4.3.3 Pro-apoptotic Bcl-2-family proteins after acquisition of cisplatin-resistance (IV)

In comparison with the pro-survival Bcl-2-family proteins, there are much fewer studies performed on the role of the pro-apoptotic Bcl-2-family proteins in malignant mesothelioma resistance to apoptosis (180-184) and in cisplatin-resistance (183, 184, 226, 227). There is one study on the expression of Bcl-2-family proteins in malignant mesotheliomas which showed varying degrees of loss of expression of Bad, Bid, Bim, Bax and Bak (177). Puma expression is important for cisplatin-induced apoptosis (183, 226, 227), as is Bax and Bak expression (226, 228-231). However, there are few studies on the effect of cisplatin exposure on the expression of pro-apoptotic Bcl-2-family proteins (227).

The pro-apoptotic Bcl-2-family proteins can be sub-divided into potent and weak pro-apoptotic proteins (Figure 6). The three potent pro-
apoptotic proteins, tBid, Puma, Bim, can bind to and inhibit all of the pro-survival proteins. Of these, truncated Bid was not detected in the P31 and P31res1.2 cells either during control conditions or after cisplatin exposure (IV,V). Of the weak pro-apoptotic proteins, Bad, Bmf, Bik and Hrk can bind to and inhibit Bcl-2 or Bcl-Xl (Figure 6). Hrk content was not determined in this study.

**The effect of acquired cisplatin-resistance on the potent pro-apoptotic proteins Puma and Bim**

The potent pro-apoptotic protein Puma is induced by p53 (85, 232) (Table 2), and cisplatin exposure can induce Puma expression (183, 227). There are two BH3-containing isoforms of Puma – Pumaα and Pumaβ (85). The P31 cells expressed both isoforms, but the levels varied over time. Pumaα decreased, and Pumaβ increased during the 6-h exposure. In the P31res1.2 cells, Pumaα levels were stable over time, and there were very low levels of Pumaβ. Cisplatin can induce Pumaα in rat kidney proximal tubular cells, but there was no effect on Pumaβ (227). This could due to the time-related response seen in the P31 cells – in the rat kidney proximal tubular cell study the effect of cisplatin was only investigated after 24 h of exposure. In the P31 cells, cisplatin transiently increased Pumaβ after 0.5 h of exposure, and increased Pumaα after 2-6 h of exposure. Interestingly, there was no increase in Pumaα or β due to cisplatin exposure in the P31res1.2 cells. In fact, it appeared as if Pumaα was reduced after 6 h of cisplatin exposure. That the Puma isoforms exerted their pro-apoptotic effect during the initial hours of cisplatin exposure was further suggested by the observation that in both cell lines, a 24-h cisplatin exposure resulted in almost no remaining Pumaα and β levels (not shown; Poster presentation Janson & Granqvist 2006 3rd Focused Meeting on PI3K signalling and disease, Bath, England).

There are several isoforms of the potent pro-apoptotic protein Bim. Originally, three isoforms were described – BimEL (∼23 kDa), BimL (∼15 kDa) and BimS (∼12 kDa) (233, 234). Of these, BimEL and BimL are bound to the dynein motor complex and inactive until apoptosis-inducing stimuli
cause them to be released (88) (Table 2). The BimEL levels were increased in the P31res1.2 cells, although the BimL levels remained the same as in the P31 cells. BimS is not bound to the dynein motor complex, and appears not to be expressed in healthy cells (88). Instead, transcription of BimS is increased after apoptotic stimuli such as cytokine withdrawal. There were low levels of BimS present in both cell lines under control conditions. The role of Bim in cisplatin-induced cell death is not yet clear, it is only described that down-regulation of BimEL does not protect neuroblastoma cells from cisplatin-induced apoptosis (235). In the P31 cells, cisplatin had no effect on BimEL, but transiently increased BimL after 0.5 h and BimS after 0.5 and 2 h of exposure. In contrast, cisplatin decreased BimEL, BimL and BimS in the P31res1.2 cells.

**The effect of acquired cisplatin-resistance on the weak pro-apoptotic proteins Bad, Bmf and Bik**

Bad is a weak pro-apoptotic protein that can bind to and inhibit both Bcl-XL and Bcl-2 (Figure 6). It is negatively regulated through phosphorylation by PKB and other proteins, and phosphorylated Bad is released from Bcl-XL or Bcl-2 and sequestered to the cytosol by 14-3-3 proteins, thus Bad’s pro-apoptotic effect is inhibited (81, 84, 93, 94) (Table 2). Inhibition of Bad phosphorylation sensitises ovarian cancer cells to cisplatin (236). The P31res1.2 cells had more Bad than the P31 cells under control conditions, and more phosphorylated Bad. Cisplatin exposure decreased Bad in the P31res1.2 cells and transiently increased Bad in the P31 cells, thus the average Bad content during cisplatin exposure was similar in both cell lines. However, the amount of Bad available to inhibit Bcl-2 or Bcl-XL during cisplatin exposure was lower in the P31res1.2 cells because of the increased phosphorylation of Bad in these cells.

Bmf is another weak pro-apoptotic protein that also can bind to and inhibit Bcl-XL and Bcl-2 (Figure 6). In viable cells Bmf is sequestered to filamentous actin, and this released from actin after certain stress stimuli, including release of cells from their substrate (237) (Table 2). Others have
reported that cisplatin did not change Bmf transcription or expression, and
knock-down of Bmf did not affect the extent of cisplatin-induced apoptosis
(238, 239). The P31res1.2 cells had at least three times more Bmf than the
P31 cells both under control conditions and after cisplatin exposure. In the
P31 cells there appeared to be a decrease in Bmf content after cisplatin
exposure. In the P31res1.2 cells, however, there was a transient increase
after 0.5 h of cisplatin exposure, followed by a gradual decrease. However,
these changes are only relevant if Bmf is released from actin and available
for interactions with the target pro-survival proteins.

Bik is the third weak pro-apoptotic protein that was investigated in
this study. It can bind to and inhibit Bcl-X\textsubscript{i}, but not Bcl-2 (48, 49). There
are no previous reports on the role of Bik in cisplatin-resistance, or the
effect of cisplatin on Bik expression or activity. There are also no reports on
the expression or activity of Bik in MPM. I did not find any differences in
Bik expression in P31 and P31res1.2 cells, and there was no effect of
cisplatin exposure.

4.3.4 Acquisition of cisplatin-resistance increased basal
caspase-3 activity and the cleavage of caspase-8
and -9 (IV)

As already discussed, the main apoptotic signalling pathway targeted by
cisplatin in P31 and P31res1.2 cells was the mitochondrial signalling
pathway. Decreased caspase-9 (240, 241) and caspase-3 (242, 243)
activities can be cisplatin-resistance mechanisms. The regulation of caspase
activities is complex and differs for the different caspases (Figure 5 and 7)
(44, 54-57, 62, 105, 106, 108, 244-246).

Caspase-3 activity

A surprising finding was that the basal caspase-3 activity was increased
about six-fold after acquisition of cisplatin-resistance [III,V]. This increase
was consistent at all time-points investigated, from 0.5 h to 24 h after
change to fresh, cisplatin-free medium. The caspase-3 activity was
determined by detection of cleavage of a synthetic DEVD-AFC substrate. Caspase-7 has similar substrate specificity in vitro, although in vivo substrate specificity differs (44). PARP cleavage is dependent on caspase-3 activity, and caspase-3 controls DNA fragmentation and the morphological changes of apoptosis (44). In the P31res1.2 cells there was cleavage of PARP under control conditions, consistent with the increased basal activity of caspase-3. However, it was not possible to detect any cleaved fragments of caspase-3 during control conditions, despite the use of two caspase-3 antibodies from different companies (not shown).

The P31res1.2 cells were viable and proliferated, showing that the caspase-3 activity levels were below the threshold level necessary to execute apoptosis. The majority of cells with caspase-3 activity that results in < 30% substrate cleavage do not progress to apoptosis (56). Consistent with this, PARP cleavage was < 30% of total PARP under control conditions in the P31res1.2 cells, but increased to > 30% after cisplatin exposure.

In both cell lines, cisplatin exposure increased caspase-3 activity and cleavage after 24 h. In the P31 cells, the increase in caspase-3 activity from 16.2 ± 2.4 to 23.9 ± 2.5 fluorescent units per 100 µg protein was significant (p = 0.037). In paper II the increase in caspase-3 activity from 11.2 ± 1.2 to 21.2 ± 1.4 protein was significant (p = 0.004), and in paper III the increase from 12.1 ± 1.8 to 36.5 ± 12.3 was almost significant (p = 0.055). Based on the fact that the increased caspase-3 activity did not result in increased PARP cleavage after 6 h, I concluded that the caspase-3 activation of P31 cells after 6 h of cisplatin exposure was still insufficient for execution of apoptosis. This differed from the P31res1.2 cells, in which a 6-h cisplatin exposure increased PARP cleavage to 39.1 ± 9.5% of total PARP and caspase-3 activity from 53.9 ± 7.7 to 114.7 ± 14.0 (p = 0.036). The increase in caspase-3 activity was also found in paper III; from 65.8 ± 7.8 to 137.5 ± 14.1 (p = 0.006).

Despite these differences in caspase-3 activation after 6 h of cisplatin exposure, the proportion of cells that exhibited DNA fragmentation at 72 h
after the end of a 6-h cisplatin exposure was similar – about 50% in both cell lines (also found in papers II, III, IV and V). This suggested that the caspase-3 activity of the P31 cells somehow “caught up” with the caspase-3 activity of the P31res1.2 cells. When the levels after 24 h were compared to the levels after 6 h, the final caspase-3 activities after 24 h of exposure were similar in both cell lines (p = 0.631), as was the extent of PARP cleavage. Thus it appeared as if the caspase-3 activity increased more rapidly in the P31 cells than in the P31res1.2 cells. In paper III, the P31 cells had a higher caspase-3 activity after 24 h of cisplatin exposure (p = 0.003). However, it could not be definitely concluded whether the P31 cells had a more rapid increase in caspase-3 activity or a higher final caspase-3 activity.

**Caspase-8 activity**

The basal caspase-8 activities were similar in both cell lines up to 6 h after change to fresh, cisplatin-free medium, when determined by detection of cleavage of a synthetic IETD-AFC substrate. After 24 h, the P31res1.2 cells had higher basal caspase-8 activity than the P31 cells (p = 0.025). Again, these results were not consistent with the Western blotting of caspase fragmentation. Although there was some cleavage of caspase-8 in the control P31 cells, the P31res1.2 cells had much more cleavage products. This might be a consequence of the increased caspase-3 activity, since caspase-8 is a substrate for caspase-3 (64). It appeared as if the full-length Bid content was lower in the P31res1.2 cells, but truncated Bid was never detected in any of the cell lysates, not even after 24 h of cisplatin exposure. In both cell lines, a significant increase in caspase-8 was concomitant with increased cleavage of the protein, and the caspase-8 activity after 24 h of cisplatin exposure was similar in both cell lines (p = 0.749). Because of the relatively low caspase-8 activity, and the lack of Bid cleavage, the caspase-8 activation and activity was not further investigated in this study.
Caspase-9 activity

Similar to the basal caspase-8 activity, the basal caspase-9 activities were similar up to 6 h, and higher in the P31res1.2 cells than in the P31 cells after 24 h in control medium (p = 0.004). Caspase-9 activity was determined by detection of cleavage of synthetic LEHD-AFX substrate. Just like for caspase-8, there was more cleavage products detected in the control P31res1.2 cells. There was no evidence of caspase-9 cleavage under control conditions in the P31 cells. In the P31res1.2 cells, the 37 kDa fragment was predominant, but there were also detectable amounts of the 35 kDa fragment. Caspase-9 activation occurs after cytochrome c activation of the apoptosome (54, 55, 247). The scaffold protein of this multimere is Apaf-1 (55, 248), and it appeared as if the P31res1.2 cells had increased content of Apaf-1. In addition, the whole-cell content of cytochrome c was much higher in the P31res1.2 cells. However, it must be noted that the release of cytochrome c to the cytosol remains to be determined.

Just like the cisplatin-induced activation of caspase-3 and -8, a 24-h cisplatin exposure resulted in significantly increased caspase-9 activity and cleavage in both cell lines. The caspase-9 activities after 24 h of cisplatin exposure were similar (p = 0.337). In parallel to the caspase-3 activation, a 6-h cisplatin exposure increased caspase-9 cleavage in the P31res1.2 cells. However, the increase in activity from 10.0 ± 1.5 to 13.6 ± 1.0 fluorescent units per 100 µg protein was not significant (p = 0.150). But, the P31res1.2 6-h cisplatin-induced caspase-9 activity was higher than in the P31 cells (p = 0.037).

Caspase-9 is a substrate for caspase-3 (249). Proteolytic cleavage of caspase-9 yields a 37 or 35 kDa fragment and a 12 kDa fragment. Caspase-3-mediated cleavage of caspase-9 yields a 10 kDa fragment instead of the 12 kDa fragment, but similar long fragments. The caspase-3-mediated cleavage results in higher caspase-9 activity than the proteolytic cleavage. Unfortunately the caspase-9 antibody used in this experiment did not detect the short 10 or 12 kDa fragment. It was notable that the whole-cell content of cytochrome c is increased in the P31 cells after 24 h of cisplatin
exposure. This observation, together with the previously discussed effects on Bcl-2 proteins (IV), suggested that cisplatin-induced activation of caspase-9 was mediated through cytochrome c release to the cytosol in the P31 cells. In contrast, the cisplatin-resistance of the pro-apoptotic proteins in the P31res1.2 cells (IV) and relatively high whole-cell content of cytochrome c that remained unaffected by cisplatin exposure suggested that the cisplatin-induced activation of caspase-9 occurred without mitochondrial membrane permeabilisation and cytochrome c release. This suggestion was supported by the concurrent caspase-3 activation and increased caspase-9 cleavage in P31res1.2 cells after 6 h of cisplatin exposure. However, these differences in caspase-9 activation between the cell lines remain mere hypotheses until cisplatin effects on mitochondrial membrane permeabilisation and cytochrome c release to the cytosol has been studied.

A potential role for the X-linked inhibitor of apoptosis protein
Increased expression or inhibited down-regulation of the X-linked inhibitor of apoptosis protein (XIAP) definitely has a role in cisplatin-resistance (250-256). XIAP binds to and inhibits caspase-3 and caspase-9 (Figure 5) (257, 258). Western blotting showed that the two cell lines had similar contents of full-length XIAP. However, there was cleavage of XIAP in both cell lines. The XIAP fragments were between 38 and 50 kDa in size, and in the P31res1.2 cells, a shorter fragment was the main product. The increased cleavage of XIAP could be a consequence of the increased basal caspase-3 activity, but then the fragments should be about 30 kDa (79). Furthermore, XIAP cleavage was reduced after 24 h of cisplatin exposure, when caspase-3 activity was highest in both cell lines. HtrA2/Omi protease activity can result in XIAP fragments of 30-45 kDa (259), i.e. similar to those found in this study. Although we did not determine if the HtrA2/Omi protease activity differed between the cell lines, the P31res1.2 cells had an increased
HtrA2/Omi expression and thus at least had the potential for increased activation of HtrA2/Omi.

4.4 The role of PI3K/PKB signalling in acquired cisplatin-resistance (VI)

Many studies have investigated the effect of inhibiting PI3K in order to inhibit PKB signalling, and to study the role of PKB signalling in cancer cell therapy resistance (186, 260-267). PKB activation is frequently found in cisplatin-resistant cell lines and tumour tissues, including malignant mesothlioma (9, 186, 231, 236, 250, 253, 268-273). Inhibition of PI3K can sensitise cells to cisplatin (186, 231, 268, 270, 273), although this is not always the case (271). Cisplatin-induced activation of the epidermal growth factor receptor (EGFR) results in PKB activation (272). However, the PKB isoforms are differentially regulated by epidermal growth factor (EGF) stimulation (143). It is suggested that specific PKB isoforms might be involved in cisplatin-resistance (253, 268), but further studies are necessary to determine if this is the case. Because PKB is such an attractive target for cancer therapy (153), much effort is now put into developing specific PKB inhibitors in order to increase the efficiency and decrease the toxicity of potential therapeutic drugs (153, 274).

4.4.1 PI3K-independent, constitutive activation of PKB in P31 and P31res1.2 cells

A time-resolved study of PKB phosphorylation showed that in both P31 and P31res1.2 cells PKB was constitutively activated. The P31res1.2 cells had increased phosphorylation of PKB compared to the P31 cells. Incubation of the cells in serum-free medium decreased phosphorylation of PKB, but it was never completely inhibited. In fact, the phosphorylation of PKB increased between 24 and 72 h of serum deprivation, although it never returned to the levels in control cells. The changed activity of PKB was supported by the effects on GSK3β.
Constitutive activation of PKB is described in other cisplatin-resistant cell lines (186, 275). Inhibition of PI3K with LY294002 enhanced chemotherapy-induced apoptosis in non-small-cell lung cancer cells, including cisplatin-induced apoptosis (275). The effect of LY294002 on cell survival and cisplatin-induced apoptosis in the two cell lines was therefore investigated. Initially, concentration-response curves showed that 10-100 µM LY294002 had very similar cytotoxicity in both cell lines. However, it proved very difficult to inhibit phosphorylation of PKB with LY294002. The IC50 of LY294002 is 1.4 µM (276) or 10 µM (277). In the P31 and the P31res1.2 cells, neither 12.5, 25 or 50 µM LY294002 were sufficient for extended inhibition of PKB phosphorylation. If 50 µM LY294002 was used, PKB phosphorylation was reduced to very low levels during up to 6 h of exposure. This concentration did not increase DNA fragmentation after 72 h of exposure. A 24-h exposure to 50 µM LY294002 caused cell cycle arrest at the G1/S transition of the cell cycle. This explained the cytotoxic effect after both 24 and 72 h. Phase-contrast images of the cell cultures supported that LY294002-exposure only caused cell cycle arrest and not cell death. LY294002-exposed cells did not grow to the same density as control cells. Additionally, there were no changes in cell morphology, and there were no changes in the proportion of floating cells. I therefore drew the preliminary conclusion that the constitutive PKB signalling in the P31 and P31res1.2 cells was PI3K-independent.

However, I was concerned that LY294002-inhibition of PI3K was ineffective, or that constitutive PI3K activation was the reason for the constitutive PKB activation. To test this, the cells were stimulated with EGF during the last ten min of a 6-h exposure. Not only is EGFR-mediated activation of PI3K well-described (278), it has also been shown that cisplatin can activate EGFR and thus PI3K-signaling (272, 279). EGFR-stimulated PI3K signalling differentially activated the PKB isoforms (143). There was no constitutive activation of EGFR in either cell line. EGF stimulation enhanced PKB phosphorylation in serum-starved P31 and P31res1.2 cells, but not in cells exposed to LY294002. This showed that
LY294002 efficiently inhibited PI3K activity. Cisplatin exposure did not activate EGFR, as shown in other cell lines (272, 279). Thus, the constitutive activation of PKB signalling in P31 and P31res1.2 cells was not the result of either constitutive activation of EGFR or cisplatin-induced activation of EGFR with subsequent increased PI3K/PKB signalling.

Protein kinase A (PKA) (280, 281), Ca\(^{2+}\)/calmodulin-dependent kinase II (282) and HSP27 (283-285) are reported to activate PKB independently of PI3K (98). I investigated the activation of HSP27 in the two cell lines. There was no increased HSP27 activity in the P31 cells to explain the PI3K-independent, constitutive activation of PKB. In the P31res1.2 cells, the HSP27 activity was increased. Cisplatin-resistance has been associated to p38-mediated increases in HSP27 activity (39, 286). However, only p38\(\delta\) was increased after acquisition of cisplatin-resistance. There were differences in the effect of cisplatin and LY294002 on p38 isoform activation, but they were not consistent with increased HSP27 activity. Thus, it appeared as if the increased HSP27 activity of the P31res1.2 cells was p38-independent. Furthermore, although some of the results suggested that increased HSP27 activity was associated with increased PKB activation, the results were not consistent. Therefore, it remains to be determined if HSP27 (283-285), or any of the other suggested proteins (280-282), have a role in the PI3K-independent, constitutive activation of PKB in P31 cells.

### 4.4.2 Different PKB isoform activity in P31 and P31res1.2 cells

Constitutive activation of PKB\(\beta\) confers cisplatin-resistance to ovarian cancer cells (231, 252, 287), and inhibition of PKB\(\beta\) rendered cisplatin-resistant cells susceptible to cisplatin-induced apoptosis (231, 287). Furthermore, PKB\(\beta\) and \(\gamma\) were implicated in cisplatin-resistance of uterine cancer cells (268). In contrast to these results, Gagnon et al (2008) recently showed that overexpression of constitutively active forms of each isoform does not confer cisplatin-resistance to uterine cancer cells (253). The P31
cells had significant activation of PKBα (p = 0.001) and PKBγ (p = 0.009) under control conditions. Due to a rather large variation in PKBβ activation, the average PKBβ activity of the P31 cells did not differ from the background levels during control conditions (p = 0.112). In the P31res1.2 cells, however, there was significant activation of all three PKB isoforms under control conditions (α p = 0.005; β p = 0.014; γ p = 0.020). In addition, the PKBα activation was about twice that in the P31 cells (p = 0.006) and the PKBγ activation was about three times that in the P31 cells (p = 0.042). Thus, acquisition of cisplatin-resistance increased the activation of all three PKB isoforms.

Cisplatin exposure can affect PKB in several ways; increased PKB activity (269, 272, 288, 289), decreased PKB activity (253), cleavage of PKB (250) or no effect (289) are responses that have been identified. In the P31 and the P31res1.2 cells, a 6-h cisplatin exposure had no effect on any of the PKB isoforms.

As discussed above, LY294002-inhibition of PI3K activity only transiently inhibited PKB activity in P31 and P31res1.2 cells. The effects on each separate isoform were also investigated. In both cell lines LY294002 exposure efficiently inhibited PKBβ activity, but did not affect PKBα activity. In the P31 cells LY294002 exposure actually increased PKBγ activity, although this was not seen in the P31res1.2 cells. Despite the general idea that PI3K activity activates all three PKB isoforms in a similar fashion, there is differential regulation of the isoforms (143, 144, 290, 291). EGF stimulation activated PKBα, β, γ differently in esophageal squamous cancer cell lines of the same origin (143). In adipocytes, insulin-stimulated PKBβ activity was twice the PKBα activity, and in hepatocytes insulin-stimulated PKBα activity was three-four times higher than the PKBβ activity, and there was no effect of insulin on PKBγ activity in either cell line (144). PKB signalling can be terminated through direct dephosphorylation by PP2A-type phosphatases (148), and by a recently discovered phosphatase, PH domain leucine-rich repeat protein phosphatase – PHLPP (290, 292). There are two isoforms of PHLPP, and
they regulate the amplitude of PKB signalling. PHLPP1 specifically modulates phosphorylation of HDM2 and GSK3α through inactivation of PKBβ. PHLPP2 specifically modulates the phosphorylation of p27 through PKBβ. The results of my study indicate that the PKBα and β isoforms are regulated in a similar manner in both P31 and P31res1.2 cells. The PKBα activation was PI3K-independent and the PKBβ activation was PI3K-dependent. Although the PKBγ activity was PI3K-independent in both cell lines, there was the unexpected observation that LY294002 exposure increased the PKBγ activity in the P31 cells but not in the P31res1.2 cells.

4.4.3 The increased PKB activity of P31res1.2 cells was not involved in the cisplatin-resistant phenotype

The lack of effect of cisplatin and LY294002 on PKB activity was disappointing. However, when cisplatin and LY294002 were combined, the PKB activities in the P31res1.2 cells became similar to the PKB activities found in the cisplatin-exposed P31 cells. Thus, if the increased PKB activity in P31res1.2 cells was important for the cisplatin-resistant phenotype, the addition of LY294002 to cisplatin should increase the cell death induced by cisplatin. This was not the case. Therefore I concluded that the increase in PKB activity that was subsequent to acquisition of cisplatin-resistance was not essential for the cisplatin-resistant phenotype.

It was interesting, however, that the combination of cisplatin and LY294002 reduced the PKBα activity, when neither of the drugs had a significant effect on their own. In fact, cisplatin exposure tended to increase PKBα activity, although it was not significantly increased compared to control cells (p = 0.052). Although LY294002 is considered a PI3K-specific inhibitor, it can inhibit other kinases with an IC₅₀ similar to that for PI3K (277, 293). It cannot be excluded that there was off-target effects of the high concentration of LY294002 used. However, the almost identical cytotoxicity of 10-100 µM LY294002 during up to 72 h of exposure in both cell lines suggests that any off-target effects at least are irrelevant for
There was also no difference in apoptosis induction by 25 or 50 µM LY294002 (unpublished data).

4.5 The whole picture

In this thesis, I have in six papers investigated the role of K⁺ transporters, apoptotic signalling pathways and PKB signalling in apoptosis of malignant pleural mesothelioma cells. To bring these results together, this section will provide a brief discussion of the whole picture.

All the studied proteins that were involved in, or could be affected by, K⁺ transport were deregulated after acquisition of cisplatin-resistance (Figure 11). However, I have not investigated the effect of K⁺ transport on all of these proteins. Therefore I only suggest that de-regulated K⁺ transport may have an important regulatory role for these proteins in as yet unidentified ways. For instance, some of the changes found in the P31res1.2 cells might be associated with the de-regulated K⁺ transport: increased Bcl-2 expression [IV], increased cleavage of caspase-9 without increased activity [V], increased cytochrome c expression [V], decreased ERK1/2 activity [III], increased PKB activity [VI]. However, increased PKB activity can result in inhibition of caspase-9 (157), decreased ERK1/2 activity (294), increased Bcl-2 expression (98) and increased Bcl-2 phosphorylation (98). Furthermore, there is accumulating data suggesting that Bcl-2 and Bcl-Xl can regulate Na⁺K⁺ATPase activity and the intracellular K⁺ and Na⁺ homeostasis (135). Thus, the interactions between these “signalling systems” are complex and await further investigation.

Acquisition of cisplatin-resistance also resulted in changes in the apoptotic proteins that were affected by PKB signalling but not K⁺ transport (Figure 11). The increased phosphorylation of GSK3α/β at serine 9 and Bad at serine 136 were direct effects of the increased PKB signalling [VI]. Some of the other changes might also be consequences of the increased PKB signalling in the P31res1.2 cells [VI], e.g. the decreased expression of Puma and Bim isoforms [IV].
It is noteworthy that almost all BH3-only proteins investigated in this thesis were affected by the acquisition of cisplatin-resistance. This suggested to me that the regulation of mitochondrial membrane permeabilisation was an important target for cisplatin, especially when I take into consideration the potential interactions between the Bcl-2 family of proteins and K⁺ transport and PKB signalling. Although NKCC1 abrogation was not a cisplatin-resistance mechanism, and increased PKB activity was not strongly associated with the acquired resistance to cisplatin, these two “signalling systems” appear to be important for the cisplatin-induced apoptosis in the P31 and P31res1.2 cells, in particular with reference to the Bcl-2 family of proteins.

**Figure 11.** Summary of the effects of cisplatin on proteins involved in, or affected by, the three “signalling systems” investigated in this thesis: apoptosis signalling, PKB signalling and K⁺ transport. Proteins investigated in this thesis are indicated by *, proteins that are framed were deregulated in P31res1.2 cells, proteins targeted by cisplatin are enhanced in **bold**, and proteins involved in cisplatin-resistance are enhanced with *italics*. The proteins are grouped roughly according to function, as indicated by the gray shading.
5 Conclusions

This thesis has increased the knowledge concerning cisplatin-resistance in human malignant mesothelioma cells by showing that the consequences of acquisition of cisplatin-resistance include changes in K⁺ transport, pro-apoptotic proteins, and pro-survival proteins. The identification of these changes depended on the time-resolution used in almost all studies. If only a single time-point, or two, had been investigated, most of the findings in this thesis had not been identified. One novel potential target for therapeutic intervention in malignant pleural mesothelioma was identified [IV], three novel cisplatin-resistance phenotypes were described [III,IV,V], and additional information on the role of PKB signalling in malignant mesothelioma cells and cisplatin-resistance was presented [VI].

The thesis also demonstrates that very early cisplatin-induced morphological changes of malignant mesothelioma cells are dissociated from the execution of apoptosis, despite the apoptosis-like appearance of the changes [II]. These cisplatin-induced changes, but not the execution of apoptosis, were dependent on NKCC1 activity.

This thesis has also increased the knowledge concerning the role of K⁺ transport in apoptosis of adherent lung cancer cells. Inhibition of both Na⁺K⁺ATPase and NKCC1 uptake induced apoptosis [I], but abrogation of NKCC1 activity did not induce apoptosis per se or enhance cisplatin-induced apoptosis [III].

The novel potential target for therapeutic intervention, and a novel cisplatin-resistance phenotype, was [IV]:

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Cisplatin-resistant pro-apoptotic BH3-only proteins; after acquisition of cisplatin resistance the cellular content of BH3-only proteins either remained unchanged or decreased during cisplatin exposure. These proteins are essential for regulating mitochondrial membrane permeabilisation in the mitochondrial signalling pathway of apoptosis.

Additional novel phenotypes of cisplatin-resistance were:

Acquisition of cisplatin-resistance resulted in abrogated NKCC1 activity [III].

Deregulation of caspases [V]; after acquisition of cisplatin-resistance malignant mesothelioma cells had higher basal caspase-3 activity, increased proteolytic processing of caspase-8 and -9, earlier cisplatin-induced activation of caspase-3 and earlier cisplatin-induced cleavage of caspase-9.

Additional information on PKB signalling in malignant mesothelioma cells and cisplatin-resistance included [VI]:

Both malignant mesothelioma cell lines had PI3K-independent, constitutively activated PKBα and γ activity. The activity of these isoforms was increased after acquisition of cisplatin-resistance. In addition, PKBβ was activated in a PI3K-dependent manner in the P31res1.2 cells.

Inhibition of the increased PKB activity after acquisition of cisplatin-resistance did not increase cisplatin-induced apoptosis.
6 Future prospects

In addition to the new knowledge already discussed, this thesis has generated numerous new questions. Some of these pertain to a more clinical perspective; others concern more basic cell and molecular biology perspectives.

The discovery of the apparently cisplatin-resistant BH3-only proteins in the P31res1.2 cells suggest that BH3-only proteins could be valid targets in combination therapy with cisplatin, or as a second-line treatment after failure of platinum-based therapy. In particular since the caspases were functional in these cells. In the past years, BH3 mimetics have gained much interest as single-agent or in combination therapy for different solid tumours, and there are drugs in phase II studies (295, 296). Studies on the effect of cisplatin on BH3-only protein activation, and expanded studies in other cell lines and tumour types will help to further understand the mechanisms involved in the deregulation of BH3-only proteins. By using the available BH3 mimetic drugs, the potential of targeting BH3-only proteins in cisplatin-resistant cells can be further evaluated.

The finding that the P31 and P31res1.2 cells have PI3K-independent constitutive activation of PKB emphasises the importance of selectively targeting PKB, and possibly PKB isoforms, rather than PI3K. There is intensive research ongoing regarding selective inhibitors of PKB (153, 274). Although PKB is an attractive target, the results of this thesis suggest that it might be difficult to achieve relevant inhibition of PKB in a clinical setting. In particular, a better understanding of the regulation and functions of the different isoforms is necessary if predictive results are to be achieved.

Finally, K+ transport is shown to be a potential therapeutic target. Functional K+ transport is necessary for cell survival, and can be involved in both apoptotic and PKB signalling. There are several drugs in clinical use
that modulate activity of K⁺ transporters. This thesis showed that NKCC1 activity was not important for cisplatin-resistance and we have unpublished data showing that inhibition of Na⁺K⁺ATPase sensitises P31res1.2 cells to cisplatin.

The thesis has also yielded several results that raised questions on a more basic cell and molecular biology level. One aspect is the activation of BH3-only proteins, already discussed above. Another is the mechanisms of PKB activation, particularly PI3K-independent activation. Finally, the thesis has raised questions on the mechanisms of activation of caspases, and the difference between caspase activation and caspase activity. The results in this study show that cisplatin-resistant cells can have increased basal caspase activity or cleavage of caspases. This has implications for the interpretation of results from staining of e.g. tumour tissues - samples with increased staining for e.g. caspase-3 or cleavage of caspase-8 or -9 could be falsely interpreted as increased apoptosis. This emphasises the importance of continued investigations on mechanisms of caspase activation, activity, and the inhibition of these two processes.


Lungcancer kan behandlas med cisplatin, liksom de mer ovanliga tumörer som bildas i lungsäcken s.k. malignt mesoteliom eller lungsäckstumör. Lungsäckstumörer är mycket elakartade och väldigt okänsliga för behandling. Patienter som fått diagnosen lungsäckstumör
överlever sällan längre än ett år efter att de fått diagnos, även om
behandling sätts in.

I denna avhandling har jag studerat vilken effekt cisplatin har på
celler från en lungsäckstumör. Jag har jämfört en mer känslig cellinje som
heter P31 med en mer okänslig cellinje som heter P31res1.2. Den okänsliga
cellinjen har vi skapat på laboratoriet genom att odla P31 celler tillsammans
med låga doser cisplatin. På så vis har vi kunnat isolera cisplatinokänsliga
celler och etablerat dem som en ny cell-linje. De två olika tumörcellinjerna
har behandlats med cisplatin och så har jag jämfört deras reaktioner på
cisplatinbehandlingen, i första hand har jag tittat på signaler förknippade
med celldödsformen apoptos. För att få en uppfattning om
signaleringsförloppet har jag avbrutit cisplatinbehandling efter olika
tidpunkter och sedan analyserat cellerna. På så sätt får jag en tidsupplöst
bild av cisplatinbehandlingens effekter. I och med detta tillvägagångssätt
har jag identifierat ett antal förändringar i de cisplatinokänsliga cellerna.
Några av dessa förändringar var inte förknippade med deras okänslighet för
cisplatin, medan andra skulle kunna ha stor roll.

För att celler skall överleva måste de kunna styra sitt eget innehåll av
kaliumjoner genom att aktivera sk kaliumjontransportörer. Jag har visat att
om man bara blockerar de två viktigaste kaliumjontransportörerna i P31
celler så kommer en relativt stor andel att dö genom apoptos. I de
cisplatinokänsliga P31res1.2 cellerna hade den ena kaliumjontransportören
mycket låg aktivitet, och jag trodde först att detta kunde vara ett skäl till att
cellerna var så okänsliga för cisplatin. Dock visade det sig att just denna
caliumjontransportör inte var viktig för cellernas överlevnad och dess
aktivitet påverkade inte cellernas känslighet för cisplatin.

För att tumörcellera skall dö genom apoptos så krävs det att vissa
signaler blir aktiverade. I mina studier visade det sig att flera av dessa
signalsystem var förändrade i de resistenta cellerna. Bland annat uttryckte
P31res1.2 cellerna s.k. apoptosstimulerande protein som knappt blev
påverkade alls av cisplatinbehandling, trots att de blev det i de känsliga P31
cellerna. Dessa apoptosstimulerande protein, s.k. BH3-proteiner, är viktiga
för att aktivera ett signalsystem som leder till att cellerna dör genom apoptos. Det är därför sannolikt att denna defekt gör P31res1.2 celler okänsliga för cisplatin, även om det återstår en del arbete för att bevisa det. Det som gör detta fynd extra intressant är att droger som härmar effekten av dessa BH3-proteiner är under utveckling, och det finns studier som har visat lovande resultat vid behandling av bl.a. lungcancer med dessa droger. I förlängningen så kan det alltså komma att visa sig att cisplatin med fördel kombineras med dylika BH3-protein droger, men det återstår mycket arbete innan några sådana slutsatser kan dras.

Inom samma signalsystem var även andra s.k. apoptosstimulerande protein förändrade i P31res1.2 cellerna. I detta fall var det proteiner som kallas för caspaser som var förändrade. Caspaser är viktiga för att apoptosen utförs, till skillnad från de s.k. BH3-proteiner som är viktiga för att signalsystemet aktiveras. I de mer okänsliga cellerna hade det viktigaste caspaset, caspas 3, en ökad aktivitet i obehandlade celler. Detta var ett ovanligt fynd. Okänslighet för cisplatin har snarast varit förknippad med minskad caspasaktivitet. Trots denna ökade caspas 3 aktivitet i obehandlade celler så var det ingen skillnad mellan P31 och P31res1.2 celler i högst uppmätta caspas 3 aktivitet, eller i caspas 8 och 9 aktivitet, efter cisplatinbehandling. Dock skedde aktivering av caspas 3 snabbare i P31res1.2 cellerna, den var mätbar redan efter 6 h, medan det dröjde till 24 h innan den var mätbar i P31 cellerna. Det kan vara så att det är P31res1.2 cellernas ökade caspas 3 aktivitet och tidigare aktivering som kompenserar för att deras BH3-proteiner inte fungerar som de ska, dock måste det visas med ytterligare experiment.

Slutligen kunde jag visa att ett viktigt överlevnadsprotein var överaktivt i de två cellinjerna. Överlevnadsproteinet kallas för PKB eller Akt (i denna avhandling har jag använt PKB), och det reglerar massor av processer inuti cellerna. Bland annat blockerar det en del av apoptos-signalsystemet. Det är väldigt vanligt att PKB är överaktiverat i tumörceller och det pågår mycket arbete för att utveckla droger som kan blockera PKB’s aktivitet. Ett nytt fynd i dessa cellinjer var att PKB aktiviteten var i stort sett
frikopplad från den normala aktiveringsprocessen. PI3K heter ett protein som normalt aktiverar PKB. Jag kunde blockera PI3K’s aktivitet, men ändå var PKB aktiverat i de båda cellinjerna. Dessutom kunde jag visa att P31res1.2 cellerna hade ytterligare ökad PKB aktivitet jämfört med P31 cellerna, men att den inte orsakade någon okänslighet för cisplatin i den signalering som aktiverades under de första 6 h av cisplatinbehandling.

Sammanfattningsvis har detta avhandlingsarbete resulterat i en ökad förståelse för hur cisplatin kan döda tumörcellerna, hur tumörcellerna reagerar på cellgiftet och vilka signalprotein som kan vara inblandade i att tumörceller blir eller är okänsliga för cisplatinbehandling. Dessa resultat kan komma till nytta när nya droger skall provas i kombination med cisplatin, och kan i förlängningen bidra till förbättrade behandlingsresultat.
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