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# THESIS AT A GLANCE

<table>
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</tr>
</thead>
</table>
| I     | Characterize K⁺ flux mechanisms in U1690 and P31 wt cells. | • Ouabain inhibits Na⁺, K⁺, ATPase pump activity, and K⁺ uptake.  
• Bumetanide inhibits Na⁺, K⁺, 2Cl⁻ cotransport, with transient effects on K⁺ uptake.  
• Amphotericin B and nigericin increased K⁺ efflux effectively. | The $^{86}$Rb⁺ method was functional when measuring K⁺ fluxes over the cell membrane. It was difficult to estimate changes in intracellular K⁺ content. Other methods are needed to determine changes in intracellular K⁺ content after K⁺ flux modulation. |
| II    | Manipulate K⁺ fluxes to reduce intracellular K⁺ content. Investigate whether K⁺ flux modulation induces apoptosis in P31 wt and U1690 cells. | • PBFI-AM can be used in 96-well plates to determine intracellular K⁺ content over 3 h.  
• Ouabain, bumetanide, and amphotericin B reduced intracellular K⁺ content in P31 wt and U1690 cells.  
• K⁺ flux modulation induced apoptosis in P31 wt cells. | Ouabain and bumetanide induced apoptosis in P31 wt and U1690 cells, but without changes in intracellular K⁺, but when combined with amphotericin B, intracellular K⁺ content was reduced and the effect on apoptosis was enhanced. |
| III   | Compare P31 wt cells with a cisplatin-resistant sub-line (P31 res) with respect to K⁺ content and apoptosis after K⁺ flux modulation. | • Ouabain induced apoptosis in both P31 wt and P31 res cells.  
• Ouabain-induced apoptosis in P31 wt cells via caspase-3 enzyme activity. Amphotericin B enhanced apoptosis by caspase-9 activation.  
• Ouabain-induced apoptosis in P31 res cells via caspase-3 enzyme activity and increased expression of pro-apoptotic Bak. | Ouabain-induced apoptosis in P31 wt and P31 res cells was executed via caspase-3 enzyme activity, but only P31 res cells exhibited increase in pro-apoptotic Bak expression. Apoptosis was enhanced in P31 wt cells by amphotericin B via caspase-9 activity. |
| IV    | Elucidate if K⁺ flux modulation can enhance cisplatin-induced apoptosis in P31 wt and P31 res cells. | • 10 mg/L cisplatin in P31 wt cells equals 40 mg/L cisplatin in P31 res cells (regarding DNA fragmentation)  
• Ouabain increased cisplatin-induced apoptosis in P31 wt cells.  
• Ouabain sensitized P31 res cells to cisplatin. | Ouabain enhanced cisplatin-induced apoptosis in P31 wt cells, and P31 res cells were sensitized to cisplatin by ouabain. Cisplatin-induced apoptosis was enhanced by ouabain and amphotericin B via active SAPK/JNK. |
AIMS

The aims of this thesis were to:

- Characterize K\(^+\) fluxes in a human malignant mesothelioma (P31 wt) and a small-cell lung cancer (U1690) cell line.

- Manipulate K\(^+\) fluxes in P31 wt and U1690 cells and measure changes in intracellular K\(^+\) content.

- Investigate whether manipulation of K\(^+\) fluxes induced apoptosis in U1690, P31 wt, and a cisplatin-resistant sub-line (P31 res) of the parental P31 wt cell line.

- Investigate if cisplatin-induced apoptosis in P31 wt and P31 res cells could be enhanced by K\(^+\) flux modulation.

- Investigate signalling pathways involved in ouabain- and cisplatin-induced apoptosis in P31 wt and P31 res cells.
LIST OF PAPERS


IV. Andersson B, Janson V, Moharer J, Behnam-Motlagh P, Grankvist K. The Na⁺, K⁺, ATPase inhibitor ouabain sensitizes resistant human malignant pleural mesothelioma cells to cisplatin by increased Bak expression. In manuscript.
ABBREVIATIONS

K⁺  Potassium ion
RVI  Regulatory volume increase
RVD  Regulatory volume decrease
Caspase  Cystein-aspartic-acid-proteases
Apaf-1  Apoptotic protease activating factor
TNF-α  Tumour necrosis factor-α
FasL  Fas ligand
TNFR  Tumour necrosis factor receptors
Fas  Fas receptors
FADD  Fas-associated death domain
DISC  Deach inducing signalling complex
MAPK  Mitogen activated protein kinase
MAPKK  Mitogen activated protein kinase kinase
SAPK/JNK  Stress activated protein kinase/jun n-terminal kinase
p-SAPK/JNK  Phosphorylated- Stress activated protein kinase/jun n-terminal kinase
PI3K  Phosphoinositide-3 kinase
NMDA  N-methyl-D-aspartate
MPM  Malignant pleural mesothelioma
MEM  Minimal essential medium
LDH  Lactate dehydrogenase
PBFI-AM  Potassium-binding benzofuran isophtalate acetomethylester
TUNEL  TdT-mediated dUTP nick end labeling
TdT  Terminal deoxynucleotidyl transferase
BCA  Bicinchorinic acid
TEA  Tetraethylammonium
INTRODUCTION

"The goal of all life is death..." as Sigmund Freud (1856-1939), a well known neurologist and the father of psychoanalysis once stated. This is true for the many cells that constitute our body. The balance between cell death and survival is crucial for tissue homeostasis and, when disrupted, proliferative or degenerative diseases may arise. Tumours arise when cell proliferation is uncontrolled, often due to lack of cell death. Apoptosis is a process in which intra- or extra-cellular signals trigger an inherent suicide mechanism. This can be a protection for the organism against cells transforming into cancer cells, but also during normal processes, as apoptosis is necessary to remove unwanted cells. Cancer cells often carry mutations or mechanisms that make them resistant to apoptosis and thereby non-responsive to treatment. Extensive research is therefore needed to increase the knowledge of the apoptotic process, what initiates it – what pathways are involved – and how to induce it in cancer cells.

Ion fluxes

In the normal situation, the cell volume is rather constant due to a balance in the movement of water and solutes across the plasma membrane. The potassium ion (K⁺) is the most abundant cation inside the cell, with a concentration of about 140-170 mmol/L intracellularly and 5 mmol/L extracellularly. This makes cytoplasmic ion homeostasis and cell volume sensitive to fluxes of K⁺. When placed in a hyperosmotic environment (more solutes outside the cell than on the inside) shrinkage occurs due to water efflux. To counteract the shrinkage, regulatory volume increase (RVI) mechanisms are induced. The cell volume is restored by activation of influx mechanisms such as the Na⁺, K⁺, 2Cl⁻-cotransporter (Fig. 1) and Cl⁻, HCO₃⁻ exchangers which increases the intracellular concentration of Na⁺ and Cl⁻ and enables water influx. The opposite effect is triggered by a hypotonic environment (less solutes outside the cell than on the inside) where the cell volume increases due to influx of water. Regulatory volume decrease (RVD) mechanisms leads to a net loss of K⁺ and Cl⁻ which forces water efflux and restores the cell volume (Gomez-Angelats and Cidlowski 2002).
Apoptosis

Apoptosis was in 1972 defined as “a general mechanism of controlled cell deletion which is complementary to mitosis in the regulation of animal cell populations” (Kerr et al. 1972). In other words; an in-built mechanism for the deletion of unwanted cells in order to maintain tissue homeostasis. Apoptotic cells were described already in 1885 by Walther Flemming (reviewed in (Lockshin and Zakeri 2001)), although the term apoptosis was not used until 1972 (Kerr et al. 1972). In a thesis defended by Richard A. Lockshin, in the year of 1963 (as described in (Lockshin and Zakeri 2001)), programmed cell death was described as a sequence of controlled steps towards their own destruction. Apoptosis is Greek and stands for “falling off” or “dropping off” (as leaves from trees), the term then refers to the morphological changes (i.e. apoptotic bodies) that is particular for this type of cell death. First, the cell shrinks due to efflux of $K^+$ and loss of cellular water. The chromatinn is condensed and the DNA is fragmented. Later on, membrane blebs are formed that ultimately separate and form apoptotic bodies. The cell membrane remains intact during formation of the blebs and therefore no leakage of cytoplasmic content occurs as in necrosis (cell death by rupture of the cell membrane). Apoptotic cell death is not stressful to surrounding cells because it does not give rise to an inflammatory response due to the engulfment and phagocytosis of the apoptotic bodies by macrophages.

Caspases

Considerable enzyme activity is necessary in order for apoptosis to proceed from the initial cell shrinkage to the final death. Cystein-aspartic-acid-proteases (caspases) with enzymatic activities are involved in apoptosis. Initiator caspases – caspase-2, -8, and -9 cleaves and activates the other type – executor caspases – caspase-3, and -7. All caspases must be cleaved in order to become activated (Riedl and Shi 2004).

Signalling pathways involved in apoptosis

Extensive research has been done on the molecular mechanisms of apoptotic cell death, including studies of the nematode *C. elegans* in 1986 by the Nobel Prize winners Brenner, Horvitz, and Sulston, but still there are unanswered questions. There are two major pathways that in the end lead to apoptotic cell death, the intrinsic pathway where a mitochondrial pathway is activated, and the extrinsic pathway where extra-cellular ligands bind to death receptors on the plasma membrane. Both pathways merge to similar activation of executor caspases, and to the same morphological characteristics.
The intrinsic pathway (Fig. 2) can be initiated by stress such as growth factor withdrawal, mitochondrial stress, or DNA damage. Upon inclusion of pro-apoptotic molecules (such as Bax and Bak) in the mitochondrial membrane, cytochrome c can be released from the mitochondria (Liu et al. 1996; Danial and Korsmeyer 2004). Cytochrome c together with apoptotic protease activating factor (Apaf-1) and caspase-9 forms the apoptosome (Li et al. 1997) which enables caspase-9 to self-process and activate the executor caspase-3.

The extrinsic pathway (Fig. 2) is initiated by binding of death ligands, such as tumour necrosis factor-α (TNF-α) or Fas ligand (FasL) to tumour necrosis factor receptors (TNFR) or Fas receptors (Fas) situated on the cell surface (Riedl and Shi 2004). The receptors are then aggregated and form complexes that recruit cytosolic factors such as Fas-associated death domain (FADD) and caspase-8 to form a death inducing signalling complex (DISC). When in the DISC complex, caspase-8 becomes active and cleaves and activates caspase-3.

**Fig. 2: Extrinsic and extrinsic pathways of apoptosis. Both pathways merge in caspase-3 activation.**
Caspase-8 can also cleave the pro-apoptotic molecule Bid (Luo et al. 1998) which causes release of cytochrome c from the mitochondria. Hence cross-talk between the two pathways can occur.

Mitogen activated protein kinases (MAPKs) is a family of protein kinases that are involved in regulation of many cellular events. They exert their effect by phosphorylation of target proteins and are activated themselves by MAPK kinases (MAPKKs) (Fig. 3). Stress activated protein kinase/jun n-terminal kinase (SAPK/JNK) are proposed to be activated by different stress stimuli (Ip and Davis 1998). The active form of SAPK/JNK (p-SAPK/JNK) can favour both cell survival and cell death. An inhibitor of SAPK/JNK has been implicated to be of importance in resistance against anticancer drugs (Wang et al. 2006). SAPK/JNK phosphorylates Bcl-2 (Srivastava et al. 1999), an anti-apoptotic molecule that is known to inhibit the release of cytochrome c (Kluck et al. 1997).

![Figure 3: SAPK/JNK can be activated by MAPKKs.](image)

**K⁺ involvement in apoptosis**

Several studies report the involvement of K⁺ in apoptosis. Most studied are neurons, which die of apoptosis if cultured in conditions with low (5 mmol/L) concentration of K⁺. They require around 25 mmol/L of extracellular K⁺ to survive (D'Mello et al. 1993). Neuronal apoptosis has reported to be induced by enhancement of K⁺ efflux (Yu et al. 1997) more specifically by activity of N-methyl-D-aspartate (NMDA) receptor-gated channels which are permeable to K⁺ (Yu et al. 1999).

Other cell types have also been studied. Thymocytes undergoing apoptosis exhibited reduced intracellular K⁺ (Dallaporta et al. 1998) with levels of 56 mmol/L K⁺ (Hughes et al. 1997) which is comparable with apoptotic fibroblasts that contained 50 mmol/L K⁺ (Barbiero et al. 1995). Caspase activity and DNA fragmentation was restricted to cells with low K⁺ level due to inhibitory effects of normal K⁺ concentrations on enzyme activity (Hughes et al. 1997).
and the formation of the apoptosome (Cain et al. 2001). If the cytoplasmic K\(^+\) concentration is restored, apoptosis is not executed and the cells will survive (Bortner et al. 1997). Suggested mechanisms behind low intracellular K\(^+\) during apoptosis have been proposed. Cytochrome c has been shown to activate K\(^+\) channels leading to depletion of intracellular K\(^+\) and cell shrinkage (Platoshyn et al. 2002), and inhibition of K\(^+\) channels using various channel blockers, affected cytochrome c release (Maeno et al. 2000).

The role of K\(^+\) channels in cancer cells have been studied and it has been suggested that they could be used as tumour markers (Stuhmer et al. 2006). K\(^+\) channel openers were shown to increase proliferation of breast cancer (Abdul et al. 2003) and colon cancer cells (Abdul and Hoosein 2002) whereas K\(^+\) channel inhibitors reduced proliferation. Clearly, K\(^+\) channel modulation plays a crucial role in apoptosis of several cell types including cancer cells.

**K\(^+\) modulators**

**Ouabain**

Ouabain is a digitalis-like substance that is extracted from the plants *Strophanthus gratus*, *Acokanthera ouabaio*, or through chemical preparation. Ouabain is a specific blocker of the Na\(^+\), K\(^+\), ATPase pump, and drugs with this effect is used in treatment of heart failure (Wasserstrom and Aistrup 2005). Digitalis slows the pulse as well as the conduction of nerve impulses in the heart (FASS). Endogenous ouabain which has a close structural similarity to ouabain extracted from plant, are found in mammalian tissue (Kawamura et al. 1999) and is under control of epinephrine and angiotensin II.

**Amphotericin B**

Amphotericin B is an antifungal drug derived from the bacteria *Streptomyces nodosus*. It form pores in the cell membrane, which disrupt the osmotic integrity and intracellular solutes leak out. Amphotericin B is the drug of choice for many serious systemic fungal infections, including those frequently occurring during cancer chemotherapy or treatment with immuno-suppressive drugs (Barrett et al. 2003). Unfortunately, the clinical use is limited by severe side effects such as nephrotoxicity (Porter and Bennett 1981; Hartsel and Bolard 1996). Amphotericin B has a higher affinity for ergosterol-containing membranes (such as fungi) than cholesterol-containing human cell membranes. The affinity explains the antifungal effects of the drug, however the adverse effects seen when administering the drug proves this affinity not to be absolute. The same amount of amphotericin B is needed for formation of ion channels in ergosterol- and cholesterol-containing membranes. The channels formed differs slightly in conductance but the main difference lies in the lifetime of the channel where
channels in ergosterol-containing membranes have about one hundred times longer lifetime than in cholesterol-containing membranes (Brutyan and McPhie 1996).

**Bumetanide**

Bumetanide is derived chemically and is a potent inhibitor of the $\text{Na}^+$, $\text{K}^+$, 2$\text{Cl}^-$-cotransporter and a diuretic (increases the amount of urine secretion) used to treat high blood pressure and reduce swelling and fluid retention caused by for example heart or liver disease. Adverse effects of administration are dehydration and low levels of potassium, sodium, and magnesium in the blood (FASS).

**Cisplatin**

Cisplatin (cis-diamminedichloroplatinum) is widely used as chemotherapeutic drug in treatment of several cancer types such as testicular, ovarian, non-small-cell- and small-cell lung cancers. Non-small-cell lung cancer is often intrinsically resistant to cisplatin whereas small-cell lung cancer can develop acquired resistance already after one cycle of treatment (Fuertes et al. 2003).

Cisplatin is cytotoxic due to DNA-intercalation and induction of either apoptosis or as with higher doses – necrosis (Chu 1994; Fuertes et al. 2003). The intercalated cisplatin makes it difficult for the cell to transcribe and replicate its DNA, which can trigger apoptosis. Binding to non-DNA targets, such as RNA, proteins, microfilaments and other molecules also disrupts the normal functions and can induce apoptosis (Gonzalez et al. 2001; Fuertes et al. 2003).

**Cisplatin resistance**

When cells become resistant to cisplatin, higher doses are required and this increases the risk of side effects. Therefore, it is of great importance to overcome the resistance and increase treatment efficacy of tumour cells.

There are numerous mechanisms for cells to circumvent cytotoxicity and acquire drug resistance. These include DNA repair, reduced drug accumulation, and disruption of signalling pathways (Siddik 2003) all of which reduce apoptosis and promotes tumour growth. Cisplatin-induced cell death has been found to be associated with an increase in $\text{Cl}^-$ channel activity since inhibition of $\text{Cl}^-$ channels made the cells resistant to cisplatin treatment (Ise et al. 2005). Over-expression of $\text{K}^+$ channels in cisplatin-resistant epidermal and liver carcinoma cells have been found, but the channels did not interfere with the resistance (Liang et al. 2005).
Although cisplatin has limitations, due to the development of resistance and also severe side effects, it is still clinically useful. However, it is of importance to develop new or use current pharmaceuticals to increase treatment efficacy of cisplatin.

**Lung cancer**

According to the National Board of Health and Welfare in Sweden, 3,000 patients were diagnosed with lung cancer in the year of 2003, making it the fourth most frequently diagnosed form of cancer. The introduction of continuously better treatment approaches has improved the prognosis considerably. This includes improved localised therapy with surgery and radiotherapy, new chemotherapeutics, and recently access to novel drugs, such as EGFR inhibitors and antibodies against VEGF. However, still the estimated 5-years survival is less then 15 % for those diagnosed with lung cancer. Lung cancer is divided in two main subtypes, non-small-cell lung cancer and small-cell lung cancer.

Small-cell lung cancer is a fast proliferating tumour with early development of metastases. This explains why the disease often is at an advanced stage at the time of diagnosis. Patients with untreated disease have a survival of 1-2 months; however with combination treatment (chemotherapy and radiation) the median survival is 12-16 months when the disease is limited.

Malignant pleural mesothelioma (MPM) is an aggressive form of cancer most often caused by asbestos exposure. There is a latency period of 15 to 50 years or more between initial exposure and development of the disease with the average being 30 to 40 years. Although MPM is not a common disease, the incidence is expected to increase (Ismail-Khan et al. 2006) despite a ban of asbestos use in the Western World. The prognosis of most MPM is poor due to resistance against treatment (Leard and Broaddus 2004).
MATERIALS AND METHODS

Cell lines and test substances

P31 wt is a human malignant pleural mesothelioma cell line derived from excess fluid in the pleural space (pleural effusions) (Marklund et al. 1982) and was used in papers I-IV. A cisplatin-resistant sub-line (P31 res) was established by culturing P31 wt cells in increasing concentrations of cisplatin with final concentration of 1.2 mg/L cisplatin. P31 res cells were used in paper III and IV. U1690 is a small-cell lung cancer cell line extracted from pleural effusion (Bergh et al. 1985) and were used in paper I and II. The cells were maintained as monolayer cultures in full medium; Eagle’s minimal essential medium (MEM) with Earl’s salts, supplemented with 10% foetal calf serum, 200 µmol/L L-glutamine, and 50 mg/L gentamycin. Both during maintenance and experiments, the cells were kept at 37 ºC in a humidified atmosphere containing 5 % CO₂. Test substances discussed in paper I-IV are presented in Table 1.

Table 1: Summary of cell lines, test substances, and concentrations discussed in paper I-IV.

<table>
<thead>
<tr>
<th>Test Substance</th>
<th>Paper</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
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</thead>
<tbody>
<tr>
<td><strong>Amphotericin B</strong> (mg/L)</td>
<td>P31 wt</td>
<td>3, 6, and 9</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>P31 res</td>
<td>-</td>
<td>-</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>U1690</td>
<td>3</td>
<td>3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Nigericin</strong> (µmol/L)</td>
<td>P31 wt</td>
<td>2.5, 5, and 7.5</td>
<td>5</td>
<td>-</td>
<td>-</td>
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<tr>
<td></td>
<td>P31 res</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>U1690</td>
<td>5, and 7.5</td>
<td>5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Ouabain</strong> (µmol/L)</td>
<td>P31 wt</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>P31 res</td>
<td>-</td>
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<td>10</td>
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<tr>
<td></td>
<td>U1690</td>
<td>10</td>
<td>10</td>
<td>-</td>
<td>-</td>
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<tr>
<td><strong>Bumetanide</strong> (µmol/L)</td>
<td>P31 wt</td>
<td>10</td>
<td>10</td>
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<td>-</td>
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<tr>
<td></td>
<td>P31 res</td>
<td>-</td>
<td>-</td>
<td>10</td>
<td>-</td>
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<td></td>
<td>U1690</td>
<td>10</td>
<td>10</td>
<td>-</td>
<td>-</td>
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<tr>
<td><strong>Cisplatin</strong> (mg/L)</td>
<td>P31 wt</td>
<td>-</td>
<td>-</td>
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<td>-</td>
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<tr>
<td></td>
<td>P31 res</td>
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<td>-</td>
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<td>10 and 40</td>
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<td>U1690</td>
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</table>

*In paper I, a set of K⁺ channel inhibitors (4-aminopyridine, charybdotoxin, iberiotoxin, margatoxin, and quinidine), and ionophores (beauvericin, digitonin, gramicidin D, nonactin, nystatin, tetraethylammonium, and valinomycin), were screened, for details see paper I.*
Materials and methods

**$^{86}$Rb$^+$ uptake (I)**

P31 wt cells (25,000 cells/well) or U1690 cells (40,000 cells/well) were plated into 96-well culture plates (Isoplate, Perkin Elmer, Wallac, Turku, Finland) and maintained in full medium for 48 h. After aspiration of media, 100 µL of full medium with or without test substances, alone or in combination, and with $^{86}$Rb$^+$ (0.75 mCi/L) were added to the wells. After each time interval, the supernatants were aspirated and the cells washed twice with 200 µL NaCl. Liquid Scintillation Cocktail (150 µL) was added and intracellular $^{86}$Rb$^+$ was counted for using a MikroBeta counter. The result of $^{86}$Rb$^+$ uptake was expressed as percentage of untreated control cells for each time point.

**$^{86}$Rb$^+$ efflux (I)**

P31 wt cells (25,000 cells/well) or U1690 cells (40,000 cells/well) were seeded into 96-well culture plates (Isoplate, Perkin Elmer Wallac) and maintained in full medium overnight. On the following day, $^{86}$Rb$^+$ (0.75 mCi/L in full medium) was added and the cells again incubated overnight. After aspiration of media, 100 µL of medium with or without test substances were added to the wells. At the end of each time interval the supernatants were transferred to 96-well plates (Flexible plate, Perkin Elmer Wallac). After addition of 150 µL Optiphase SuperMix Liquid Scintillation Cocktail (Perkin Elmer Wallac), the $^{86}$Rb$^+$ content of the cells and the supernatants was counted using a MikroBeta counter (Perkin Elmer Wallac). The percentage of $^{86}$Rb$^+$ efflux was calculated in relation to total cellular content of $^{86}$Rb$^+$, according to following formula:

\[
\text{% efflux} = 100 \times \frac{\text{efflux}}{(\text{efflux} + \text{remain})}
\]

where “efflux” is the counts-per-minute (cpm)-value in the efflux solution and “remain” is the cpm-value of $^{86}$Rb$^+$ remaining in the cells.

**Cytotoxicity – LDH release (II)**

A lactate dehydrogenase (LDH) release test (CytoTox 96, Promega Corporation, Madison USA) was used to determine the cytotoxicity of test substances after 3 h incubation. P31 wt cells (15,000 cells/well) or U1690 cells (40,000 cells/well) were plated into 96-well culture plates and maintained in full medium overnight. On the following day, medium was replaced with medium and test substances. Wells with untreated cells were used to set background LDH release, and eight wells with untreated cells were lysed with 10 µL enclosed lysate buffer to determine maximum LDH release. The assay solution was mixed with 50 µL
supernatant and incubated in the dark for 30 min at room temperature. Before absorbance at 490 nm was determined in a micro-plate reader, 50 µL stop solution was added (Molecular Devices, USA). Results were corrected for background absorbance and presented as percent of maximal LDH release.

**Determination of intracellular K⁺ using PBFI-AM (II-IV)**

P31 wt cells (15,000 cells/well), P31 res cells (20,000 cells/well), or U1690 cells (40,000 cells/well) were plated into 96-well culture plates (Black & White IsoPlate, Perkin Elmer) and maintained in full medium overnight. On the following day, the medium was changed to medium with low serum (1%) and incubated 45 min. The cells were then loaded with potassium-binding benzofuran isophtalate acetomethylester (PBFI-AM) by adding 50 µL of PBFI-AM (10 µmol/L) (Invitrogen, Carlsbad, CA, USA) with 0.04% Pluronic F-127 (Sigma Chemical Company, St. Louis, MO, USA). After 100 min, excess PBFI-AM was removed by washing with low-serum medium, test substances were added and the experiment was terminated after 1, 2, or 3 h by washing the cells with physiological NaCl solution. Excitation ratios of fluorescence at 344 and 387 nm, measured at emission 500 nm were used to determine the intracellular content of K⁺. Results were corrected for background fluorescence and presented as percent of untreated control.

**Quantification of apoptosis by TUNEL-staining (II-IV)**

P31 wt, P31 res, or U1690 cells were incubated with culture medium to about 80% confluence; medium was then changed to fresh medium containing test substances. Incubation continued for 3 h, medium was thereafter changed to fresh medium and the cells were left for proliferation. After 48 h, cells were harvested with 0.2% EDTA and any floating cells were collected by centrifugation of the medium. Apoptosis was quantified with an “In situ cell death detection kit, TMR red” (Roche, Mannheim, Germany), a TUNEL- (TdT-mediated dUTP nick end labelling) assay detecting nuclear DNA fragmentation. Free 3’-OH terminals were labelled with modified fluorescence-labelled nucleotides by catalysis of TdT (terminal deoxynucleotidyl transferase). The assay was performed as described in the manufacturers’ manual. Analysis was performed in a FACSCalibur™ flow cytometer (BD Biosciences, San Jose, CA) using the FL-3 channel for detection of TUNEL-positive signal. Results were presented as percent of total events. TUNEL-negative cells were considered viable and TUNEL-positive cells were considered apoptotic.
**Cell lysates (II-IV)**

P31 wt, P31 res or U1690 cells were incubated with culture medium to about 80% confluence, medium was then changed to fresh medium containing test substances. Incubation continued for 3 h, then the medium was changed and proliferation continued for 24 h. Floating and adherent cells were collected with 0.2% EDTA and re-suspended in cold lysis buffer (supplemented with EDTA free protease inhibitor cocktail tablets, Roche Diagnostics GmbH, Mannheim, Germany) for 30 min. Total protein content was determined with Bicinchoninic acid (BCA) protein assay kit (Pierce biotechnology Inc. Rockford, IL, USA). Cell lysates were used in the determination of caspase enzyme activity and for detection of pro- and anti-apoptotic molecules with Western blot.

**Determination of caspase enzyme activity (II-IV)**

Caspase-3, -8, and caspase-9 fluorometric assay kits (R&D Systems, Minneapolis) were used to determine caspase enzyme activity in P31 wt, P31 res, and U1690 cells. U1690 cells were only tested for caspase-3 activity. Caspase-3, caspase-8, or caspase-9 fluorogenic caspase-specific substrate was incubated with 200 µg protein at 37 °C for 2 h. Fluorescence was measured at excitation wavelength 400 nm and emission 505 nm in a Luminescence Spectrometer LS 50B (Perkin Elmer). Results were presented as fluorescence per µg protein.

**Detection of pro- and anti-apoptotic molecules by Western blot (III-IV)**

Cell lysate of P31 wt or P31 res cells (19.5 µL of each, diluted so that the same amount protein was loaded), 7.5 µL NuPAGE 4X LDS sample buffer, and 3 µL NuPAGE reducing agent was mixed and incubated for 10 min at 95-100 °C. The samples were run on a 12.5% Tris-HCl SDS-PAGE criterion Precast gel (BioRad, Hercules, CA, USA) using NuPAGE 1X MOPS buffer and 100 µL NuPAGE antioxidant, for approximately 75 min at 150 V. Full Range Rainbow RPN800 standard (GE Healthcare, Buckinghamshire, UK) was used as a molecular weight marker. Blotting was performed 1.5 h at 30 V using a tank blotting apparatus onto Immune-Blot PVDF membranes, (BioRad). The buffers, reducing agent and antioxidant were from Invitrogen (Carlsbad, CA, USA). The membrane was then blocked in TBS buffer containing 0.2% Tween® 20, 1 mol/L Tris pH 7.4, 5 mol/L NaCl and 5% milk over night at 4 ℃. Thereafter the membrane was incubated for 1 h with the primary antibodies diluted (1:1000) in 5% milk in TBS buffer and 0.25% Tween 20. After repeated washings with TBS buffer, the membrane was incubated for 1 h with the secondary antibody diluted
Materials and methods

1:5000 in 5% milk in TBS buffer and 0.25% Tween 20. The membrane was again washed three times with TBS buffer. Detection of antibody binding was performed by enhanced chemiluminescence staining (Amersham ECL Advance Western Blotting Detection Kit, GE Healthcare). Anti-actin antibody (Chemicon International, Temecula, CA) was used to ensure equal loading of protein. Results were presented as representative blots of at least three independent experiments.

Statistics

Statistical significance was tested with one-way ANOVA. The level of significance for rejecting the null hypothesis of zero treatment effect was p = 0.05.
RESULTS AND DISCUSSION

There is a general acceptance of the theory that K\(^{+}\) deprivation is a prerequisite for apoptosis. Provoked K\(^{+}\) efflux due to activation of K\(^{+}\) channels, or by K\(^{+}\) ionophores promotes apoptosis (Yu et al. 1997; Yu et al. 1999; Krick et al. 2001). The work in this thesis was initiated by Linda Marklund who studied the effect of K\(^{+}\) modulators on apoptosis induced by amphotericin B and cisplatin (Marklund et al. 2001; Marklund et al. 2001; Marklund et al. 2004). The initial project raised the question whether more effective K\(^{+}\) uptake inhibitors and K\(^{+}\) ionophores (Fig. 4) could be used to decrease the intracellular K\(^{+}\) concentration and induce apoptosis induction?

![Fig 4: The theoretical principle for K\(^{+}\) modulation. Inhibitors of K\(^{+}\) uptake together with effective K\(^{+}\) efflux stimulators might be used to achieve low intracellular K\(^{+}\) and induce apoptosis.](image)

In this thesis we show that K\(^{+}\) flux modulators (i.e. ouabain and amphotericin B) induced apoptosis per se in parental human pleural mesothelioma cells (P31 wt) and a cisplatin-resistant (P31 res) sub-line via expression of pro-apoptotic molecules, caspase-3 and/or -9 enzyme activity, and DNA fragmentation. K\(^{+}\) modulators, primarily the Na\(^{+}\), K\(^{+}\), ATPase pump inhibitor ouabain, was shown to sensitize P31 res cells to cisplatin-induced apoptosis to the same level as P31 wt cells. We conclude that ouabain with or without amphotericin B induced apoptosis per se and augmented cisplatin-induced apoptosis in P31 wt and P31 res cells.

Characterization of K\(^{+}\) fluxes (I)

In order to characterize P31 wt and U1690 cells with respect to K\(^{+}\) fluxes, we used \(^{86}\)Rb\(^{+}\) as a K\(^{+}\) analogue. We described in detail the main K\(^{+}\) flux mechanisms of two different lung cancer cell lines.
**Results and discussion**

**Uptake of K⁺ was effectively inhibited by ouabain and bumetanide**

Earlier studies of P31 wt cells shows that the Na⁺, K⁺, ATPase pump and the Na⁺, K⁺, 2Cl⁻-cotransport are the main mechanisms for the initial (15 min) uptake of K⁺ (Sandstrom et al. 1994). The inhibitor of big conductance K⁺ channels; tetraethylammonium (TEA), also affects the initial uptake of K⁺ in P31 wt cells (Sandstrom et al. 1994). Expanding the time interval and studying ⁸⁶Rb⁺ fluxes up to 6 h revealed that bumetanide had a transient effect, and after 6 h there was no inhibition of ⁸⁶Rb⁺ uptake. Ouabain alone and combined with bumetanide, inhibited the ⁸⁶Rb⁺ uptake during the 6-h test period with the combination being more effective at 30 min. After 6 h, the inhibiting effect on ⁸⁶Rb⁺ uptake by ouabain combined with bumetanide was solely due to Na⁺, K⁺, ATPase pump inhibition by ouabain.

The ion flux mechanisms in U1690 cells have not been characterized until now. The results were similar as those described for the P31 wt cells. Bumetanide initially inhibited the ⁸⁶Rb⁺ uptake, but as in P31 wt cells, the effect was transient. Ouabain combined with bumetanide was initially (at 1 h) more effective on ⁸⁶Rb⁺ uptake than ouabain alone, but as in P31 wt cells, after 4 h the inhibiting effect on ⁸⁶Rb⁺ uptake was due to ouabain.

**Specific K⁺ channels could not be inhibited in P31 wt and U1690 cells**

A set of different K⁺ channel inhibitors (see footnote to Table 1) were tested for regarding effect on ⁸⁶Rb⁺ efflux. In initial studies (up to 30 min incubation), efflux of ⁸⁶Rb⁺ in P31 wt cells was inhibited by iberiotoxin, a specific blocker of BK-channels. In U1690 cells, quinidine had an initial effect suggesting the presence of TWIK1/TASK2 or hERG type of K⁺ channels. However, when the time interval was prolonged to 6 h, there was no inhibition of ⁸⁶Rb⁺ efflux in either cell line. Hence, the efflux of K⁺ was difficult to inhibit, most likely due to unspecific leakage of K⁺.

**Efflux of K⁺ could be stimulated by nigericin and amphotericin B**

We could not obtain specific inhibition of K⁺ efflux; however that was not the primary aim, the aim was to elucidate whether or not the efflux of K⁺ could be stimulated in order to deprive the cells of K⁺. Of the tested ionophores (see footnote to Table 1), amphotericin B and nigericin were shown to provoke efflux of K⁺ in both P31 wt and U1690 cells.

When testing the effect of amphotericin B and nigericin on ⁸⁶Rb⁺ uptake, amphotericin B had an initial inhibiting effect on ⁸⁶Rb⁺ uptake in P31 wt cells. In U1690 cells, uptake of ⁸⁶Rb⁺ was inhibited at the end of the test period, and initially there was rather an increase in ⁸⁶Rb⁺ uptake. Incubation with nigericin combined with bumetanide and ouabain were later found to be toxic to the U1690 cells (II) and therefore not used in further studies of the U1690 cell line.
Unexpected effects, such as uptake inhibition of postulated efflux stimulators, demonstrate the importance of characterization of $K^+$ fluxes in cells or cell lines studied.

**Reduction of intracellular $K^+$ content in P31 wt and U1690 cells (II-V)**

The $^{86}\text{Rb}^+$ assay was a useful method when screening substances and studying separate flux mechanisms over the cell membrane. However, due to the differences in experimental setup, no conclusions could be drawn regarding intracellular $K^+$ content. In order to further elucidate the effects of $K^+$ flux modulators on cellular $K^+$ content in P31 wt, P31 res and U1690 cells, we developed a 96-well plate method using PBFI-AM. PBFI-AM has been widely used for determining intracellular $K^+$ content using different applications, such as cover slips (Kasner and Ganz 1992), 24- or 6 well plates (Li et al. 2003), or flow cytometry analysis (Bortner et al. 1997; Scoltock and Cidlowski 2004). We found the 96-well application to be more efficient and cost effective.

A combination of $K^+$ uptake inhibition and efflux stimulation was required for intracellular $K^+$ reduction

Amphotericin B and nigericin was not only effective efflux stimulators, but they also had an effect on uptake of $K^+$ with an initial (30 min) stimulation followed by a distinct inhibition of uptake (I). These results further argue for the importance of studying changes of intracellular $K^+$ content. $K^+$ efflux stimulation with amphotericin B was not enough to effectively decrease PBFI fluorescence in P31 wt (III), P31 res cells (III) or U1690 cells (II), probably due to activation of effective reuptake mechanisms. Inhibition of uptake alone, with ouabain alone or in combination with bumetanide, did not change the PBFI fluorescence during the test period. However, when combining ouabain and amphotericin B, with (II) or without bumetanide (III), there was a decrease in PBFI fluorescence in P31 wt (II, III), and U1690 (II) cells, but not for P31 res cells (III).

Cisplatin alone or in combination with ouabain did not change the PBFI fluorescence in P31 wt cells (IV). Combining amphotericin B and ouabain with cisplatin reduced the PBFI fluorescence but with less effect than without cisplatin. P31 res cells were unaffected by 10 mg/L or 40 mg/L cisplatin alone or in combination with ouabain regarding intracellular $K^+$ content. When adding 10 mg/L cisplatin to ouabain and amphotericin B, there was a minor decrease in PBFI fluorescence.

When interpreting the results, one has to keep in mind that 1) the depletion of $K^+$ is only measured for up to 3 h, 2) only the initial effects are studied, and 3) that $K^+$ depletion is an ongoing process since apoptosis is nevertheless induced in P31 wt and P31 res cells.
Results and discussion

Induction of apoptosis by K\(^+\) modulation (II, III)

In order to elucidate whether K\(^+\) modulation *per se* could induce apoptosis, (III) TUNEL-staining (DNA fragmentation) was used to determine apoptosis in P31 wt (II-III) and P31 res cells, 48 h after a 3 h incubation with test substances. TUNEL-staining was also done with U1690 cells, however due to technical difficulties, the U1690 cell line was not suitable for the method and was excluded for further studies.

*Inhibition of Na\(^+\), K\(^+\), 2Cl\(^-\)-cotransport activity did not induce apoptosis*

The combination of ouabain and bumetanide inhibited K\(^+\) uptake (I), and induced apoptosis (II), but did not change intracellular K\(^+\) content. Bumetanide did not induce apoptosis *per se*, or in combination with amphotericin B, nor did it enhance ouabain-induced apoptosis in P31 wt or P31 res cells, rather it seemed to have a minor protecting effect of apoptosis in P31 wt cells, and was therefore excluded from further studies.

*Inhibition of Na\(^+\), K\(^+\), ATPase pump activity induced apoptosis*

When separating Na\(^+\), K\(^+\), ATPase pump inhibition from Na\(^+\), K\(^+\), 2Cl\(^-\)-cotransport inhibition it was clear that the DNA-fragmentation seen when ouabain and bumetanide were combined, was solely due to the effect of Na\(^+\), K\(^+\), ATPase pump inhibition (III). Ouabain induced apoptosis to the same extent in P31 wt cells and P31 res cells (III) suggesting that Na\(^+\), K\(^+\), ATPase pump activity was not coupled to cisplatin-resistance. Since there was no change in intracellular K\(^+\) content, ouabain-induced apoptosis was probably due to other effects than K\(^+\) depletion. Ouabain has been shown to induce apoptosis in prostate cancer cells, by increasing the levels of intracellular Ca\(^{2+}\) (Yeh et al. 2001). Ouabain-induced changes in the conformation of the Na\(^+\), K\(^+\), ATPase pump, activates several pathways involving the Na\(^+\), K\(^+\), ATPase pump in signal transduction (Xie and Cai 2003).

*K\(^+\) efflux stimulation enhanced ouabain-induced apoptosis*

Amphotericin B alone did not induce apoptosis in P31 wt (II, III) or in P31 res cells (III). However, when combined with ouabain, the number of apoptotic cells was increased in both cell lines (III). Although amphotericin B is important for cancer patients, which are more susceptible for fungal infections, the clinical use is limited due to nephrotoxicity (Porter and Bennett 1981). It is of importance that the amphotericin B-enhanced effect on apoptosis outweighs the disadvantages of side effects.
Enhancement of cisplatin-induced apoptosis by K\(^+\) modulation (IV)

Since cisplatin is used in the treatment of pleural mesothelioma, but with limitations due to acquired cisplatin-resistance, we investigated whether K\(^+\) modulation could enhance cisplatin-induced apoptosis in P31 wt and P31 res cells. Cisplatin alone induced apoptosis in both cell lines; however the P31 res cells required four times more cisplatin (40 mg/L) to induce DNA fragmentation to the same extent as in P31 wt cells incubated with 10 mg/L cisplatin.

**Inhibition of Na\(^+\), K\(^+\), ATPase pump activity enhanced cisplatin-induced apoptosis**

Ouabain enhanced cisplatin-induced apoptosis in both P31 wt and P31 res cells. In P31 res cells, the combination of ouabain and 10 mg/L cisplatin yielded about the same percentage of apoptotic cells as if incubated with 40 mg/L cisplatin. Na\(^+\), K\(^+\), ATPase pump activity has been suggested to facilitate cisplatin accumulation (Andrews et al. 1991), and ouabain-induced inhibition of the Na\(^+\), K\(^+\), ATPase pump has been reported to reduce the sensitivity of prostate cancer cells to cisplatin treatment (Blok et al. 1999). These reports are contrary to our findings where inhibition of Na\(^+\), K\(^+\), ATPase pump activity by ouabain sensitized cisplatin-resistant cells to cisplatin.

**K\(^+\) efflux stimulation enhanced cisplatin-induced apoptosis**

Amphotericin B is known to increase the anti-tumour efficacy of cisplatin (Morikage et al. 1991; Sharp et al. 1994), which was also seen in P31 wt and P31 res cells. Since amphotericin B itself did not induce apoptosis, the enhanced effect on cisplatin-induced apoptosis could be due to increased accumulation of the drug, as proposed earlier (Morikage et al. 1993; Poulain et al. 1997). However, the combination of cisplatin and amphotericin B has been shown to be severely toxic and induced nephrotoxicity in a mouse model, which suggests clinical limitations since there was no increase in the treatment efficacy of cisplatin (Bergstrom et al. 1997).

**Signalling pathways in apoptotic P31 wt and P31 res cells (III, IV)**

Caspases are enzymes with key functions in apoptotic signalling. Depending on the pathway activated (Fig. 2), different initiator caspases are involved. Crosstalk between the different pathways is possible and renders analysis more difficult. In addition to caspase enzyme activity, different pro- and anti-apoptotic molecules are activated in order to promote or
inhibit apoptosis. We investigated apoptosis signalling pathways in P31 wt and P31 res cells 24 h after a 3 h incubation with the Na\(^+\), K\(^+\), ATPase pump inhibitor ouabain and/or K\(^+\) efflux stimulator amphotericin B with or without cisplatin.

**Caspase-8 was not activated in P31 wt or P31 res cells**

In response to external signals, death receptors in the membrane are activated which in turn recruits and activate cytoplasmic proteins. Caspase-8 is the initiator caspase for the extrinsic pathway, which is activated via cytoplasmic proteins, and in turn activates executor caspases. Apoptosis induced by ouabain alone (III), or by cisplatin, alone or in combination with ouabain (IV), did not induce caspase-8 enzyme activity in P31 wt or P31 res cells which suggested that apoptosis was not executed via the extrinsic pathway.

**Caspase-9 was activated in P31 wt cells but not in P31 res cells**

Another main pathway to apoptosis is activated via internal signals where pro- and anti-apoptotic Bcl-2 family members interact via the mitochondria to release cytochrome c which in turn activates caspase-9, the initiator caspase in the intrinsic apoptotic signalling pathway. In P31 wt cells, ouabain (III), or cisplatin alone (IV), did not activate caspase-9, but when combined, caspase-9 enzyme activity was increased. The activity was further increased in P31 wt cells by addition of amphotericin B (III, IV). In contrast, P31 res cells exhibited no increase in caspase-9 activity (III, IV). Together with the comparable levels of DNA fragmentation in cisplatin- and ouabain-exposed P31 wt and P31 res cells, the lack of caspase-9 activity suggested that apoptosis was inefficiently executed in both cell lines when incubated with cisplatin or ouabain alone.

**Caspase-3 was the executor caspase in both P31 wt and P31 res cells**

Caspase-3 is the executor caspase in the intrinsic as well as the extrinsic apoptotic signalling pathway. Both P31 wt and P31 res cells exhibited caspase-3 enzyme activity (II-IV). In P31 cells, ouabain induced caspase-3 activity almost to the same extent as 10 mg/L cisplatin (III, IV). Only when combining cisplatin, ouabain, and amphotericin B there was a further increase in caspase-3 activity (IV). In P31 res cells, 10 mg/L cisplatin alone or combined with ouabain or amphotericin B did not increase caspase-3 activity (IV). However, the combination of 10 mg/L cisplatin, ouabain, and amphotericin B increased caspase-3 activity to the same extent as 40 mg/L cisplatin alone (IV). We conclude that caspase-3 was the executor caspase in both P31 wt and P31 res cells and that the level of caspase-3 activity together with levels of caspase-9 activity could determine the outcome of apoptosis.
Expression of pro-apoptotic molecules was different in ouabain- or cisplatin-induced apoptotic P31 wt and P31 res cells

Upstream of caspase-9, there are several possibilities to activate initiator caspases. Screening a set of pro- (Bax, Bak, Bim, Bid, and Bad) and anti-apoptotic molecules (Bcl-2 and Bcl-X<sub>L</sub>) showed that Bak expression was increased in ouabain-induced apoptotic P31 res cells but not in P31 wt cells (III). Bak can be controlled by Bcl-X<sub>L</sub> and Mcl-1, and upon apoptotic stimulation both have been shown to be inactivated to ensure Bak-promoted apoptosis (Willis et al. 2005). However, no changes in expression of Bcl-X<sub>L</sub> were detected in either cell line. Bak has been shown to act independently of other pro-apoptotic molecules, however the release of cytochrome c was shown to be enhanced when Bak was expressed in combination with Bax and Bid (Wang et al. 2001). In P31 wt cells, minor or no increase of Bax expression was detected and no changes that could be correlated with the Bak activation were noted in P31 res cells (III, IV). The expressions of Bid, Bim, and Bad were not changed in P31 wt or in P31 res cells.

Active SAPK/JNK was expressed in P31 wt and P31 res cells when ouabain, amphotericin B, and cisplatin were combined (IV). Cisplatin-induced activation of the SAPK/JNK pathway has been shown in human lung cancer cells, and the cells were made resistant to cisplatin by knocking out molecules upstream of SAPK/JNK (Wang et al. 2006). Ouabain, with or without amphotericin B, or cisplatin alone did not activate SAPK/JNK (III, IV) in P31 wt or in P31 res cells. Since the activation of SAPK/JNK correlated to the high levels of DNA fragmentation it was suggested that the combination was more stressful to the cells.

We concluded that there were differences in signalling pathways between apoptotic P31 wt and P31 res cells and depending of apoptotic stimulation.
CONCLUSIONS

- The Na⁺, K⁺, ATPase pump inhibitor ouabain inhibited K⁺ uptake in P31 wt and U1690 cells. The Na⁺, K⁺, 2Cl⁻-cotransport inhibitor bumetanide had a transient effect on K⁺ uptake in P31 wt and U1690 cells. The antifungal ionophore amphotericin B and the antibiotic nigericin stimulated K⁺ efflux in P31 wt and U1690 cells.

- K⁺ modulation with ouabain and amphotericin B, with or without bumetanide, reduced intracellular K⁺ content in P31 wt and U1690 cells as measured with the fluorescent potassium-binding probe PBFI-AM after 3 h incubation.

- Ouabain induced apoptosis in P31 wt and P31 res cells. Amphotericin B enhanced ouabain-induced apoptosis in both cell lines but had no effect on its own. Bumetanide alone or in combination with ouabain or amphotericin B did not contribute to apoptosis.

- Ouabain enhanced cisplatin-induced apoptosis in P31 wt cells, and P31 res cells were sensitized to cisplatin-induced apoptosis by ouabain. Amphotericin B enhanced apoptosis induced by cisplatin and ouabain in both cell lines.

- Ouabain-induced apoptosis was executed through caspase-3 enzyme activation in both P31 wt and P31 res cells, but only P31 res cells exhibited an increase in pro-apoptotic Bak expression. Amphotericin B enhanced the effect of ouabain-induced apoptosis in P31 wt cells through caspase-9 activation. Cisplatin-induced apoptosis was enhanced by ouabain and amphotericin B through activation of SAPK/JNK in P31 wt and P31 res cells.


Resultaten i denna avhandling bygger på forskning där P31 wt och P31 res celler använts som modell för att studera malignt mesoteliom och cisplatinresistens. Syftet med studierna var att studera och manipulera K⁺ flödena i cellerna för att tömma dem på K⁺ och på så sätt inducera apoptos. Manipulering av K⁺ flöden kombinerades även med cisplatin för att förstärka effekten av cisplatinbehandling.


Vi drog slutsatsen att manipulation av K⁺ flöden kan inducera apoptos i P31 wt och P31 res celler, samt förstärka effekten av cisplatin vid utvecklandet av resistens.

Resultaten är grunden för fortsatta experimentella studier. På längre sikt kan läkemedel som påverkar cancercellers K⁺ balans ersätta eller förbättra effekten av de cellgifter som idag används vid behandling av malignt mesoteliom, men även andra former av cancer.
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