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APOPTOSIS, CELLULAR DIVISION OR MITOTIC CATASTROPHE?

EFFECTS OF KINASE INHIBITION AND DNA DAMAGE IN LUNG CANCER CELLS

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Stockholm 2007
Vilka tror vi att vi är?

här famlar vi omkring
och fattar ingenting

bo kaspers orkester
LIST OF PUBLICATIONS

I.  Apoptosis-inducing factor determines the chemoresistance of non-small-cell lung carcinomas.


A suggestion for the readers of this thesis

The regulation of life and death of various types of lung cancer cells is extremely complex. Therefore, no one knows when, or if, there will be a successful cure for lung cancer diseases. One positive thing is that it is quite easy to significantly reduce the risk for obtaining lung cancer. Please, take care of yourselves. Avoid smoking.
ABSTRACT

Non-small cell lung cancer (NSCLC) cells are generally characterized by a low response to conventional anti-cancer agents, such as DNA-damaging drugs. Treatments that induce DNA damage relies on cellular signalling resulting in the induction of growth arrest and apoptotic cell death. Deregulation of apoptosis contributes to resistance and failure of death execution may occur even in cases when all components of the apoptotic machinery are present in the cell. One possible reason for the inhibition of cell death is aberrant signalling of kinases, favouring cell survival. The main goal of this project was to understand the function of the apoptotic machinery in human lung cancer cells resistant to anti-cancer treatment and to investigate if the efficiency of the killing of these cells could be modified by using inhibitors of phosphorylation-based signalling. The NSCLC cell line U1810 is resistant to induction of apoptosis by conventional DNA damage-inducing anti-cancer treatments; however these cells are sensitive to the action of the broad range protein kinase inhibitor staurosporine (STS).

The two STS analogues PKC 412 and Ro 31-8220 are more specific protein kinase C (PKC) inhibitors compared to STS. The anti-tumour effects of PKC 412 and Ro 31-8220, when used alone or in combination with DNA-damaging treatments, were investigated. Ro 31-8220 neither induced apoptosis when used alone, nor sensitized cells to treatment with the DNA-damaging drug etoposide. PKC 412 induced death of a small number of U1810 cells, and sensitized cells to gamma radiation and etoposide. The difference in cell death induction upon treatment with Ro 31-8220 and PKC 412 could not be explained by a difference in the potency of PKC inhibition of the two STS analogues. Interestingly, Ro 31-8220 increased, whereas PKC 412 decreased, activity-related phosphorylation of the protein kinase Akt. Moreover, etoposide increased phosphorylation of Akt, which could be reversed by PKC 412. Inhibitors (wortmannin and LY-294002) of the Akt upstream activator PI3-kinase had a similar effect on U1810 cell as PKC 412. LY-294002, wortmannin and PKC 412 promoted apoptosis in a cell cycle dependent manner. LY-294002 and wortmannin induced apoptosis in late mitosis, whereas PKC 412 treatment provoked apoptosis in early mitosis and also induced formation of multinucleated cells. LY-294002 and wortmannin, in a similar manner as PKC 412, sensitized U1810 as well as H157 NSCLC cells to etoposide. PD 98059, which is an inhibitor of the protein kinase MEK, could not induce apoptosis when used alone in neither of the two NSCLC cell lines. However, in H157 cells PD 98059 enhanced apoptosis induced by treatment with etoposide in combination with wortmannin or LY-294002.

STS, PKC 412, etoposide or etoposide in combination with PKC 412 induced apoptosis at different time points and with different potency in U1810 cells. All four treatments resulted in cellular activation of caspase-3-like activity. Caspases cleave many proteins and are often determining apoptosis. Nevertheless, irrespective of the type of apoptosis-inducing treatment used, an inhibitor of caspase activity could not prevent apoptosis-related nuclear condensation in U1810 cells. Instead, upon all four different treatments, nuclear apoptosis was dependent upon release of Apoptosis inducing factor (AIF) from mitochondria. When etoposide and PKC 412 were combined, there was an increase in the release of AIF from mitochondria of U1810 cells. By using inhibitors of Poly(ADP-ribose) polymerase (PARP) we could show that release of AIF from mitochondria upon treatment with etoposide alone, or in
combination with PKC 412, demands PARP activity. Single treatment with PKC 412 induced AIF release and nuclear apoptosis independently of PARP. PKC 412 decreased inhibitory phosphorylation of the protein Bad, a protein which upon activation is known to promote release of mitochondrial proteins.

Collectively, our data suggest that etoposide and PKC 412 complement each other for the induction of apoptosis in NSCLC cells. Both drugs, in different manners, induce upstream mitochondrial events that promote increased release of mitochondrial AIF which determines apoptosis. PKC 412, possibly through inhibition of PI3-kinase downstream signalling, increases death of NSCLC cells through provoking mitotic catastrophe, and possibly also through activation of Bad. Etoposide increases cell death through induction of PARP-dependent release of AIF from mitochondria.
SAMMANFATTNING


I denna studie har vi använt odlade celler som har sitt ursprung i lungtumörer och i dessa undersökt mekanismer som krävs för att apoptos ska kunna aktiveras. Främst har vi använt U1810-celler, som är av typen icke små-cellig lungcancer, vilket är den vanligaste lungcancerformen. Dessa celler är relativt resistenta mot gammastrålning och den DNA-skadande medicinen etoposid (VP-16) som används i cancerbehandling. Dock dör de genom apoptos efter behandling med stauroporin (STS), som är en kraftig hämmer av en mängd olika kinaser som behövs för olika processer i cellen. STS kan inte användas för behandling av patienter, men ett av dess derivat, PKC 412, är i klinisk prövning. PKC 412 och STS-derivatet Ro 31-8220 hämmar inte lika många olika typer av kinaser som STS. I likhet med STS är dock PKC 412 och Ro 31-8220 goda hämningar av Protein kinas C-aktivitet, som på olika sätt är inblandad i tumörbildning. Vi kunde visa att PKC 412, men inte Ro 31-8220, aktiverade apoptos i ett antal U1810 celler, vilket inte kunde förklaras med olikheter i grad av Protein kinas C-hämmning medierad av de två STS-derivaten. Däremot fann vi att PKC 412 minskade, medan Ro 31-8220 ökade, aktivitetsrelaterad fosforylering av kinasen Akt. Det är känt att Akt motverkar apoptos i många olika celltyper. Genom att använda hämningar av den kinas som normalt aktiverar Akt (PI3-kinas), kunde vi visa att minskad aktivitet av denna signalväg gav upphov till ökad apoptos i U1810-cellkulturer. PKC 412 och PI3-kinashämmaren LY-294002 framkallade apoptos när cellen var i mitos, och celldöden berodde därför antagligen på att cellen stördes i sin delningsprocess, vilket kallas "mitotisk katastrof". H157 celler av icke små-cellig lungcancertyp uppvisade liknande mitosförändringar som U1810 celler efter behandling med PKC 412 och PI3-kinashämmare, vilket indikerar att PI3-kinassignalering generellt skulle kunna vara viktig för överlevnad när lungcancerceller delar på sig.

Etoposidbehandling gav en ökning av cellulär aktivitetsrelaterad Akt-fosforylering. När PKC 412 eller PI3-kinashämmare, men inte Ro 31-8220, kombinerades med etoposid ökade apoptosen i kulturer av U1810- och H157-celler. Cellernas skydd mot DNA-skadande etoposid skulle alltså kunna bero på en aktivering av PI3-
kinassignalering, vilken ger signaler om att cellen ska överleva. H157-celler kunde ytterligare sensibiliseras för kombinationen etoposid och PKC 412 eller PI3-kinas-hämmare om även en hämmare (PD 98059) av kinasen MEK inkluderades. PD 98059 gjorde ej U1810-cellerna känsliga för etoposid i kombination med PKC 412 eller PI3-kinas-inhibitor, vilket tyder på skillnader i signalreglering och eventuellt på en skillnad i mekanismen för apoptosresistens, mellan de två celllinjerna.

Vi undersökte också på vilket sätt etoposid, PKC 412 och STS påverkar det apoptotiska maskineriet i U1810 celler. Trots stora skillnader i tidpunkt för, och kvantitet av, apoptos framkallad av etoposid, PKC 412, STS eller kombinationen PKC 412 och etoposid, var den slutgiltiga apoptosen i samtliga fall beroende av mitokondriellt utsläpp av proteinet ”apoptosis inducing factor” (AIF). Aktivering av kaspaser, som leder till klyvning av en mängd protein, och ofta är nödvändig för apoptos, var dock inte avgörande för U1810-celldöd. När etoposid och PKC 412 kombinerades ökade det mitokondriella utsläppet av AIF. För att behandling med etoposid skulle leda till mitokondriellt utsläpp av AIF krävdes aktivitet av Poly(ADP-ribos) polymeras (PARP). PARP är ett enzym som fungerar som katalysator av många processer i cellen. Mitokondriellt utsläpp av AIF som framkallades av PKC 412-behandling krävde dock ej PARP-aktivitet. Däremot minskade PKC 412 aktivitetshämmande fosforylering av proteinet Bad som efter aktivering verkar för mitokondriellt utsläpp av protein. Våra resultat tyder på att PKC 412 och etoposid behandlingsmässigt kompletterar varandra genom att höja det mitokondriella utsläppet av AIF och därmed öka apoptosnivån. PKC 412 har också en viktig, möjligtvis genom dess hämmande effekt på Akt, negativ effekt på celldelning. PKC 412 gör celldelningen mer känslig, vilket resulterar i mitotisk katastrof som kan leda till att cellen aktiverar apoptos. Etoposid, däremot, höjer aktiviteten av Akt, vilket antagligen ökar chansen för överlevnad.
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<table>
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<th>Description</th>
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<tbody>
<tr>
<td>AIF</td>
<td>apoptosis inducing factor</td>
</tr>
<tr>
<td>APC</td>
<td>anaphase-promoting complex</td>
</tr>
<tr>
<td>ATM</td>
<td>ataxia telangiectacia mutated</td>
</tr>
<tr>
<td>ATR</td>
<td>ATR and rad3-related</td>
</tr>
<tr>
<td>Cdk</td>
<td>cyclin-dependent kinase</td>
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<tr>
<td>DAG</td>
<td>diacylglycerol</td>
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<tr>
<td>GSK</td>
<td>glycogen-synthase kinase</td>
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<tr>
<td>DNA-PK</td>
<td>DNA-dependent protein kinase</td>
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<tr>
<td>EGFR</td>
<td>epidermal growth factor receptor</td>
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<tr>
<td>ERK</td>
<td>extracellular-regulated kinase</td>
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<tr>
<td>FAK</td>
<td>focal adhesion kinase</td>
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<tr>
<td>IAP</td>
<td>inhibitor of apoptosis protein</td>
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<td>JNK</td>
<td>c-jun NH2-terminal kinase</td>
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<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
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<tr>
<td>MEK</td>
<td>mitogen-activated protein kinase/extracellular-regulated kinase</td>
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<tr>
<td>MPT</td>
<td>membrane permeability transition</td>
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<tr>
<td>mTOR</td>
<td>mammalian target of rapamycin</td>
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<tr>
<td>NSCLC</td>
<td>non-small cell lung cancer</td>
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<tr>
<td>PARP</td>
<td>poly(ADP-ribose) polymerase</td>
</tr>
<tr>
<td>PI</td>
<td>phosphoinositol</td>
</tr>
<tr>
<td>PI3-kinase</td>
<td>phosphoinositol 3-kinase</td>
</tr>
<tr>
<td>PIKK</td>
<td>PI3-kinase-like protein kinase</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
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<tr>
<td>PtdIns</td>
<td>phosphatidylinositol</td>
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<tr>
<td>RB</td>
<td>retinoblastoma</td>
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<tr>
<td>SCLC</td>
<td>small cell lung cancer</td>
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<td>STS</td>
<td>staurosporine</td>
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1 INTRODUCTION

The survival of a multicellular organism depends on the intactness of size and function of the different organs. The homeostasis is orchestrated by information kept in genes. Absence of proper genetic information or its deregulation may lead to different diseases.

More than hundred years ago Charles Darwin presented his theory of evolution, explaining how different species evolve due to the fact that “the fittest survive”. According to this theory, if for example a large bill is advantageous for obtaining food in a harsh environment, birds having a large bill will get more food compared to their relatives with small ones. Birds with large bills will thus survive and the trait will be inherited by the bird-ancestors and a population of birds with large bills will evolve.

Also in the body of a multi cellular organism an “evolution” can take place. A cell that manages to get a growth advantage due to deregulation of genes, will be better fit as compared to its surrounding cells, and might evolve into a tumour. Depending on the cell type, the environment, and the types of deregulation of cellular information, the reasons why cells evolve into cancer cells are different. Thus, a cancer disease is recognized as a growing tumour, but the underlying reasons for its occurrence and the ideal medicine for elimination of the cancer, might be different. However, in all tumours the information regulating cellular growth and death are deregulated. Therefore, there is a general requirement for increased knowledge about mechanisms regulating life and death in different tumour cells, and knowledge on how these mechanisms can be manipulated by various types of medicines is essential.

1.1 LUNG CANCER

Worldwide, every year, more than 1.35 million new patients are diagnosed for lung cancer and 1.18 million lung cancer deaths appear every year (Parkin et al., 2005), which means that tumours of the lung are the cancer diseases with the least favourable diagnosis. The main reason why people get lung cancer is through inhalation of toxic molecules produced by combustion of tobacco. Smoking is estimated to be the cause of 21% of the total global cancer death incidence and 60% of these smoking-related deaths is due to lung cancer (Ezzati et al., 2005). Since many people in the world smoke, aware or non aware of the risks, in combination with the lack of effective treatment of lung cancers at late stages, these diseases are a major problem for the affected and for the society.

1.1.1 Non-small and small cell lung cancer

Based on histopathological features lung cancer is divided into two main groups; non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC). SCLC and NSCLC cells also exhibit differences in mutation spectra and hot spots for loss of heterozygosity, which is consistent with the different biological and clinical features of the two lung cancer types (Wistuba et al., 2001). Of these two types, NSCLC is the most common (80-85%). NSCLC is further sub-divided into adenocarcinoma, large cell carcinoma and squamous carcinoma, of which adenocarcinoma has glandular features
and squamous carcinoma shows characteristics of epithelial cells. SCLC normally expresses properties of neuroendocrine cells, characteristics that are not often seen in NSCLC cells (Wistuba et al., 2001) and the cells are typically oat-cell like.

1.1.2 Resistance to conventional treatment is a characteristic of NSCLC cells

Early diagnosed NSCLC (stage I and II), where disease is confined to the lung, is cured by surgery in 40-70% of patients (Chang & Sugarbaker, 2003; Mountain, 2000). A main problem is that about 50% of NSCLC patients present with advanced-stage disease (Chang & Sugarbaker, 2003; Govindan, 2003). Late diagnosed NSCLC is associated with metastasis and typically shows resistance to various treatments (Chang & Sugarbaker, 2003). The majority of late-diagnosed NSCLC patients die within one to two years (Schiller, 2001).

SCLC is one of the most aggressive types of cancer and most often relapses after a successful initial therapy (Schiller, 2001). The few long-term survivors with SCLC are at significant risk of developing second primary lung cancer, usually of the NSCLC type within five years after the first diagnosis (Comis et al., 1998).

The resistance to anti-cancer treatment of NSCLC and the better responsiveness of SCLC observed in patients is also evident in cell lines derived from the respective tumour types (Joseph et al., 2000; Sullivan et al., 1996). Therefore, lung cancer cell lines provide a powerful tool for thorough studies of resistance mechanisms. Also, since there is no effective cure for lung cancer at late stages, new treatment strategies have to be developed.

To improve survival of NSCLC patients many attempts have been made to find effective combinatory treatments of conventional anti-cancer agents. In cases when surgery is a suboptimal initial approach, or in cases when tumours are unresectable, but suitable for local treatment by radiation, concurrent treatment with radiation and chemotherapy has been shown to improve survival. Treatment of advanced lung cancer often involves combinatory treatment of a DNA-cross linking compound of the platinum family, such as cisplatin, with a topoisomerase inhibitor (e.g. etoposide) or a microtubule inhibitor (e.g. paclitaxel and vinorelbine) (Spira & Ettinger, 2004).

Reduction of tumour size mediated by anti-cancer medicines relies on growth arrest and the induction of cell death (Coulats & Strasser, 2000).

1.2 APOPTOSIS - THE MOST FATAL DECISION OF THE CELL AND THE SAVIOUR OF THE ORGANISM

Cells have the capacity to change morphology and function in programmed manners, which is a prerequisite for development and maintenance of multicellular organisms. The initiation of these changes may take place due to influence from surrounding cells, endocrinial factors or the signal may emanate from the cell itself. Examples of programmed cellular changes are the continuous production of the different types of blood cells from pluripotent hematopoietic stem cells (Pazdernik, 1980), and the gradual changes that take place in a skin cell as it moves from the basal membrane towards the outer part of the skin and, eventually, fall off as a terminally differentiated cell (Watt, 1983; Watt, 1989). An important topic of this thesis is the programmed
process called apoptosis. When this process is initiated and undisturbed, eventually the cell will be eliminated.

The observation that many features of apoptosis are evolutionary conserved from yeast to human, (Jin & Reed, 2002) and the fact that apoptosis is important for the shaping of the embryo (Baehrecke, 2002) are interesting aspects of cell biology, which are beyond the scope of this thesis. Instead, I focus on the role apoptosis plays in removal of cells that are on their way to become too abnormal for being beneficial for the organism (Brown & Attardi, 2005). For example, many of the skin cells that are damaged by UV light from the sun, or lung cells damaged by tobacco, are believed to be removed by apoptosis and thereby survival and propagation of abnormal cells that might evolve into cancer cells is prevented. A tumour cell has lost its capacity to settle its own death, because it is producing inaccurate information that it should survive and divide, and the cell also has defects in the machinery essential for the execution of death (Hanahan & Weinberg, 2000).

Anti-cancer medicines are often strong triggers of apoptosis. Many of these drugs induce DNA damage, which is a trigger of the cell death machinery (Roos & Kaina, 2006). Reduction of tumour size mediated by anti-cancer treatments thus relies on the cellular capability of recognizing the death signal mediated by the medicine and also on the capability of the cell to undergo cell death. However, if cells cannot respond to the death trigger from a DNA damaging agent, cells, now possibly even more abnormal due to the increased risk for mutations associated with DNA damage, continue to survive and divide.

Since many lung cancer cells are resistant to conventional anti-cancer drugs, to improve treatment response of patients, it is important to find more information about essential apoptotic mechanisms in lung cancer cells, as well as to find out in which manners the activation of these mechanisms can be achieved.

### 1.2.1 Markers of apoptosis

As mentioned above, apoptosis is a programmed type of death, which means that once activated it follows a predictable scheme of morphological (Kroemer et al., 2005) and biochemical changes (Bras et al., 2005; Robertson et al., 2000) and is energy consuming (Bras et al., 2005). Since specific mechanisms are activated, apoptosis can be distinguished from a non-programmed death referred to as “necrosis”. This accidental cell death may follow due to severe damage to essential cellular systems, and is not energy consuming (Bras et al., 2005).

To determine the mode of death it is important to use markers for several types of cell death. For example, even though a cell dies in an apoptosis-like manner, it may not activate all mechanisms that are apoptosis-related. Moreover, especially in vitro, at late stages of apoptosis, it might be difficult to distinguish this type of death from necrosis. Further, some biochemical features of apoptosis are also seen in other processes of programmed death, for example when a keratinocyte is terminally differentiating (Fernando & Megeney, 2006). Morphological markers for apoptosis are; rounding up of the cell, retraction of pseudopodes, reduction of cellular volume, condensation of the chromatin, membrane blebbing and maintenance of an intact plasma membrane until late stages, fragmentation of the nucleus and appearance of so-called “apoptotic bodies” ((Kroemer et al., 2005). Examples of apoptosis-related biochemical features are;
activation of a type of proteases called caspases, appearance of cleaved proteins upon processing by caspases, flip of phosphatidyl serine from inside of plasma membrane to outside while the membrane is still intact, cleavage of DNA at repeated sites forming a so-called “DNA-ladder”, disturbance of the mitochondrial transmembrane potential and appearance of mitochondrial proteins, such as cytochrome c and AIF, in the cytosol (Bras et al., 2005; Robertson et al., 2000; Vance & Steenbergen, 2005).

1.2.2 The intrinsic and the extrinsic pathways

Apoptosis can be activated through extrinsic or intrinsic pathways (Sprick & Walczak, 2004), thus either by stimulation of receptors on the cellular membrane, or by means of intracellular mechanisms, activated, for example, upon DNA-damage (Norbury & Zhivotovsky, 2004).

Both the intrinsic and extrinsic pathways are characterized by caspase activation (Sprick & Walczak, 2004). Upon oligomerization-mediated activation, initiator caspases (e.g. caspase -2, -8, -9, -10) cleave and thereby activate executioner caspases (e.g. -3, -6, -7). The executioner caspases cleave many different proteins which in this way are activated or inactivated, favouring apoptosis (Earnshaw et al., 1999). The extrinsic pathway depends upon the activation of caspase-8 which is initiated by stimulation of death receptors on the plasma membrane, (Sprick & Walczak, 2004). Activation of the intrinsic pathway relies on the disturbance of the outer membrane of the energy-producing cellular organelles called mitochondria. This leads to the release of mitochondrial proteins, including cytochrome c, into the cytosol. Once in the cytosol cytochrome c forms a complex with apaf-1 and caspase-9, which lead to activation of downstream caspases (Sprick & Walczak, 2004). Both caspase-8 and caspase-9 cleave the executioner caspase-3, which is indispensable for some apoptosis-related processes (Porter & Janicke, 1999). Between the two pathways, there is crosstalk; triggering of the intrinsic pathway may lead to activation of caspase-8, and downstream of death receptor stimulation there may be activation of the Bcl-2 family member Bid, which eventually leads to the release of mitochondrial proteins resulting in a stronger apoptotic response (Sprick & Walczak, 2004).

Since most roads seem to lead to mitochondria it is not surprising that the integrity of this organelle is tightly regulated. The Bcl-2-family contains numerous proteins that are either pro- (e.g. Bid, Bad, Bax, Bcl-XS) or anti-apoptotic (e.g. Bcl-2, Bcl-XL) (Fadeel et al., 1999). Depending on the combination and status of different Bcl-2 family proteins in the cytosol and at different organelles, including the mitochondria, apoptosis can be either promoted or inhibited (Fadeel et al., 1999). Release of proteins from mitochondria can be achieved by different mechanisms including mitochondrial permeability transition (facilitated by Ca^{2+} accumulation in the mitochondrial matrix) (Zamzami & Kroemer, 2001), or through pore formation in the outer mitochondrial membrane due to oligomerization of Bcl-2 family members, such as bax (Er et al., 2006), and is sometimes dependent on lysosomal release of cathepsins (Chwieralski et al., 2006).

Depending on the mode of mitochondrial permeabilization, in addition to release of cytochrome c there might also be release of other proteins influencing apoptosis progression. One of these proteins is called Apoptosis Inducing Factor (AIF). This protein is able to induce apoptosis independently of caspase activity and will be
discussed more thoroughly in the next section. Downstream of mitochondrial events apoptosis-promoting proteins may be inhibited by different mechanisms. For example, Inhibitor of apoptosis proteins (IAPs) can bind to caspases and thereby stop their activities (Riedl & Shi, 2004).

The ultimate cause of apoptotic death is the processing of DNA (Robertson et al., 2000). During the process of apoptosis the structural integrity of the euchromatin and the nuclear lamina is lost, which eventually lead to nuclear condensation. This condensation takes place independent of or due to caspase-mediated actions. Upon entering the nucleus caspases are also involved in the activation of nucleases that cleave DNA at repeated sites forming so-called low and high molecular weight fragments. DNA fragments can also be formed in caspase-independent manners (Robertson et al., 2000). To enable clearance of apoptotic cells the flip of phosphatidyl serine to the outer part of the plasma membrane acts as a signal to surrounding cells that the apoptotic cell should be engulfed and digested (Vance & Steenbergen, 2005).

Fig. 1. DNA damage (1) induces growth arrest (2A). If the damage is severe there is activation of the intrinsic apoptotic pathway (2B), which involves release of mitochondrial proteins. Upon mitochondrial release of cytochrome c (Cyt c) (3A), this protein forms a complex with caspase-9 and Apaf-1 which lead to activation of downstream caspases which cleave many proteins. Release of apoptosis inducing factor (AIF) from mitochondria (3B), is followed by its translocation to the nucleus. The entrance of caspases (4A) and/or AIF (4B) into the nucleus results in nuclear condensation and fragmentation (5).
1.2.3 Apoptosis inducing factor

In 1996, the group of Guido Kroemer (Susin et al., 1996) discovered that the mitochondrial intermembrane protein fraction contains an apoptosis inducing activity, and some years later they purified a protein which maintained its bioactivity in the presence of the pan-caspase inhibitor z-VAD-fmk (Susin et al., 1999). The protein, which was called “Apoptosis inducing factor” (AIF), contains a mitochondrial localization sequence in its amino-terminal and in the carboxyterminal there is an oxidoreductase domain (Lorenzo et al., 1999). Some of the structural features of the oxidoreductase domain are important for the apoptogenic effect of AIF, whereas its oxidoreductase activity is not (Susin et al., 1999).

In normal cells, AIF is located in the mitochondrial intermembrane space, or bound to the inner membrane, and upon treatment with apoptosis-inducing agents, such as the kinase inhibitor staurosporine or DNA-damaging agents, AIF is released to the cytosol (Cregan et al., 2004). This process can be inhibited by Bcl-2 (Susin et al., 1996) and Bcl-XL (Otera et al., 2005).

Upon release, AIF translocates to the nucleus and induces nuclear chromatin condensation, large scale DNA fragmentation, and exposure of phosphatidyl serine on the plasma membrane surface (Daugas et al., 2000). If microinjected into the cytoplasm of normal cells, AIF induces a Bcl-2-independent drop in mitochondrial membrane potential, followed by release of cytochrome c (Daugas et al., 2000).

There is also an AIF homologue called AMID which upon overexpression, in resemblance with AIF, induces a caspase-independent apoptosis (Wu et al., 2002). Moreover, recently, one more death-inducing AIF homologue, AIFL, was discovered (Xie et al., 2005).

1.2.4 The apoptotic machinery in lung cancer cells

Aberrant expression or localization of proteins of the apoptotic machinery can contribute to resistance of cells to anticancer treatments. Resistance mechanisms may also involve aberrant post-transcriptional modifications of these proteins, influencing their activity. There are probably also mechanisms of resistance that more indirectly affect the apoptotic machinery. For example, deregulation and strengthening of cellular signalling pathways favouring growth and survival might “hide” weaker signals that promote apoptosis. Survival signalling may also more directly target the apoptotic machinery through regulation of expression levels, or post-transcriptional modification of, apoptosis-related proteins. Examples of aberrant signalling contributing to resistance will be discussed in later sections, and here I focus on resistance mechanisms related to the expression level and localization of apoptotic machinery proteins in lung cancer cells.

Since Bcl-2 family members play a central role in regulation of apoptosis, many efforts have been made to clarify their role in SCLC and NSCLC resistance. In general, focus of the studies has been directed towards Bcl-2. Expression of the Bcl-2 protein seems to shorten survival of SCLC patients; although this correlation is not always seen (Koutsami et al., 2002). In NSCLC, the link between Bcl-2 expression and survival prognosis is unclear. There are reports of Bcl-2 expression correlating with shorter NSCLC patient survival (Groeger et al., 2004; Huang et al., 2003; Lai et al., 2002). However, Bcl-2 expression has also been associated with prolonged survival (Cox et
al., 2001; Fokkema et al., 2006; Kren et al., 2004; Martin et al., 2003), as well as being a non-prognostic factor (Krug et al., 2003; Yaren et al., 2006). In experiments with both SCLC (Sartorius & Krammer, 2002; Zangemeister-Wittke et al., 1998; Ziegler et al., 1997) and NSCLC (Ke et al., 2004; Koty et al., 1999) cells, expression of the Bcl-2 protein correlates with increased resistance to apoptosis –inducing stimuli. However, in NSCLC clinical samples, Bcl-2 overexpression is associated with spontaneous apoptosis (Hanaoka et al., 2002), and SCLC cell resistance has been correlated with a decreased Bcl-2 level (Henness et al., 2002; Kumar Biswas et al., 2004).

As mentioned above, the interplay between different Bcl-2 family members is important. There are many studies showing that death of NSCLC cells is associated with down-regulation of anti-apoptotic Bcl-2 family members, including Bcl-2, Bcl-XL and/or up-regulation of pro-apoptotic members, such as Bax (Chou et al., 2003; Khanna et al., 2003; Nguyen et al., 2003; Oizumi et al., 2002). Also has been shown that overexpression of Bcl-XL inhibit death of cultured NSCLC cells (Ke et al., 2004). Investigations of clinical samples show that, within the NSCLC group, Bcl-2 expression is higher in squamous- compared to adeno-carcinoma (Hanaoka et al., 2002; Lai et al., 2002). Bcl-2 expression level also differs between SCLC and NSCLC cell lines; SCLC cells exhibit a higher expression of Bcl-2 protein as compared with NSCLC cells (Joseph et al., 1999).

Except for the difference in the protein level of Bcl-2 in SCLC and NSCLC cells, there seems to be a general divergence also in caspase expression. SCLC cell lines, but not NSCLC cells, have a frequent loss of caspase -1, -4, -8 and -10 (Joseph et al., 1999). However, at current it is not clear if the differences in Bcl-2 and caspase expression levels influence the resistance to conventional anti-cancer treatment of the two lung cancer types. SCLC cells also have different levels of some IAPs (inhibitor of apoptosis proteins) when compared with NSCLC cells (Ekedahl et al., 2002). In similarity with the uncertainties concerning in which cellular contexts Bcl-2 determines resistance to apoptosis in lung cancer cells, the involvement of IAPs in resistance is not completely clear. Experimentally induced changes in IAP expression influence the outcome of caspase-dependent apoptosis in lung cancer cells (Fennell, 2005). However, treatment response, in general, is not always explained by altered IAP levels. NSCLC cell resistance is sometimes correlated with inhibited caspase activation, which, in addition to IAP expression, can be due to, for example, decreased caspase-3 protein level (Fennell, 2005). Another possible mechanism for NSCLC resistance is inhibition of caspase-3 translocation into the nucleus (Joseph et al., 2001). Relief of this resistance was seen only after mitochondrial release of AIF and its nuclear translocation (Joseph et al., 2002).

As discussed by Shivapurkar (Shivapurkar et al., 2003), the knowledge concerning efficiency/failure of apoptosis in lung cancer is lagging behind, compared to many other types of cancer. To enable understanding on how to reactivate apoptosis in lung cancer cells, there is a need for deeper understanding on to what extent different apoptotic proteins may substitute for each other. For example, in skin keratinocytes, a low Bcl-2 level has been found to correlate with a high expression of Bcl-XL (Wrone-Smith et al., 1995), a protein that is known to have a similar anti-apoptotic function as Bcl-2 (Fadeel et al., 1999). Similar phenomena might be found in lung cancer cells.

There is also a requirement for increased knowledge on how different apoptotic pathways and mechanisms may interact and substitute for each other. For example, in a
certain type of cell, upon drug treatment, if there is no caspase activation, maybe it is possible to reactivate a caspase independent cell death mechanism?

In order to relieve the resistance of lung cancer cells to therapeutic attempts, it would also be of value to find out more about lung cancer sub type-specific aberrations in regulation of apoptosis. And not to forget, resistance of lung cancer cells, as well as of other types of cancer cells, cannot be explained solely by defects in the apoptotic machinery.

1.3 THE CELL DIVISION CYCLE

To maintain the correct genomic information, structure and function of the organism, cells duplicate and divide in a structured manner. In tumour cells the cell cycle machinery, in similarity with apoptosis, is deregulated, favouring uncontrolled growth. The cell division-cycle encloses doubling of cellular size, and the duplication of the DNA and organelles of a cell, as well as the division of this cell into two daughter ones. This cycle is divided into four phases; G1-, S-, G2- and M-phase (Caputi et al., 2005). A G1-phase cell of a diploid organism, such as ourselves, is half the size of a G2-phase cell and harbours two copies of each chromosome (except of the sex chromosomes in males of which there is one Y and one X chromosome). When a cell leaves G1-phase and enters S-phase it starts to double its size, as well as to duplicate its DNA, centrosomes and organelles, a process which is ended when the cell reaches G2-phase (Nigg, 2001; Polymenis & Schmidt, 1999). Thus, in G2-phase the cell harbours two copies of all chromosomes, each consisting of two sister chromatids. In M-phase the double-sized cell divides, which is a course of events that includes mitosis and cytokinesis, the process of nuclear and cytoplasmic division, respectively, ending with the formation of two cells in G1-phase (Nigg, 2001).

Mitosis can be divided into different phases, namely prophase, prometaphase, metaphase, anaphase and telophase (Nigg, 2001). In G1-, S- and G2- phase the cell is said to be in interphase. This phase is characterized by appearance of diffuse chromatin. In prophase, which is the transition from G2 to M-phase, the chromatin condenses into well-defined chromosomes, each of them consisting of two identical sister chromatids. Moreover, in prophase a bipolar structure, the mitotic spindle, which is assembled outside the nucleus between separating centrosomes and is composed of microtubules and associated proteins, begins to form. Prometaphase starts with disruption of the nuclear envelope and microtubules of the mitotic spindle can now bind to the chromosomes by the aid of protein complexes forming kinetochores. In metaphase the chromosomes are arranged in one plane, forming a so-called “metaphase plate”, halfway between the spindle poles. The two identical chromatids of each chromosome are separated and pulled towards the spindle poles in anaphase, and each chromatid now becomes a separate chromosome. During anaphase also cytokinesis starts by formation of an actin-based contractile ring which causes furrowing of the plasma membrane in the plane of the metaphase plate (Nigg, 2001). In telophase a new nuclear envelope forms around each of the two sets of daughter chromosomes, and cytokinesis is completed by the cleavage furrow eventually getting so deep so that the two cells are separated (Straight & Field, 2000).
The cell division cycle consists of interphase (G₁, S and G₂-phase) and mitosis (M-phase). In a time perspective the shortest phase is mitosis. When the cell is in prophase the two microtubule-producing centrosomes (illustrated as black dots in the figure) start to separate, forming two microtubule asters. In prometaphase the microtubules of the mitotic spindle asters can interact with the chromosomes (the 46 chromosomes of the human genome are represented by two chromosomes in the figure). When the cell is in metaphase the chromosomes are lined up half way between the spindle pools. In anaphase the chromatids of each chromosome are separated (and thus defined as two chromosomes), and pulled towards the spindle poles. At this stage also cytokinesis, the cytoplasmic division, starts. In telophase cytokinesis is ended by cleavage of the cell into two. The newly formed cells can then enter interphase and start to grow.

**1.4 THE LIFE AND DEATH OF A CELL IS REGULATED BY A COMPLEX SIGNALLING NETWORK INVOLVING PROTEIN KINASES**

Regulation of cellular life and death is extremely complicated, involving extra- and intra-cellular networks of different interacting signalling systems, of which phosphorylation-based signalling is one.

A protein kinase is an enzyme that transfers a phosphate group from a high-energy phosphate (most often ATP) to specific amino acid residues (serine and threonine or tyrosine) in a protein (Al-Obeidi & Lam, 2000; Shen et al., 2005). This type of phosphate transfer is an important part of intracellular pathways regulating catalysis of various chemical reactions. In addition, also extracellular signals are often transmitted by phosphorylations. These phosphorylations are generated by receptor kinases that enable transmission of the external signal to the intracellular response machinery (Tibes et al., 2005).

Cellular homeostasis, growth, division and cell death all relies on a kinase signalling network, which is sensitive to any single event that might disturb the
accuracy of the two daughter cells. Abnormalities in kinase signalling are believed to play an important role in development and maintenance of tumours (Dancey & Sausville, 2003; Nigg, 2001).

Fig. 3. Binding of extracellular ligands to receptors at the cell surface leads to receptor activation (A). When activated these receptors initiate activation of phosphorylation-based intracellular signalling pathways that form a complex network (B). The activated kinases of intracellular signalling pathways are illustrated as smiling faces with bound phosphate (illustrated as a P). Each arrow in the small scale left side of the picture (B) represents the event illustrated in (C). An activated kinase transfers phosphate groups from high-energy donor molecules, such as ATP (which thereby is converted to ADP), to specific target molecules (illustrated as sleeping faces), such as other kinases (C). Upon phosphorylation these kinases in turn phosphorylate other kinases or other target molecules. Many of the target molecules can be phosphorylated at different sites by different types of kinases (D), and thereby the function of the target molecule can be delicately adjusted. Kinase-mediated phosphorylation of target molecules may also lead to inhibition of molecular activity (E). A defined sequence of phosphorylation events may for example lead to transcription of specific genes.

1.4.1 Apoptosis regulation involves phosphorylation-based signalling

Activation of phosphorylation-based signalling pathways in cancer cells may ultimately lead to initiation of transcription of survival-related genes as well as to repression of death-related genes (Brunet et al., 2001).
Phosphorylation-events also regulate the apoptotic machinery on a post-transcriptional level. The initial apoptosis triggering signal, mediated by receptor binding (Holmstrom & Eriksson, 2000), or DNA damage (Kastan & Bartek, 2004) relies upon phosphorylation-based signalling. Downstream of the apoptosis initiation signal various events, for example mitochondrial integrity, are regulated by phosphorylation and dephosphorylation of e.g. proteins of the mitochondrial membrane (Horbinski & Chu, 2005). As mentioned, cell death can be regulated upstream or at the level of mitochondria, due to modification of anti- and pro-apoptotic Bcl-2 family members. The inhibition or activation of some Bcl-2 family proteins are known to be mediated through phosphorylation by kinases that are also involved in regulation of proliferation (Blagosklonny, 2001; Gross, 2006; Horbinski & Chu, 2005). For example, in NSCLC cells nicotine triggers multi-site phosphorylation of the pro-apoptotic Bcl-2 family member Bad, leading to its sequestration by 14-3-3 protein in the cytosol (Jin et al., 2004). This inhibition of Bad is the result of survival-related signalling, possibly activated through nicotine-mediated stimulation of beta-adrenergic receptors. Some kinases, e.g. Death-associated protein kinase (DAP-kinase ) (Cohen & Kimchi, 2001), are known to be involved in tumour suppression through stimulation of apoptosis. In lung cancer DAP-kinase expression is frequently inhibited by gene-silencing through methylation (Toyooka et al., 2003).

Kinase involvement has also been observed in the execution phase of apoptosis. Some of the pro-apoptotic kinases, such as p21-activated kinase 2 (PAK2) (Jakobi, 2004) and protein kinase Cδ, are activated by caspase cleavage (Emoto et al., 1995).

Thus, phosphorylation-based signalling directly interferes with, and participates in, the apoptotic machinery, through pre- or post-transcriptional regulation of apoptotic machinery-proteins.

1.4.2 Phosphorylation-based signalling drives the cell cycle

Kinase signalling has an important role in cell cycle regulation (Murray, 2004). The main players of this process are the cyclin-dependent protein kinases (Cdks) and their “controllers”, the cyclins that bind to Cdks and thereby activate them. The levels of different cyclins fluctuate in an ordered manner throughout the phases of the cell cycle, when reaching the highest peaks driving the cell into the next phase (Murray, 2004).

In G1-phase cyclin D associates with Cdk4 and Cdk6 (Obaya & Sedivy, 2002). These complexes mediate the phosphorylation of the proteins of the retinoblastoma family (RB) that upon phosphorylation cannot inhibit E2F-mediated transcription of genes favouring proliferation. For example, RB phosphorylation leads to E2F transcription factor activation needed for centrosome duplication and DNA replication during S-phase. E2F transcription factors activate transcription of cyclin A and E. At the G1/S transition, Cdk2, when associated with either cyclin A or E, phosphorylate downstream targets, which lead to progression into S-phase (Obaya & Sedivy, 2002).

M-phase entry requires increased levels of cyclin B and it’s binding to Cdk1 (Ohi & Gould, 1999). The activity of the resulting Cdk1-cyclin B complex is positively regulated by phosphorylation at Thr167, which is mediated by the Cdk-activating kinase (CAK). Negative regulation is supplied through phosphorylation by Myt and Wee1 of two residues, Thr14 and Tyr15, respectively, in the ATP-binding site of Cdk1. Upon
activation of Cdc25C, this phosphatase dephosphorylates Thr\textsuperscript{14} and Tyr\textsuperscript{15}, and the Cdk1-cyclin complex becomes fully active (Ohi & Gould, 1999).

Activated Cdk1-cyclin complexes phosphorylate different targets such as nuclear lamins, microtubule-binding proteins and condensins, which are important events for nuclear envelope breakdown, separation of centrosomes and assembly of the mitotic spindle and chromosome condensation, respectively (Nigg, 2001). Cyclin B, together with other mitosis proteins, are degraded by the anaphase-promoting complex, which is necessary for proper exit from anaphase (Nigg, 2001).

Except for Cdk-mediated phosphorylations there are numerous examples of phosphorylation events that regulate the progression of the cell cycle. For example chromosome condensation correlates with massive phosphorylation of histones and other proteins, which is mediated by e.g. Never in mitosis A (NIMA) and Aurora family members. These two kinase families as well as Polo-like kinases are also involved in centrosomal microtubule nucleation activity and centrosome separation in early M-phase. The event of de-phosphorylation is also important for progression of the different phases of the cell cycle. For example, the mentioned phosphatase Cdc25C is crucial for progression from G2- to M-phase (Nigg, 2001).

All tumour cells exhibit aberrations in their regulation of proliferation. Lung tumours frequently have aberrant expression and regulation of RB proteins, upregulated cyclin levels, inhibition of various inhibitors of Cdks and overexpression of the phosphatase Cdc25A which affect G\textsubscript{1}-S progression. In NSCLC there is also overexpression of Cdk1 and cyclin B, which increases the probability of G\textsubscript{2}-M progression (Caputi et al., 2005).

1.5 CELL CYCLE CHECKPOINTS - THE REFEREES NEEDED FOR A SUCCESSFUL CONTINUATION OF THE CYCLE

In between and within the different phases of the cell cycle, there are so-called “check points” where the process of cell division can be halted if conditions are not optimal for proceeding into the next phase (Kastan & Bartek, 2004).

Checkpoints can be found in G\textsubscript{1}-, early S-, late G\textsubscript{2}- and M-phase (the spindle-checkpoint) (Kastan & Bartek, 2004; Musacchio & Hardwick, 2002). For example, check points can be activated due to aberrations in replication in S-phase or improper alignment of chromosomes to the mitotic spindle or due to extensive DNA damage (Kastan & Bartek, 2004).

Depending on the severity of the underlying reason for stopping the cell cycle, the cell can decide to halt until the problem is removed e.g. through repair of DNA damage. However, if the abnormalities are substantial, the check point system will activate the cell death programme. In this manner, potential harmful cells harbouring mutations due to DNA damage, or cells with an abnormal amount of chromosomes due to failure of mitosis, will be removed from the organism (Kastan & Bartek, 2004).

An important regulator of the checkpoints in G\textsubscript{1}-, S- and G\textsubscript{2}-phase is the extensively studied protein p53 (Damia & Broggin, 2004; Gottifredi & Prives, 2005). Since this protein is frequently mutated in tumour cells (Liu & Gelmann, 2002), it is believed to be extremely important for preventing tumour formation. In 50 % of NSCLC tumours, one allele of p53 is mutated (Brambilla et al., 2003). In cases when p53 is not functional, cells with defects seem to escape G\textsubscript{1} checkpoints (Dixon &
Norbury, 2002). However, even though p53 is important for the accuracy of cellular activities during S- and G2-checkpoints, in cases when p53 is not functional, cells can still arrest in S- and G2-phase, resulting in a delayed M-phase entry (Dixon & Norbury, 2002; Gottifredi & Prives, 2005).

Phosphorylation is very important in the regulation of the checkpoint systems. Ataxia Telangiectacia Mutated (ATM) and ATM and Rad3-related (ATR) are two important checkpoint kinases primarily involved in the response to DNA-damaging agents and replication problems such as stalled replication forks, respectively. Upon activation these kinases phosphorylate proteins involved in DNA repair and cell cycle regulation, e.g. p53 (if present). ATM and ATR also signal to the checkpoint mediators Chk2 and Chk1 which further propagate signals to proteins involved in DNA repair, apoptosis and cell cycle regulation (Kastan & Bartek, 2004). For example, both Chk2 and Chk1 phosphorylate Cdc25C and thereby inhibit the entrance into mitosis (Donzelli & Draetta, 2003). Several studies show that Chk2 expression is reduced in NSCLC (Caputi et al., 2005).

At metaphase, as long as not all kinetochores are bound to microtubules/as long as there is lack of tension in any of the kinetochores due to absence of microtubule binding, the spindle checkpoint is activated (Pinsky & Biggins, 2005). One of the kinases involved in sensing the microtubule-mediated tension at the kinetochore is Ipl1 (Aurora) kinase (Musacchio & Hardwick, 2002).

The proteins of the spindle checkpoint inhibit the activation of proteins needed for the entrance of the cell into anaphase. Many important proteins for proper spindle checkpoint function belong to the Mad and Bub families. Until proper microtubule binding to the kinetochore, Mad2, Bub3 and BubR1 form a complex with the anaphase-promoting complex (APC) regulating factor Cdc20. Upon release of this factor APC is activated which lead to degradation of securin. When securin is degraded its binding partner separase is released and is now free to act on the cohesion that hold sister chromatids together, ultimately leading to their separation. Inhibition of anaphase onset also involves phosphorylation mediated by for example Polo-like kinases (Musacchio & Hardwick, 2002).

Checkpoint signalling is important for protecting the organism from propagation of cells with potential damages in their genome. Aberrant check point regulation thus influences both the development of cancer, as well as the outcome of treatment with anti-cancer medicines, such as DNA-damaging or microtubule-interfering agents.

1.6 MITOTIC CATASTROPHE

As described in previous sections, in a cell there is an intimate interplay between forces of survival and death, and in the majority of tumour cells the survival side wins. However, fast, unscheduled propagation without sufficient breaks makes the delicate process of division even more vulnerable. The uncontrolled mitosis leads to more mistakes such as loss or gain of chromosomes, and often also to an increase in the number of dying cells, even though most cells survive to maintain and increase the size of the tumour. If cell death stimuli are strengthened, for example by extensive DNA damage through anti-cancer treatment, even if apoptosis cannot be triggered at the G2 check point, exit from G2-phase might be fatal for the cell.
In cells that have an intact spindle checkpoint, upon DNA damage and escape from G2-phase there is a delay at metaphase. Rieder and Maiato (Rieder & Maiato, 2004) define the outcome of such a delay at metaphase of DNA-damaged cells prematurely entering metaphase as mitotic catastrophe. The outcome of the delayed mitosis may be; death by apoptosis, death by necrosis, production of two or more aneuploid cells or exit from mitosis as a double-sized cell with twice the genetic material of a normal cell. The latter occurrence might be followed by senescence, death from apoptosis or, in the case of later cell division, to different types of aneuploidy (Rieder & Maiato, 2004). Another, more recently formed, definition of mitotic catastrophe describes the phenomenon as an apoptotic cell death occurring either as a result of DNA damage or incomplete DNA replication upon failure to activate the checkpoints in G2- and M-phase (Castedo et al., 2004). If, in these cells, the apoptotic machinery is suppressed, this may lead to asymmetric cell division resulting in the generation of aneuploid offspring (Castedo et al., 2004).

There are many different scenarios that, according to the above-mentioned definitions, would lead to mitotic catastrophe. Cells that have a deficiency in their DNA structure checkpoint in G2-phase are probably at high risk for mitotic catastrophe upon DNA damage. Strengthening this assumption, there are examples of different types of manipulation of the G2 checkpoint, with or without combination with DNA damage, leading to mitotic catastrophe (Castedo et al., 2004).

Aberrations in expression and localization of cell cycle regulatory genes have also been shown to lead to mitotic catastrophe. For example, premature entering of Cdk1/cyclin B complexes into the nucleus leads to cell death. Overduplication of centrosomes, or failure in centrosome segregation, also leads to difficulties for the cell to divide (Castedo et al., 2004). Recently, the existence of a centrosome inactivation checkpoint that may provoke mitotic catastrophe through centrosome amplification or fragmentation was suggested (Loffler et al., 2006). Direct inhibition or stabilization of the mitotic spindle, which inhibits entering or exit from metaphase, induced by the hyper- or depolymerisation of microtubules by drugs like taxanes or vinca alkaloids also lead to the induction of nuclear morphologies typical for mitotic catastrophe (Castedo et al., 2004). Furthermore, repression or bypass of the spindle checkpoint due to aberrant expression of spindle-checkpoint constituents as well may lead to mitotic catastrophe (Castedo et al., 2004).

Resistance of tumour cells to DNA-damaging drugs correlates with an increase in DNA repair capacity (Gatti & Zunino, 2005), a phenomenon which is also observed in lung cancer cells (Sirzen et al., 1999). Thus, it is believed that DNA-damaged tumour cells have the capacity to stop in S- and G2-phase, bypass death induction, and instead repair the damage to such an extent that the cell cycle can go on without lethal outcome. Probably, an increased survival-related signalling contributes to the successful propagation of DNA-damaged tumour cells. One possibility to trick these tumour cells to die could be to prematurely drive them out of G2, before DNA is repaired, and to reduce survival-related signalling, which would increase the probability of mitotic catastrophe.
Fig. 4. DNA damage or incomplete DNA replication in combination with check point failure may lead to premature entering of these cells (without proper repair of the damage) into M-phase (A). Dysfunction of important mitosis-related kinases may also lead to aberrant entrance into mitosis (A). Depending on the status of the constituents of the spindle checkpoint the damaged cells in (A) may leave metaphase fast or upon a metaphase delay (B). Cells with an intact spindle check point may activate apoptosis, or if the stop at metaphase is prolonged, since gene transcription is silenced during mitosis, cell death may ensue because the cell cannot carry out its vital functions (C). Alternatively, the cell leaves metaphase without cytokinesis or through non-symmetrical division as a tetraploid cell or in the latter case alternatively as two aneuploid cells (C). In the case of tetraploidy, if p53 is present, in G1-phase cell death may be induced at the polyploidy checkpoint (C). Other vice, in the next cell division the polyploid cell may produce aneuploid cells or die due to difficulties in mitosis due to the polyploidy (D). Aneuploid cells may continue to cycle, although the sensitivity for such cells at mitosis is higher compared to normal cells (D). Surviving aneuploid cells may contribute to tumour development.

1.6.1 Where apoptosis and cell division meet

There are many morphological similarities between apoptosis and cell division. As discussed above, mitotic catastrophe often results in apoptosis. In these cases, it is possible that the underlying cause of cell death - the aberrant mitosis - is overlooked. For example, an early mitosis marker is the condensation of chromosomes, thus, in cases when apoptosis is initiated at early mitosis, this mitosis marker might be hidden by chromatin condensation due to apoptosis. Further, both apoptosis and early mitosis are characterized by degradation of the nuclear membrane although the underlying mechanism for the breakdown is not the same (Buendia et al., 2001). Moreover,
apoptosis, as well as mitosis, are recognized by rounding up of the cell and lost of adherence.

The interplay between the cell cycle and the apoptotic machinery, leading to mitotic catastrophe, is far from elucidated, yet there are known proteins that are involved in both these two processes, and thus are likely to play a role in mitotic catastrophe.

**1.6.1.1 Cdk1 and survivin**

Cdk1 and Survivin are two examples of proteins clearly involved in both apoptosis and mitosis. Cdk1 activation, which is required for progression of cells from G2- to M-phase, has also been reported in cells undergoing apoptosis, suggesting its involvement in the death process (Castedo et al., 2002; Golsteyn, 2005). Indeed, there are numerous reports of cases when apoptosis has been stopped by Cdk-inhibitors. However, there are also many reports on Cdk-inhibition triggering apoptosis, suggesting a need for clarifying the role of Cdns in different cellular contexts (Castedo et al., 2002; Golsteyn, 2005).

The protein Survivin has been implicated in mitosis and apoptosis (Altieri, 2001). Survivin is a member of the Inhibitor of apoptosis (IAP) protein gene family which includes proteins that inhibit caspase activation (Nachmias et al., 2004). It is not clear whether Survivin on its own can suppress caspase activity. However, Survivin can promote a reduced caspase activity by complex formation with, for example, the IAP XIAP (Nachmias et al., 2004).

The expression of Survivin is regulated in a cell cycle-dependent manner. Survivin level is low during G1-, S- and G2-phase and increases at G2-M transition (Li et al., 1998). In addition, Survivin is a substrate of Cdk-1 (O'Connor et al., 2000). Survivin regulates microtubule dynamics and contributes to proper spindle formation (Altieri, 2006). Moreover, Survivin contributes to the spindle checkpoint since it is required for a sustained mitotic arrest in response to lack of tension at the kinetochore (Lens et al., 2003). Survivin is often overexpressed in different types of cancer, a condition that correlates with increased survival of cells and resistance to therapy (Altieri, 2003b). In lung cancer Survivin mRNA level is increased compared to normal lung cells (Falleni et al., 2003; Monzo et al., 1999), and a decrease in Survivin expression sensitizes cultured lung cancer cells to apoptosis (Olie et al., 2000).

**1.6.1.2 Poly(ADP-ribose) polymerase is involved in apoptosis and mitosis**

Poly(ADP-ribose) polymerase (PARP) activity regulates many cellular processes, such as DNA repair, gene transcription, cell death, checkpoint function, mitosis and trafficking of endosomal particles (Burkle, 2005). Upon activation PARP catalyzes the cleavage of NAD+ into nicotinamide and ADP-ribose, of which the latter is polymerized onto acceptor proteins, such as histones, transcription factors or, in a self-regulating manner, to PARP itself (Burkle, 2005). Except for activation/inhibition of different proteins through PARP-mediated covalent binding of ribose-polymers, poly(ADP) ribose can also act non-covalently with certain proteins (Pleschke et al., 2000).
In human cells there are at least 18 isoforms of PARP (Ame et al., 2004), of which the majority have unknown specific function. The most studied PARP isoform is PARP-1, which is recognized as a nuclear DNA-nick-sensor enzyme, even though it is now known to be activated also by other stimuli, such as undamaged DNA (Burkle, 2005). In the absence of single- and double-strand breaks, poly (ADP-ribosyl)ation is a rare event in the cell (Juarez-Salinas et al., 1979). However, upon DNA damage this activity can increase 100-fold and 90% is mediated by PARP-1 (Shieh et al., 1998). Upon activation PARP-1 induces DNA repair through modification and/or activation of proteins involved in DNA repair, such as p53, histones, topoisomerases and DNA-dependent protein kinase (Burkle, 2005). Overall, it seems that PARP-1 acts as a negative regulator of DNA damage-induced genomic instability and prevents cancer development. Except from PARP-1, PARP-2 is the only known homolog to be activated by DNA strand breaks (Burkle, 2005).

There have been observations of an initial burst of PARP activation in apoptosis (Scovassi et al., 1998; Simbulan-Rosenthal et al., 1998), suggesting requirement for PARP in apoptosis, at least in some cellular contexts. However, since PARP-1 is cleaved by caspase-3 and -7, inhibition of PARP might be necessary for proceeding of the apoptotic process. Since apoptosis is ATP-dependent (and thus indirectly NAD⁺ dependent) and PARP-mediated NAD⁺ depletion leads to necrosis, one hypothesis is that PARP, merely due to its influence on cellular energy stores, may serve as a molecular switch between these two modes of death (Virag & Szabo, 2002). However, PARP might also play a more direct role in apoptosis. PARP interact with the ribosomal protein S3a and Bcl-2 (Song et al., 2002). Furthermore, PARP isoforms have been identified in mitochondria (Du et al., 2003). It has also been shown that AIF translocation from the mitochondria to the nucleus is dependent upon PARP-1 activity (Du et al., 2003; Yu et al., 2002).

PARP isoforms have been detected at other locations than mitochondria and nucleus, (Burkle, 2005) including nuclear pores, centrosomes (Smith & de Lange, 1999) and centromeres, where they interact with the constitutive centromere proteins Cenpa and Cenpb as well as the checkpoint protein Bub3, and poly(ADP-ribosyl)ate them (Saxena et al., 2002a; Saxena et al., 2002b). Tankyrase-1-mediated poly(ADP-ribosylation) is required for spindle assembly and function (Chang et al., 2005) and PARP-1 seem to be involved in centrosome duplication by poly(ADP-ribosylation) of p53 (Kanai et al., 2003; Kanai et al., 2000). Interestingly, in line with the mode of regulation of many cell cycle regulatory proteins, the activation of the PARP isoform tankyrase that is found at the nuclear pores and centrosomes, is regulated by the phosphorylation state of the protein (Chi & Lodish, 2000).

As described above, different PARP isoforms are involved in DNA repair, cell death induction, spindle checkpoint regulation and centrosome duplication, indicating a possible role for PARP in mitotic catastrophe. Indeed, there are studies showing PARP-mediated protection of cells from defective mitosis. Cells derived from PARP-1⁻/⁻ mice escape a colcemid-induced mitotic stop faster as compared with cells from PARP-1⁺/⁺ mice (Halappanavar & Shah, 2004). Instead of apoptosis, which is induced in the PARP⁻/⁻ cells, cells lacking PARP show endoreplication. It has also been shown that upon DNA damage the histone H3 phosphorylating protein Aurora A associates with PARP-1 and becomes highly poly(ADP-ribosylated) and thereby inhibited (Monaco et al., 2005), indicating that PARP-1 is a propagator of inhibitory signals to the mitotic machinery upon DNA damage. On the other hand, a study on epithelial cells suggested...
that PARP might be involved in promoting mitotic catastrophe, since the premature chromatin condensation and histone H3 phosphorylation induced by reactive oxygen species were inhibited by the PARP inhibitor 3-aminobenzamide (Tikoo et al., 2001). However, 3-aminobenzamide also acts as an antioxidant (Virag & Szabo, 2002), which makes it difficult to evaluate the specific role of PARP in this study. Taking into consideration the various cellular effects mediated by PARP isoforms, in combination with the possible “stand-in” function of different isoforms as well as the side effects and unknown isoform specificity of different PARP inhibitors, it is clear that a lot of experimental effort is needed to reveal the secrets of PARP.

1.7 KINASES ARE OFTEN ABERRANTLY REGULATED IN LUNG CANCER CELLS – INCREASED KNOWLEDGE ABOUT THEIR CONTRIBUTION TO CELLULAR RESISTANCE WILL LEAD TO NEW TREATMENT STRATEGIES

There are numerous aberrations in kinase signalling that may be involved in development and maintenance of lung tumours. In the present study, initially the role of protein kinase C in NSCLC cell resistance was in focus, and eventually focus was changed towards MEK-1 and Phosphoinositide 3-kinase.

1.7.1 The Protein kinase C family

There are at least 11 members of the protein kinase C (PKC) family; the classical PKCs (cPKC) α, β and γ, the novel PKCs (nPKC) δ, ε, η and θ and the atypical PKCs (aPKC) μ, ζ, ι and ν (Basu, 1993; Koivunen et al., 2006).

The three subgroups of PKC are all serine-threonine kinases but differ in their requirements for activation. Phospholipase C activation, due to growth factor binding of a receptor, leads to generation of diacylglycerol (DAG) and inositoltriphosphate (IP3), of which the latter mediates the release of Ca^{2+} from intracellular stores. cPKC needs both DAG and Ca^{2+} for full activation, whereas nPKC only requires DAG. aPKC activation is not as explored but may involve phosphorylation by other PKCs. (Basu, 1993; Koivunen et al., 2006).

PKCs contain a regulatory and a catalytic domain and the activation step involves a conformational change leading to separation of these two domains, for example through partial insertion into membranes mediated by interaction with DAG (Basu, 1993; Yin & Ochs, 2001).

1.7.1.1 Protein kinase C and cancer

More than twenty years ago PKC was found to be the primary receptor of DAG-mimicking cancer-promoting phorbol esters (Niedel et al., 1983; Sando & Young, 1983), and therefore the involvement of PKC in cancer has been studied since. As phorbol esters, such as phorbol 12-myristate 13-acetate (PMA), upon addition first activates PKC, but after prolonged exposure decreases the expression of PKC, the evaluation of experimental data on the role of PKC in early cancer development is
complicated. In addition, nowadays, several alternative effectors of DAG are known (Brose & Rosenmund, 2002).

When activated, PKCs translocate from the cytoskeleton to the plasma membrane, cytosolic organelles or to the nucleus. PKCs are involved in regulation of gene expression, cell proliferation, differentiation and apoptosis (Martelli et al., 2006). Overall, it seems that there is variable expression of PKC isoforms in different types of cancer, and therefore, based on expression patterns, no general conclusions concerning the involvement of PKC in cancer can be drawn (Koivunen et al., 2006). About 20% of the NSCLC patients have a high expression of PKC α (Lahn et al., 2004). Most studies on various cell types favour a role for PKC α in promoting resistance to anti-cancer treatment (Koivunen et al., 2006). Indeed, inhibition of PKC α decreased the tumorigenicity of human lung cancer xenografts in nude mice (Wang et al., 1999), suggesting the involvement of PKC α in survival promotion of lung cancer cells. Of the other two cPKCs, PKC β is the most well studied and just as PKC α, this isoform seems to be predominantly tumorigenic (Koivunen et al., 2006).

Compared to the other PKC subgroups, not so much is known about the aPKCs, and of the nPKCs, the best studied isoforms are δ and ε. In most cell types PKC δ seems to have a pro-apoptotic function (Koivunen et al., 2006). Based on experiments with rat fibroblasts, it has been suggested that the tumour promoting function of phorbol esters is due to depletion of PKC δ (Lu et al., 1997). This PKC isoform seems to take part in several different apoptosis-related events. For example, PKC δ has been reported to promote apoptosis independently of its kinase activity (Goerke et al., 2002). Moreover, upon activation by caspase-3, this PKC isoform is activated and involved in lamin cleavage (Cross et al., 2000). There are several studies showing that PKC δ targets mitochondria and alters its membrane potential, leading to release of cytochrome c and caspase activation (Horbinski & Chu, 2005). However, inhibition of PKC δ, by the specific inhibitor rottlerin, stimulates AIF release in Tumor necrosis factor α–mediated death of HeLa cells (Basu et al., 2002).

Even though most reports indicate PKC δ being a pro-apoptotic kinase in various cell types, in lung cancer cells PKC δ might have the opposite role. Asbestos, which is a family of mineral fibers known to promote development of lung cancer, has been shown to induce the expression and activity of PKC δ in mice (Lounsbury et al., 2002). Moreover, in a panel of human-derived NSCLC cell lines, inhibition of PKC δ sensitized cells to DNA damage-induced apoptosis in a kinase activity-dependent fashion (Clark et al., 2003).

PKC ε in general seems to stimulate tumorigenesis (Akita, 2002). In lung cancer cells, the expression and activation of PKC ε is cell line dependent (Baxter et al., 1992; Ding et al., 2002; Kim et al., 1992) and its expression has been shown to promote survival of DNA-damaged lung cancer cells through inhibition of apoptosis (Ding et al., 2002).

Taken together, one might conclude that several members of the PKC family are implicated in resistance of lung cancer cells.

1.7.2 The MEK/ERK-pathway and its involvement in lung cancer

Many regulatory mechanisms of proliferation, differentiation and apoptosis involve mitogen-activated protein kinase (MAPK) cascades (Hagemann & Blank, 2001). These
signalling systems can be activated by membrane receptors upon stimulation on the cell surface.

Each MAPK pathway consists of three kinases. Upon receptor-mediated stimulation, the MAPK kinase kinase (MAPKKK) phosphorylates specific serines and threonines in the downstream MAPK kinase (MAPKK), which, upon this activation, phosphorylates critical tyrosine and threonine residues in its target protein, the MAPK. The MAPKs are serine-threonine kinases that phosphorylate cytosolic proteins, or translocate to the nucleus where they are involved in regulation of gene expression through phosphorylation of transcription factors (Hagemann & Blank, 2001).

Proteins of the extracellular regulated kinase (ERK) family of MAPK are activated by a MAPKK called mitogen-activated protein kinase/extracellular-regulated kinase kinase (MEK). MEK is commonly activated by Raf upon stimulation of the small G protein Ras (Hagemann & Blank, 2001). Another upstream activator is PKC (Jarvis & Grant, 1999), a family of kinases discussed above. The MEK/ERK pathway can also be stimulated by MEKK family members, although these MAPKKKs preferentially are upstream activators of the MAPKs c-Jun NH2-terminal kinase (JNK) and p38 (Hagemann & Blank, 2001). JNK and p38 are most often recognized as being pro-apoptotic (Matsukawa et al., 2004), and there are examples of a pro-apoptotic function of p38 and JNK also in NSCLC cells (Chuang et al., 2000; Thrane et al., 2001). However, there are also reports of p38 (Cosaceanu et al., 2006; Horowitz et al., 2004), and JNK (Lee et al., 2005a; Lee et al., 2003) promoting survival of lung cancer cells.

The Ras/Raf/MEK/ERK pathway is involved in events leading to both proliferation and cell cycle arrest, for example through regulation of gene transcription (Chang et al., 2003). MEK/ERK signalling can also mediate inhibitory phosphorylation of the Bcl-2 family member Bad, and, thereby, inhibit apoptosis (Scheid et al., 1999).

Aberrations in MEK/ERK signalling might play a role in NSCLC resistance. For example, constitutive ERK activity has been found in NSCLC cell lines (Brognard & Dennis, 2002) as well as in advanced tumours (Vicent et al., 2004). Further, in NSCLC, Ras was found overexpressed more often as compared to in SCLC (Joseph et al., 2000). However, it is not clear if Ras contributes to the higher resistance of NSCLC cells (Joseph et al., 2000). MEK/ERK is also acting downstream of epidermal growth factor receptor that is often aberrantly regulated in NSCLC (Raben et al., 2004).

The role of ERK in death regulation of NSCLC cells is not clear. ERK activity inhibits NSCLC cell death induced by cadmium (Chuang et al., 2000) and fluoride (Thrane et al., 2001). On the contrary, the MEK/ERK pathway is required for apoptosis induced by products isolated from plants, such as quercetin (Nguyen et al., 2004; Nguyen et al., 2003). However, ERK inhibition has no effect on apoptosis induced by the topoisomerase II inhibitor doxorubicin in NSCLC cells (Zhao et al., 2004).

There are also variable reports in NSCLC about the role of MEK/ERK for cell death induced by microtubule interacting drugs that are known to activate the spindle check point. ERK activity is necessary for paclitaxel-induced cell death (Suyama et al., 2004). However, other studies have shown that MEK inhibition (Hu et al., 2003; MacKeigan et al., 2000), or overexpression of a dominant-negative MEK (Brognard & Dennis, 2002), sensitize lung cancer cells to paclitaxel.

MEK inhibition also decreases reactive oxygen species-induced histone phosphorylation and premature condensation of chromatin in lung cells (Tikoo et al., 2001), indicating a role for MEK in mitotic catastrophe, which is in line with the
finding that MEK activity is required for lung cell cycle progression (Suyama et al., 2004; Thrane et al., 2001).

Overall, the many conflicting results about the role of MEK/ERK signalling in lung cancer tell us that this topic is far from elucidated.

1.7.3 Phosphoinositide 3-kinase signalling

Many signalling pathways involve Phosphoinositide 3-kinases (PI3-kinases) (Vanhaesebroeck & Waterfield, 1999). These kinases are cytosolic or membrane-bound and consist of two subunits: one regulatory and one catalytic.

PI3-kinases are generally not protein kinases, instead they phosphorylate the 3’OH position of the inositol ring of the lipid phosphatidylinositol (PtdIns) or of phosphoinositides (PI) which are phosphorylated derivatives of PtdIns. These phosphorylated PIs and PtdIns act as secondary messengers that activate downstream proteins, including kinases.

There are multiple isoforms of PI3-kinases that all share a homologous region in their catalytic domain, yet have different specificities for PtdIns and PIs (PtdIns(4)P and PtdIns(4,5)P2). Differences in the upstream activation of the various PI3-kinase isoforms further contribute to the complexity of PI3-kinase signalling. For example, some are regulated by receptor tyrosine kinases and others by heterotrimeric G-proteins (Vanhaesebroeck & Waterfield, 1999). Ras is involved in the regulation of some isoforms (Chang et al., 2003; Vanhaesebroeck & Waterfield, 1999). Moreover, the catalytic domains of the PI3-kinases can be combined with different adaptor domains that are interacting with various signalling pathways, which make the PI3-kinase signalling even more intricate. There are also reports about PI3-kinase isoforms being capable of auto- or inter-subunit protein phosphorylation, and there might also be other target proteins of PI3-kinases (Vanhaesebroeck & Waterfield, 1999).

PtdIns and PI function as key mediators of intracellular signalling (Vanhaesebroeck & Waterfield, 1999). The level of PtdIns(3)P remains relatively constant, whereas PtdIns(3,4)P2, PtdIns(3,5)P2 and PtdIns(3,4,5)P2 (PIP3) levels can rise upon stimulation. In addition to PI3-kinase-mediated activities, the level of different PIs can be regulated by kinases and phosphatases that act on the inositol ring (Vanhaesebroeck & Waterfield, 1999). PTEN is an important lipid phosphatase involved in removing phosphate groups from key intracellular PI signalling molecules (Sansal & Sellers, 2004).

The products of PI3-kinases and PI-phosphatases bind to two different specific sequences of target proteins (Vanhaesebroeck & Waterfield, 1999). PtdIns bind to FYVE domains consisting of 60-80 amino acids and PIs bind to protein domains of about 100 amino acids called pleckstrin homology (PH) domains. The FYVE domain is most often found in proteins involved in membrane trafficking. The PH domain is more common compared to the FYVE domain, and is found in various kinds of proteins including protein kinases, phospholipases, adaptor proteins, GTP-ase activating factors and nucleotide-exchange factors (Vanhaesebroeck & Waterfield, 1999).
1.7.3.1 PI3-kinase downstream targets involved in mitosis and apoptosis

PI3-kinase can regulate activation of PKC isoforms (Duronio et al., 1998), which might influence cancer progression. The most studied PI3-kinase downstream target involved in cellular survival is the protein kinase Akt/PKB (Datta et al., 1999). This protein is translocated to the plasma membrane by PI3-kinase signalling. At the membrane 3-phosphoinositide-dependent kinases (PDKs) activate Akt by phosphorylation of site Thr-308 and Ser-473. Once activated Akt phosphorylates downstream targets and thereby promotes survival of the cell (Datta et al., 1999).

PI3-kinase activation is required for G1/S progression (Liang & Slingerland, 2003). For example, PI3-kinase activity is known to inhibit expression of FOXO transcription factors involved in regulation of G1/S transition and to promote expression of cyclin E and D (Martinez-Gac et al., 2004).

PI3-kinase/Akt signalling may also be necessary for transition of cells from G2- to M-phase (Lee et al., 2005b; Liang & Slingerland, 2003), while downregulation of this pathway is essential for a proper passage through anaphase (Liang & Slingerland, 2003). Chk-1 is a target of the PI3-kinase/Akt pathway, although the effect of phosphorylation of Chk-1 might be site- and cell type-specific (Jin & Woodgett, 2005; King et al., 2004; Shtivelman et al., 2002). Overall, the involvement of PI3-kinase signalling in G2/M seems to be complicated, since too much as well as too little Akt activity trigger G2-arrest (Roberts et al., 2002).

Akt is a regulator of cell death (Luo et al., 2003). For example, Akt can modify apoptosis through phosphorylation of kinases involved in gene transcription. Akt negatively regulates apoptosis-related kinases, such as glycogen synthase kinase -3 (GSK-3) (Brunet et al., 2001) and p38/JNK (Franke et al., 2003) and is involved in survival-related activation of NF-kappaB (Brunet et al., 2001).

Akt can also more directly interfere with the apoptotic machinery. Just as ERK, Akt phosphorylates and thereby inhibits Bad, although Akt targets another site of this Bcl-2 family protein (Datta et al., 1997). Moreover, Akt inhibits Bax translocation to mitochondria (Tsurtu et al., 2002), and cleavage of the Bcl-2 family member Bid (Kandasamy & Srivastava, 2002). Furthermore, it has been shown that Akt function in concert with mTOR, and together increase resistance to microtubule-directed agents (VanderWeele et al., 2004) by a mechanism involving Bcl-2 phosphorylation (Asnaghi et al., 2004). There are also studies showing that Akt is involved in regulation of Survivin expression (Belyanskaya et al., 2005; Ohnishi et al., 2006). Overall, it seems that Akt acts to inhibit apoptosis.

Chemotherapy-induced mitotic catastrophe in human glioma cells is decreased by Akt overexpression (Hirose et al., 2005). Apart from the study on glioma cells, there is no thorough investigation of the involvement of Akt in mitotic catastrophe.

1.7.3.2 PI3-kinase-like protein kinases

The PI3-kinase-like protein kinases (PIKKs) contain a kinase domain exhibiting a significant homology to the core domain of PI3-kinase (Shiloh, 2003). However, instead of lipids, the substrates of these PIKKs are Ser/Thr residues of target proteins.

There are five known PIKKs; DNA-dependent protein kinase (DNA-PK), Ataxia telangiectasia mutated (ATM), ATM and Rad3-related (ATR), mammalian target of rapamycin (mTOR) and ATX.
Four of the PIKKs (all except for mTOR) are known to be involved in regulation of DNA damage response. ATM and ATR are two checkpoint kinases that share several targets, and both are activated by, and repair, DNA double strand breaks. ATR also responds to UV-light, hypoxia, and, as mentioned earlier, stalled replication forks. DNA-PK is directly involved in the non-homologous end-joining type of DNA double strand break repair (Shiloh, 2003). mTOR regulates protein translation, cell growth and cell cycle progression (Fingar & Blenis, 2004).

1.7.3.3 Involvement of PI3-kinase, PI3-kinase downstream targets and PIKKs in lung cancer

PI3-kinase is frequently overexpressed in lung cancer (Lin et al., 2001) and Akt is often phosphorylated in NSCLC (Balsara et al., 2004; Lee et al., 2002; Shah et al., 2005; Tsurutani et al., 2006), yet there are conflicting results regarding the prognostic significance of phosphorylated Akt (Shah et al., 2005; Tsurutani et al., 2006). In NSCLC (Brognard et al., 2001; Nakashio et al., 2000) as well as in SCLC (Kraus et al., 2002; Krystal et al., 2002; Moore et al., 1998) cell lines Akt is overexpressed, which increases survival and correlates with resistance to apoptosis induced by chemotherapy and irradiation. Akt activation can also increase invasion in vitro (Balsara et al., 2004).

Akt can promote survival of lung cancer cell by several different mechanisms. Tobacco components stimulate Akt activation (West et al., 2003), which lead to phosphorylation of the Bcl-2 family protein Bad (Jin et al., 2004) and NFkappaB-dependent survival (Tsurutani et al., 2005), as well as to increased growth due to Akt/mTOR signalling (Han et al., 2006). Another Akt-mediated mechanism of resistance might be expression of Survivin, since in NSCLC cells PI3-kinase inhibition leads to downregulation of this protein (Ohnishi et al., 2006) and in SCLC cells Survivin expression is regulated in an Akt-dependent manner (Belyanskaya et al., 2005). Apoptosis triggered by the extrinsic pathway is inhibited by Akt on the level of Bid (Kandasamy & Srivastava, 2002). Moreover, it has been shown that Akt activation correlates with increased activity of mTOR and Forkhead in NSCLC tumours (Balsara et al., 2004).

PIKKs are also implicated in survival of lung cancer cells. For example, a high DNA-PK content and activity correlates with radioresistance of NSCLC cells (Guo et al., 2005; Sirzen et al., 1999), and inhibition of DNA-PK sensitizes this type of cells to DNA damage (Eriksson et al., 2001; Omori et al., 2002; Sak et al., 2002). A high DNA-PK protein level gives resistance to DNA damage in SCLC (Hansen et al., 2003a; Hansen et al., 2003b), as well. However, a low DNA-PK activity is associated with an increased risk for development of lung cancer (Auckley et al., 2001) and DNA-PK repression is found at early stages of lung cancer (Iwanaga et al., 2005), suggesting a tumor stage dependent role of DNA-PK.

ATM might also be involved in development of lung cancer since the ATM genotype seems to affect the risk for appearance of this type of disease (Kim et al., 2006). There are several studies indicating involvement of mTOR in NSCLC. Inhibition of mTOR induced anti-tumour response in NSCLC patients (Gomez-Martin et al., 2005) and in lung adenocarcinomas mTOR activity correlates with gene alterations in EGFR (Conde et al., 2006), a receptor known to be involved in survival of lung cancer cells. In NSCLC cells, mTOR activity is important for Ras-dependent
growth (Wislez et al., 2005) and inhibition of mTOR sensitizes cells to DNA damage (Wu et al., 2005). mTOR inhibition in NSCLC cells can also activate Akt in a PI3-kinase dependent manner, and simultaneous inhibition of both these kinases give a better growth inhibition (Sun et al., 2005).

Taken together, it is clear that, by regulation of different mechanisms, the PI3-kinase/Akt pathway and PIKKs are involved in lung cancer cell survival.

1.8 KINASE INHIBITORS FOR LABORATORY USE AND FOR TREATMENT OF DISEASE

As exemplified in previous sections, protein kinases are important regulators of cellular life and death. The contribution of kinases to tumorigenesis has been studied for over 25 years (Cohen, 2002).

Since aberrant protein phosphorylation strongly contributes to the development of cancer, as well as to other diseases, a lot of effort has been put into identifying, and developing inhibitors of, the kinases involved (Cohen, 2002; Dancey & Sausville, 2003). At present there are numerous kinase inhibitors undergoing clinical trials. One major problem with many kinase inhibitors is the lack of specificity. More than 500 protein kinases are encoded by the human genome, and since most inhibitors exert their action through binding to the ATP-binding site, which is much conserved, the majority of inhibitors target a broad range of kinases (Cohen, 2002; Dancey & Sausville, 2003). Depending on the cell type and the cellular event, different signalling pathways interfere with each other in various ways and at different time points. This leads to further complication in the identification of kinases that are contributing to a disease.

1.8.1 Staurosporine and its analogs and other inhibitors of the MEK pathway

Staurosporine (STS) is a well studied microbial agent that was discovered almost 30 years ago (Omura et al., 1995), and was one decade later shown to be a potent inhibitor of protein kinase C (PKC) (Tamaoki et al., 1986). Since PKC was one of the first kinases identified as involved in cancer (Castagna et al., 1982), and STS was found relatively unselective in its kinase inhibition (Fabbro et al., 1999), the pharmaceutical industry started to modify the STS molecule to produce various derivatives (Cohen, 2002). These STS analogs were made as PKC specific as possible (Davis et al., 1992a; Davis et al., 1992b; Wilkinson et al., 1993).

Many of the STS derivatives have been used in numerous experimental studies, and two of them, namely, N-benzoyl staurosporine (PKC 412/CGP 412 51/ midostaurin) and 7-hydroxystaurosporine (UCN-01), have entered clinical trials for treatment of advanced cancers, including lung cancer (Cohen, 2002; Monnerat et al., 2004). However, the STS analogs are nowadays known to inhibit other targets than PKC, which means that their effects in cancer cells might be mediated by inhibition of other kinases than PKC (Cohen, 2002; Fabbro et al., 1999; Gescher, 1998; Gescher, 2000). For example, the anti-tumour effect of UCN-01 in lung cancer is believed to occur primarily through inhibition of cell cycle regulatory proteins such as Chk-1 (Busby et al., 2000; Mack et al., 2003; Senderowicz, 2000). PKC 412 is known to inhibit e.g. platelet-derived growth factor, stem cell factor and vascular endothelial
growth factor receptor at similar concentrations required for inhibition of some of the PKC isoforms (Fabbro et al., 1999).

In my studies of cell death regulation in lung cancer cells, I have used STS and two STS derivatives; PKC 412 and the bisindolylmaleimide Ro 31-8220. As described in previous sections, PKC is acting upstream of MEK/ERK. Therefore, I have also used the compound PD 98059, which suppresses the activation of MEK-1 (Davies et al., 2000). This compound, as well as another called U0126, is commonly used for inhibition of MEK-1 activation in laboratory experiments. Important inhibitors of the MEK/ERK pathway in lung cancer treatment are Gefitinib (Iressa, ZD1839) and erlotinib that are both undergoing clinical trials for applicability in lung cancer treatment (Mendelsohn & Baselga, 2006). Both these medicines are inhibitors of the epidermal growth factor receptor (EGFR) which is upstream of the Ras-dependent MAPK cascade (Blackhall et al., 2006).

![Fig. 5. The structures of Staurosporine, Ro 31-8220 and PKC 412.](image)

### 1.8.2 Inhibitors of PI3-kinase and PIKKs

The two most widely experimentally used PI3-kinase inhibitors are wortmannin and LY-294002. Wortmannin was originally isolated from *Penicillium Wortmannii* (Walker et al., 2000) while LY-294002 is a synthetic compound based on the structure of the flavonoid quercetin (Vlahos et al., 1994). Both compounds bind to the ATP-binding site of the PI3-kinase catalytic domain (Walker et al., 2000). Wortmannin most closely fills the reactive site, which is reflected in a better inhibition mediated by this compound. Since wortmannin is rather unstable in solution, even though this compound is a more specific PI3-kinase inhibitor compared to LY-294002, for long term studies the latter might be preferred (Walker et al., 2000).

LY-294002 and wortmannin also inhibit PIKKs (Davies et al., 2000; Izzard et al., 1999; Smith et al., 1999; Vanhaesebroeck & Waterfield, 1999) and rapamycin is a widely used specific inhibitor of mTOR (Davies et al., 2000). Important inhibitors of the PI3-kinase/Akt pathway in lung cancer treatment are Gefitinib (Iressa, ZD1839) and erlotinib (Blackhall et al., 2006).
1.9 KNOWLEDGE ABOUT EFFECTS OF MEDICINES ON CELLULAR PROCESSES IS DETERMINING SUCCESSFUL TREATMENT

Most lung cancer patients are not cured by the available therapy approaches. One obvious problem is that NSCLC and SCLC are not two diseases with two optimal treatments. Lung cancer evolvement involves uncontrolled growth and spread of cells, but for each tumour the underlying reason for growth might be different. Therefore, there is probably one optimal medicine/combination of medicines for every single tumour.

In clinical studies, when comparing the outcome of two different treatments in patients, even though one medicine gives a better overall outcome, it is impossible to know if some of the patients would have benefited more from the other treatment. Thus, based upon results from this type of studies, it is hard to predict which patients will gain from a specific medicine. In the future, many clinical studies will probably involve identification of gene-expression and post-translational modifications, aiming to characterize tumours that show resistance or response to a specific treatment (De Petris et al., 2006). By this approach it may be possible to tailor the treatment strategy according to the geno-phenotype of a specific tumour.

To enable improvement of lung cancer treatment, there is also a need for increased knowledge about underlying mechanisms responsible for driving the growth of tumours. Subsequent disclosure of the key constituents of such mechanisms is important for enabling the construction of medicines that are effective in disarming tumours that express these particular targets. Gleevec is an example of an effective targeted drug used in chronic myelogenous leukaemia (CML). This medicine targets the Abelson tyrosine kinase that upon chromosome rearrangement most often is the important cause of CML (Cohen, 2002). There are also ongoing studies concerning the tumour specificity of the EGFR-targeting drug Gefitinib. There are indications that specific EGFR gene mutations and/or amplification confer a particularly sensitive phenotype for Gefitinib (Cappuzzo et al., 2006).

Thus, in the future, there might be tailored treatment of lung cancer involving conventional medicines, as well as medicines specifically targeting a known aberration, such as an aberrantly regulated kinase in a signalling pathway known to cause disease.

For successful future treatment, there is also a requirement for more information about how cellular life and death regulation interfere with the action of medicines. For example, in cells with an intact p53, the M-phase blocker paclitaxel is not effective and the reason for this might be that p53 blocks mitotic catastrophe by activating a polyploidy check point (Castedo et al., 2004). Paradoxically, in this case the tumour suppressor p53 indirectly suppresses the action of the drug, just by acting normal.

DNA-damaging medicines are other examples of drugs that are highly dependent upon the status of cellular life and death regulatory systems. The efficiency of a DNA-damaging medicine relies upon the cellular status of DNA repair systems and check point proteins activated by the specific DNA damage that is induced (Roos & Kaina, 2006). To ensure treatment response by a specific DNA damaging drug, one must as well make sure that the drug is allowed to act in the phase of the cell cycle where it is efficient. For example, medicines that induce secondary DNA damage due to inhibition of proteins involved in replication, such as topoisomerase inhibitors (e.g. etoposide) are
suggested to be particularly effective in S-phase when DNA replication occurs (Kaufmann, 1998).

There is an increased interest in combining different types of drugs in order to increase treatment response. In these cases it is important to understand the underlying mechanisms of the medicines, since this information helps when developing efficient treatment protocols. For example, the anti-tumour effect of the staurosporine analog UCN-01 in combination with the DNA-damaging drug cisplatin is dependent upon the order of drug administration. In lung cancer cells UCN-01 was most effective when added after DNA damage induced by cisplatin, probably because of its inhibitory effect on Chk-1, leading to mitotic catastrophe (Mack et al., 2003).

Thus, in order to improve anti-cancer treatment, increased knowledge of how various cellular processes are regulated, in combination with information about how different medicines interact with these cellular processes, is extremely important. This information can help in the identification of cellular aberrations critical for tumour growth, which is important for enabling specification of suitable targets of future drugs. Furthermore, this knowledge gives the possibility to predict if, in a specific cell, the cellular process that the drug is supposed to activate, or inhibit, may at all be affected by the drug. Information about how medicines interact with cellular processes can also be used when evaluating the effect of medicine combinations, since if administered in the wrong order the drugs might inhibit the action of each other.
2 THIS STUDY

2.1 MAIN GOAL

The main goal of this project was to understand the function of the apoptotic machinery in human lung cancer cells resistant to anti-cancer DNA-damaging treatment and to investigate if the efficiency of the killing of these cells could be modified by using inhibitors of phosphorylation-based signalling.

2.1.1 Specific aims

Specific aims of the study were:
1) to analyze if staurosporine analogs activate common markers of apoptosis in NSCLC cells.
2) to identify survival-related phosphorylation-based signalling in NSCLC cells.
3) to investigate the possibility of modifying the efficiency of the killing of this type of tumours by combining DNA damage with kinase inhibition.
3 RESULTS AND DISCUSSION OF PAPER 1-4

In the present study the survival of lung cancer cells resistant to DNA damage-inducing agents was challenged by treatment of these cells with staurosporine, staurosporine analogs or PI3-kinase and MEK inhibitors alone, or in combination with agents inducing DNA damage. Resistance mechanisms of these cells were sought on the level of apoptosis. With time, and an increasing amount of data, it became obvious that cell death induction in most cases was cell cycle dependent, and focus was therefore also directed towards this aspect.

3.1 APOPTOSIS MECHANISMS INDUCED BY TREATMENT WITH STAuroSPORINE, PKC 412 AND ETOPOSIDE (PAPER 1, 2, 4)

3.1.1 Staurosporine-induced nuclear apoptosis of U1810 NSCLC cells is determined by apoptosis inducing factor

Staurosporine (STS) is a potent and fast inducer of apoptosis in lung cancer cells (Joseph et al., 2002). In non-lung derived cells STS-induced death has been reported to occur in a Bax-dependent manner (Kashkar et al., 2002; Tafani et al., 2001), involving Bad and mitochondrial permeability transition (MPT) (Tafani et al., 2001), suggesting that STS-induced death involves activation of the intrinsic pathway. There are reports of both caspase-dependent (Belmokhtar et al., 2001; Rocha et al., 2000) and caspase-independent (Belmokhtar et al., 2001; Joseph et al., 2002; Rocha et al., 2000; Stepczynska et al., 2001) apoptosis upon STS treatment.

Using various techniques we showed that STS-induced death of U1810 non-small cell lung carcinoma (NSCLC) cells of the large cell carcinoma subtype (Bergh et al., 1985) is dependent upon mitochondrial release of apoptosis inducing factor (AIF) and translocation of AIF into the nucleus (paper 1). Caspase inhibition could not rescue U1810 cells from STS-induced death (paper 1). These findings support the model of a resistance mechanism that functions through inhibition of transportation of caspase-3 into the nucleus (Joseph et al., 2001; Joseph et al., 2002). In U1810 cells, DNA damage induces a relatively high caspase-3-like activity, without corresponding nuclear apoptosis (Joseph et al., 2001). STS somehow increases the mitochondrial release of AIF and its translocation into the nucleus, which precedes the appearance of caspase-3 in the same location (Joseph et al., 2002).

3.1.2 PKC 412 induces caspase-independent apoptosis in U1810 cells and caspase-dependent apoptosis in U1285 SCLC cells

STS is a non-selective kinase inhibitor and therefore this compound is neither suitable for use in treatment of patients, nor for identification of survival-determining signalling in experimental studies. Nevertheless, the clue to relief of resistance seemed to be kinase inhibition. We therefore investigated the induction of apoptotic features upon treatment with the STS analogues PKC 412 and Ro 31-8220. Both these compounds...
are more specific protein kinase C inhibitors compared to STS (Davis et al., 1992a; Fabbro et al., 1999). Since PKC 412 is in clinical trials, involving lung cancer patients (Monnerat et al., 2004; Propper et al., 2001), investigation of effects mediated by this STS analogue in lung cancer cells was of particular interest.

PKC 412 has been shown to inhibit growth of a variety of tumour-derived cell lines (Fabbro et al., 1999), including small cell lung cancer (SCLC) and NSCLC (Courage et al., 1995; Courage et al., 1996; Fabbro et al., 1999; Ikegami et al., 1996a; Ikegami et al., 1996b). There are several reports of PKC 412-induced death with apoptotic features in various tumour types (Begemann et al., 1996; Ganeshaguru et al., 2002; Ikegami et al., 1995; Sedlak et al., 1995; Tenzer et al., 2001), including NSCLC (Ikegami et al., 1995), but not SCLC (Ikegami et al., 1996b).

Ro 31-8220 has been shown to reduce growth of a NSCLC cell line (Courage et al., 1995; Courage et al., 1996), and induces apoptosis in non-lung derived cells (Begemann et al., 1998; Han et al., 2000).

We found that PKC 412 was a better inducer of nuclear condensation compared to Ro 31-8220, when tested in NSCLC and SCLC cell lines U1810 and U1285, respectively (paper 2). In contrast with our data, in other lung cancer cells, Ro 31-8220 has been reported more toxic compared to PKC 412 (Courage et al., 1996). However, in the study by Courage at al., the mode of death was not investigated, and higher concentrations of Ro 31-8220 were used.

PKC 412 induced nuclear condensation in a higher percentage of U1285 cells, compared to the level observed in cultures of U1810 cells (paper 2). In both cell lines, U1810 and U1285, death was associated with a drop in mitochondrial membrane potential, release of mitochondrial cytochrome c and AIF into the cytoplasm, as well as an increase in caspase-3-like activity (paper 2). In U1285 cells, formation of condensed nuclei was caspase dependent and correlated with formation of small molecular weight DNA fragments, which was not the case in U1810 cells (paper 2). PKC 412-induced nuclear apoptosis of U1810 cells was associated with formation of high molecular weight DNA fragments, suggesting an AIF dependency of PKC 412-induced death (paper 2), which was later confirmed (paper 4). These data suggest that PKC 412-induced nuclear apoptosis of U1285 and U1810 cells relies on disturbance of the mitochondrial membrane. However, downstream of mitochondrial events there is a difference in death regulation in the two cell lines.

In U1285 cells, there was a time-dependent increase in the amount of cells exhibiting nuclear condensation, which correlated with an increase of release of mitochondrial proteins and increased caspase activity (paper 2). In contrast, in U1810 cells the levels observed of mitochondrial proteins in cytosolic fractions, caspase activity and cells exhibiting nuclear condensation were not increased after 24h (paper 2). Since caspase activation has been shown to trigger mitochondrial membrane permeability transition (Marzo et al., 1998b), a low caspase activity level might trigger a further increase in, and irreversibility of, mitochondrial destabilization. Thus, the high responsiveness of U1285 cells to PKC 412 might be explained by a caspase-mediated mitochondrial downstream feedback mechanism of this cell line, perhaps in combination with a facilitated transportation of caspase-3 into the nucleus.
3.1.3 Etoposide-induced death of U1810 cells also involves AIF

PKC 412 sensitized U1810 cells to DNA damage induced by the topoisomerase II inhibitor etoposide or gamma radiation (paper 2). Upon simultaneous treatment of U1810 cells with PKC 412 and etoposide the increase in the amount of cells exhibiting nuclear condensation correlated with a drop in mitochondrial membrane potential (paper 2) and increased release of mitochondrial proteins (paper 4). PKC 412 and etoposide co-treatment-induced nuclear condensation was at least partially dependent on AIF, but not on caspase activity (paper 4).

At a relatively late time point, etoposide, by itself, induced a drop in mitochondrial membrane potential in a fraction of cells (paper 2). The etoposide-mediated drop in mitochondrial membrane potential (paper 2) correlated with a caspase-independent nuclear condensation (paper 4). This late etoposide-induced death was at least partially dependent on AIF (paper 4).

Thus, even though STS, PKC 412, etoposide, or the combination of etoposide and PKC 412, induce death at different timepoints and at different potency in U1810 cells, in all cases AIF, but not caspases, is important for nuclear condensation. There are more examples of NSCLC cells dying in a caspase-independent manner (Broker et al., 2002; Huisman et al., 2002). However, there are also examples of caspase-dependent NSCLC death (Khanna et al., 2003; Sanchez-Alcazar et al., 2003; Weigel et al., 2000). Unfortunately, in these studies, there was no investigation of AIF-dependency for cell death to occur. There are variable reports about the demand of caspase activity for AIF release from mitochondria (Cregan et al., 2004). Since caspase activation in some cellular contexts is needed for AIF release, it could be that AIF translocation from mitochondria to the nucleus is a general determining mechanism for NSCLC cell death, but in most studies focus has been directed solely towards caspases. Keeping this in mind, one could speculate if AIF is also required for PKC 412-induced death of U1285 cells.

In a cell, nuclear apoptosis can be triggered by several different, upstream mechanisms (Leist & Jaattela, 2001). Since tumour cells have evolved in different manners, it is expected that various tumour cells have different aberrations, including variations in the intactness of death pathways. This might explain cellular differences in regulation of death mechanisms, such as the variability of caspase dependency of cell death. Thus, within the broad group “NSCLC” there might be differences in intactness of death pathways. Caspase-independent AIF release may thus represent one of several mechanisms for mitochondrial release of AIF.

3.1.4 Etoposide and PKC 412 by different mechanisms contribute to mitochondrial release of proteins

AIF-dependent death of neuronal cells and fibroblasts is dependent upon an intact activity of PARP-1 (Yu et al., 2002). We decided to investigate if this connection could also be seen in NSCLC cells. Pre-treatment of U1810 cells with the PARP inhibitor PJ-34 inhibited the induction of mitochondrial release of proteins and nuclear condensation triggered by the combination of PKC 412 and etoposide (Paper 4). The late death following etoposide treatment was also inhibited by PJ-34 (Paper 4). The same PARP inhibitor could also decrease death induced by the combination of the
kinase inhibitors LY-294002 and PD 98059 and etoposide in H157 large cell carcinoma NSCLC cells (Paper 4), suggesting a possibility of general dependency on PARP for etoposide-induced death of NSCLC cells. Pre-treatment of U1810 cells with the PARP inhibitor 3-amino benzamide (3AB) gave a similar, but less pronounced, effect on cell death as was obtained with PJ-34 (paper 4). The similar effect of PJ-34 and 3AB on death induction in NSCLC cells decreases the possibility of some unspecific effect of the PARP inhibitors. Thus, these data suggest involvement of one or more PARP isoforms in etoposide-mediated death of NSCLC cells. Absence of PARP does not protect from etoposide-induced death in thymocytes (Leist et al., 1997), suggesting cell type specificity of PARP dependency for etoposide-induced death to occur.

Interestingly, PKC-412-induced mitochondrial release of proteins and nuclear condensation in U1810 cells was not inhibited by pre-treatment of cells with PJ-34 (paper 4). Moreover, 3AB pre-treatment increased PKC-412-induced nuclear condensation (paper 4). The potentiation by PARP inhibitors of PKC-412-induced apoptosis might be due to PARP involvement in prevention of mitotic catastrophe, which is discussed below.

Importantly, our data shows that in the same cell type, there are different modes of AIF release; one DNA damage-induced PARP dependent, as well as a PARP-independent triggered by kinase inhibition.

There are numerous isoforms of PARP, and PJ-34 and 3AB are broad-range PARP inhibitors. Thus, specific investigation of the different isoforms is needed for further clarification of PARP involvement in NSCLC apoptosis. As mentioned, PARP-1 and -2 are the only PARP isoforms known to be activated upon DNA damage, placing PARP-1 and PARP-2 as the strongest candidates for PARP activity involvement in etoposide-induced death.

We also investigated in which mode PKC 412 might contribute to release of AIF from mitochondria. PKC 412 induced a decrease in inhibitory phosphorylation of Bad, an event that was not observed upon treatment with Ro 31-8220 (paper 4), suggesting involvement of Bad in PKC-412-mediated release of AIF.

A mechanism of mitochondrial permeabilization was also sought on the lysosomal level, since in some cases kinase inhibition can induce lysosomal leakage of cathepsins which precedes and determines mitochondrial release of proteins (Bidere et al., 2003; Madge et al., 2003; Yacoub et al., 2006). PKC 412-induced apoptosis correlated with diminished red staining of acridine orange in cells, indicating lysosomal leakage (paper 4). However, lysosomal leakage was not observed upon the short time treatment with STS (paper 4), suggesting that lysosomal leakage in U1810 cells might be a sign of late apoptosis, and not a determining step for release of mitochondrial proteins. This conclusion was supported by the lack of inhibition of nuclear apoptosis induced by PKC 412 alone or in combination with etoposide, by pre-treatment of cells with cathepsin inhibitors E64-d and pepstatin A (Paper 4).

3.1.5 The mechanism(s) for release of AIF from mitochondria in U1810 cells requires further investigation

Most studies indicate that release of AIF correlates with a drop in mitochondrial membrane potential (Cregan et al., 2004). This suggests that the disruption of the mitochondrial membrane potential observed in U1810 cells upon apoptosis—inducing
treatments is important for AIF release. However, irrespective of the determining mechanisms of cell death, sooner or later a drop in mitochondrial membrane potential is expected in a dying cell.

The exact details of the underlying mechanisms for AIF release from mitochondria are not clear. Its passage might in some cases demand opening of the “mitochondrial permeability transition” (MPT) pore, which is correlating with release of cytochrome c and a drop in mitochondrial membrane potential (Cregan et al., 2004). There are studies showing that a soluble pool of cytochrome c in the mitochondrial intermembrane space can be released without loss of mitochondrial membrane potential (Robertson et al., 2003). This release of cytochrome c is mediated by Bax-dependent pore formation in the outer mitochondrial membrane (Er et al., 2006). Since in vitro studies show that Bax-mediated outer mitochondrial membrane permeabilization, without involvement of MPT pore formation, might also lead to release of larger proteins than cytochrome c (Er et al., 2006), it is not impossible that AIF can be released in a similar manner. Indeed, there are reports of specific release of AIF, occurring before cytochrome c release (Daugas et al., 2000; Yu et al., 2002). AIF is cleaved by calpain, and thereby released from the inner mitochondrial membrane (Polster et al., 2005), suggesting possible targeted solubilisation of AIF.

Bongkrekic acid and cyclosporin A are known to inhibit MPT pore function by binding to proteins of the MPT pore complex (Broekemeier et al., 1989; Marzo et al., 1998a), and are therefore commonly used in experimental studies of mitochondria. In order to investigate the necessity of MPT pore formation for progression of apoptosis in U1810 cells we used both bongkrekic acid and cyclosporin A. Preliminary data indicates that neither of these MPT-blockers can inhibit the drop in mitochondrial membrane potential induced by PKC 412 or STS (data not shown). Thus, PKC 412 and STS might induce release of AIF independently of MPT pore formation. Alternatively, in the experimental settings used, bongkrekic acid and cyclosporine A were not functioning, even though the concentrations used (2-10 μM cyclosporin A and 50 μM bongkrekic acid), block MPT in other cell types (Paquet et al., 2005; Woo et al., 2003; Zamzami et al., 2000).

### 3.1.6 Summary and future perspectives of “Apoptosis mechanisms induced by treatment with staurosporine, PKC 412 and etoposide (paper 1, 2, 4)”

Taken together, these data indicates that mitochondrial release of AIF is a crucial event for cell death to occur in U1810 cells, irrespective if apoptosis is triggered by kinase inhibition or DNA damage. The combinatorial treatment of cells with etoposide and PKC 412 increases release of AIF. Etoposide and PKC 412 possibly contribute to this increase by activating different mechanisms; etoposide-mediated release of AIF demands PARP, and PKC 412-induced apoptosis may involve activation of Bad. We also showed that PKC 412-induced death of U1285 SCLC cells is caspase-dependent.

Complete reduction of the AIF protein level was not possible in siRNA-experiments (paper 4). In these experiments nuclear condensation was only inhibited with 30% by siRNA for AIF. Therefore, a more thorough investigation of the contribution of AIF to cell death induced by PKC 412 and etoposide is required. Moreover, the involvement in U1810 cell death of other caspase-independent apoptosis inducers, such as Endonuclease G and AMID, is relevant to investigate.
Evaluation of a general AIF dependency of NSCLC (and SCLC) cell death would also be interesting, which could be accomplished by using a panel of NSCLC (and SCLC) cells. Several additional questions should be addressed to understand the mechanism(s) of AIF release in U1810 cells. Does etoposide-induced PARP activation induce a completely different mechanism of AIF release, compared to the mechanism induced by PKC 412? Does PKC 412-induced activation of Bad determine PKC 412-induced release of AIF? Are there other Bcl-2 family members, such as Bax, involved in U1810 cell death? Experiments investigating the role of Bad and Bax in U1810 apoptosis could involve confocal studies of individual cells using antibodies directed towards Bad and activated Bax. Moreover, the general dependency on PARP of DNA damage-induced NSCLC apoptosis needs further evaluation, together with the identification of the PARP-isoform(s) responsible for driving cell death.

3.2 ROLE OF PHOSPHORYLATION IN NSCLC APOPTOSIS
(PAPER 2, 3, 4)

3.2.1 Inhibition of protein kinase C does not determine death of U1810 cells

Compared to STS, PKC 412 and Ro 31-8220 are more specific, but less potent, inhibitors of protein kinase C (PKC). As mentioned, there are several studies indicating involvement of PKC family members in resistance of NSCLC. When tested in U1810 and U1285 cells we found that PKC 412 was a better inducer of nuclear condensation compared to Ro 31-8220 (paper 2). The divergence in the apoptosis-inducing effect mediated by the two STS analogues in U1810 cells could not be explained by a different potency of the two compounds in the inhibition of PMA-induced ERK1/2 activity (paper 2). This supports the conclusion drawn by Gesher about the lack of involvement of PKC in PKC 412-induced cell death. On the other hand, since Gesher’s conclusion was drawn from experiments performed in other cell types, it was relevant to test if PKC inhibition determined cell death in U1810 cells. To completely rule out the involvement of PKC in resistance of U1810 cells, investigations of specific PKC isoforms should be performed.

3.2.2 PI3-kinase inhibitors, but not a MEK inhibitor, induce apoptosis in U1810 cells

We found that PKC 412, at the concentration most often used, was a better inhibitor of the basal level of ERK1/2 phosphorylation, compared to Ro 31-8220 in U1810 and U1285 cells (paper 2). In H157 cells, which are not sensitive to PKC 412, treatment with PKC 412 increased ERK1/2 phosphorylation (paper 3). In H157 cells, which are not sensitive to PKC 412, treatment with PKC 412 increased ERK1/2 phosphorylation (paper 3). These data suggested involvement of ERK signalling (in a PKC-independent fashion) in survival of U1810 cells. Furthermore, we found that inhibitory phosphorylation of Bad at Ser112 which is a target of the MEK/ERK pathway (Scheid et al., 1999), was decreased by PKC 412, but not by Ro 31-8220 in U1810 cells (paper 4). The two STS analogues also had different effect on Ser136 phosphorylation of Bad which is a target site for Akt. PKC 412, but not Ro 31-8220, decreased Ser136 phosphorylation in U1810 cells (paper 4). Inhibition of
phosphorylation of these sites of Bad has been associated with release of Bad from 14-3-3 proteins, which is necessary for translocation of Bad from cytosol to mitochondria. At the mitochondria Bad interacts with Bcl-XL, and thereby the Bcl-XL-mediated protection of mitochondria is inhibited, leading to mitochondrial release of proteins (Fadeel et al., 1999).

Despite the data suggesting involvement of ERK in survival of U1810 cells, the MEK inhibitor PD 98059 could not induce apoptosis in U1810 and H157 cells (Paper 3). PD 98059 could neither sensitize H157 cells to PKC 412 (paper 3). These data suggest that MEK activity does not determine survival of U1810 and H157 cells.

In line with the data on Ser\textsuperscript{136} phosphorylation of Bad, we found that PKC 412 and Ro 31-8220 had opposite effects on activity-related phosphorylation of site Ser\textsuperscript{473} of Akt (paper 2 and 3). PKC 412 decreased, and Ro 31-8220 increased, Ser\textsuperscript{473} phosphorylation in U1810 cells (Paper 2). A similar effect on Akt phosphorylation mediated by the two STS analogues was also seen in U1285 cells (paper 2), H157 cells (paper 3) as well as in SCLC H69 and NSCLC H23 cells (data not shown), suggesting these events being general. In fibroblasts PKC 412 induces a decrease in Akt phosphorylation, which determines cell death (Tenzer et al., 2001). This information, together with information from other studies showing involvement of Akt in survival of NSCLC cells (see introduction) made us decide to investigate the involvement of the PI3-kinase pathway in death of U1810 cells. Indeed, when used alone the PI3-kinase inhibitors wortmannin and LY-294002 induced apoptosis in U1810 cells (paper 3). A decrease in the activity of Akt might contribute to release of proteins from mitochondria. However, the level of mitochondrial release of proteins (paper 4) and nuclear apoptosis (paper 3) was not completely correlating with the degree of Akt Ser\textsuperscript{473} phosphorylation. Neither did the reduction in Akt Ser\textsuperscript{473} phosphorylation correlate with increased nuclear apoptosis in H157 cells (paper 3). When used alone, PKC 412, LY 294002 and wortmannin were poor apoptosis inducers in H157 cells (paper 3). This was unexpected, since the basal level of Akt Ser\textsuperscript{473} phosphorylation was higher in H157 cells, compared to U1810 cells (data not shown), which suggests overactive PI3-kinase signalling in the H157 cells.

There are studies showing that STS-induced cell death correlates with a decrease in Akt phosphorylation and that this death can be inhibited by activation of the PI3-kinase/Akt pathway (Akbar & Kim, 2002; Tafani et al., 2001; Wan et al., 2002). STS-induced PI3-kinase signalling-dependent death of cells is associated with activation of the Bcl-2 family members Bax (Tafani et al., 2001) and Bad (Tafani et al., 2001; Wan et al., 2002). As discussed, the requirement for U1810 cell death of AIF release from mitochondria was similar irrespectively if apoptosis was induced by STS or PKC 412. Since STS and PKC 412 have target kinases in common, the two kinase inhibitors might induce AIF release with a similar mechanism. Indeed, also in U1810 cells STS decreased Akt activity (data not shown). Thus, STS-induced death of U1810 cells may require inactivation of PI3-kinase signalling. However, in U1810 cells STS induced a much faster death compared to PKC 412, indicating that the underlying reason for cell death was not completely the same. STS has been shown to inhibit focal adhesion kinase (FAK) which is involved in loss of adhesion (Kabir et al., 2002). Since loss of adherence is a trigger for apoptosis (Kraus et al., 2002) FAK inhibition might contribute to the fast death observed upon STS-treatment.
3.2.3 PI3-kinase inhibitors sensitize NSCLC cells to etoposide

Wortmannin, LY-294002 and PKC 412 sensitized H157 and U1810 cells to etoposide (paper 3). Tapodi et al. showed that PJ-34-mediated protection of liver cells from H$_2$O$_2$-induced death was associated with an increase in Akt Ser$^{473}$ phosphorylation (Tapodi et al., 2005). Moreover, PARP inhibition-mediated survival of the liver cells involved protection of mitochondria, which could be reversed by treatment of cells with PI3-kinase inhibitors. In U1810 cells treated with etoposide alone, or in combination with PKC 412, PJ-34 inhibited release of proteins from mitochondria (paper 4). However, PJ-34 could not increase Akt Ser$^{473}$ phosphorylation (paper 4), suggesting a different mechanism for PARP inhibition-mediated protection of mitochondrial destabilization, compared to the mechanism shown by Tapodi et al. Interestingly, in normal lung cells derived from mice, PJ-34 induces an increase in Akt activity (Veres et al., 2003). In U1810 cells, etoposide induces an increase in Akt Ser$^{473}$ phosphorylation, which is decreased by PKC 412 (paper 4). Thus, in U1810 cells, one mechanism of resistance to DNA damage could be the absence of PARP-mediated Akt inhibition upon DNA damage.

The cellular response to Topoisomerase inhibitors is known to involve activation of PI3-kinase-like protein kinases (PIKKs), such as ATM, ATR and DNA-PK (Sordet et al., 2003). It is not known whether PKC 412 inhibits the activity of these kinases, but both wortmannin and LY-294002 are known to inhibit PIKKs (Abraham, 2004; Vanhaesebroeck & Waterfield, 1999). ATM is an activator of Akt by mediating increased Ser$^{473}$ phosphorylation (Viniegra et al., 2005). When PARP is inhibited, ATM is known to be activated (Bryant & Helleday, 2006; Watanabe et al., 2004). Thus, upon cellular DNA damage, if there is an abnormally low PARP activation, or a defect in the signalling between PARP and ATM, this might lead to a high Akt activity, irrespective if PARP is inhibited or not. ATM is also known to phosphorylate Bid, which leads to accumulation of cells in S-phase (Kamer et al., 2005). In the same study, it was shown that if Bid is non-phosphorylatable upon etoposide treatment, there is a decrease in S-phase accumulation of cells and an increase in apoptosis. Etoposide treatment leads to an accumulation of U1810 cells in S and G$_2$-phases of the cell cycle (paper 3). Collectively, these data suggests that ATM might be involved in resistance of U1810 cells to etoposide-induced apoptosis.

U1810 cells have a high DNA-PK activity, which correlates with increased radioresistance (Sirzen et al., 1999), suggesting its involvement in resistance to cell death. On the other hand, DNA-PK inhibition has been shown to inhibit activation of PARP-1 (Veuger et al., 2004). Keeping this in mind, if PI3-kinase inhibitors decrease DNA-PK activity, it is difficult to explain the increased PARP-dependent apoptosis in cells exposed to PI3-kinase inhibitor in combination with DNA damage (paper 3 and 4). However, since PARP fulfils many different activities in a cell (Burkle, 2005) which all must be regulated in different manners, a more thorough investigation on the connection between PARP, DNA-PK and apoptosis in U1810 cells is needed.

Except for activation of Bad (paper 4), which might contribute to sensitization of U1810 cells to etoposide, PI3-kinase inhibition can, for example, lead to activation of GSK (Beurel & Jope, 2006) and inhibition of survivin (Altieri, 2003a), which might also contribute to cell death. PI3-kinase inhibition can also induce Akt-independent cathepsin-mediated disturbance of mitochondrial membrane potential (Madge et al.,
However, as discussed earlier, lysosomal disruption does not seem to determine U1810 cell death (Paper 4).

Except for effects of PI3-kinase- and PIKK- signalling on proteins of the apoptotic machinery, this signalling is also involved in other cellular processes, and thereby, more indirectly, might regulate apoptosis (paper 3). Involvement of PI3-kinase and PIKK signalling in mitotic catastrophe will be discussed in the next section.

3.2.4 Other kinases that might be involved in PKC 412-induced apoptosis and sensitization of NSCLC cells to DNA damage

PKC 412 is known to sensitize cells to conventional anti-cancer drugs through inhibition of phosphorylation of P-glycoprotein which is a transmembrane protein known to increase resistance through pumping drugs out of the cell (Gescher, 2000). Sensitization of U1810 cells was also seen when PKC 412 was combined with gamma radiation (paper 2). Therefore, the sensitizing mechanism of PKC 412 on etoposide-treated U1810 cells is most likely not determined by inhibition of p-glycoprotein-mediated actions.

As mentioned, PKC 412 also inhibits other kinases, e.g. platelet-derived growth factor, stem cell factor (c-Kit), and vascular endothelial growth factor (Fabbro et al., 1999), which are all involved in cancer development (Fabbro et al., 1999; Tibes et al., 2005), and their inhibition might thus contribute to the effects seen in NSCLC cells upon PKC 412 treatment.

3.2.5 MEK inhibition sensitizes H157 NSCLC cells to PI3-kinase inhibition in combination with etoposide

Interestingly, even though the MEK inhibitor PD 98059 neither could induce cell death on its own, nor increase PI3-kinase-induced apoptosis, it did sensitize H157 cells to PI3-kinase inhibition when combined with DNA damage (paper 3). PD 98059 could not sensitize U1810 cells to the combination of PI3-kinase inhibition and etoposide (paper 3). However, in the U1810 cells, but not in the H157 cells, LY-294002 decreased both Akt and ERK phosphorylation (paper 3), which might explain why PD 98059 did not further increase death of U1810 cells treated with PI3-kinase inhibitor in combination with etoposide.

There are other examples when simultaneous inhibition of both PI3-kinase/Akt and the MEK/ERK pathways is important for inhibition of lung cancer cell growth or for induction of cell death. Epidermal growth factor receptor is upstream of both MEK/ERK and PI3-kinase/Akt signalling (Raben et al., 2004). It was shown that simultaneous inhibition of MEK and the PI3-kinase pathway was needed for apoptosis induction in cells resistant to EGFR inhibition (Jamaat et al., 2003). Reduced effectiveness of the EGFR inhibitor gefitinib in NSCLC cells sometimes correlates with increased Akt phosphorylation (Kokubo et al., 2005). Moreover, anti-tumour activity of gefitinib in a xenograft model was shown improved when combined with PI3-kinase inhibition (Ihle et al., 2005). Thus, cellular events are regulated by signals emanating from different regulatory pathways and it seems that simultaneous inhibition of the PI3-
kinase pathway and MEK-ERK signalling in some cases improves the killing of lung cancer cells.

3.2.6 Summary and future perspectives of “Role of phosphorylation in NSCLC apoptosis (paper 2, 3, 4)”

PKC 412-induced death of U1810 cells is not determined by inhibition of protein kinase C, but may involve activation of Bad through dephosphorylation of this protein. U1810 cells were sensitive to PI3-kinase inhibitors, whereas H157 cells were only sensitive if PI3-kinase inhibition was combined with etoposide. MEK inhibition alone had no effect on cell death, but sensitized H157 cells to apoptosis induced by etoposide in combination with PI3-kinase inhibition.

It would be interesting to investigate the respective involvement of PI3-kinase and PIKKs in apoptosis regulation of NSCLC cells. One future experiment could be to investigate the role of Akt in PKC 412-induced apoptosis, for example, by using siRNA for Akt, or by overexpression of a dominant negative or a constitutively active Akt. It would also be of interest to find out why etoposide increases Akt phosphorylation in U1810 cells. Could it be due to over activation of ATM? An investigation on the interplay between PARP, ATM and DNA-PK in U1810 cells could also be of interest. Moreover, since H157 cells and U1810 cells show different level of resistance to DNA damage, as well as differences in their regulation of signalling pathways, the signalling network in these cells needs a more detailed characterization. Interestingly, PI3-kinase signalling can inhibit Bax through phosphorylation (Er et al., 2006). Thus, phosphorylation status of Bax upon PKC 412 treatment would be interesting to investigate. Finally, there are probably many downstream targets of PI3-kinase signalling that are unknown. Important targets of PKC 412, LY-294002 and wortmannin in cells could be identified through a 2D-gel electrophoresis approach using antibodies targeting phosphorylated proteins.

3.3 INHIBITION OF PI3-KINASE SIGNALLING LEADS TO MITOTIC CATASTROPHE (PAPER 2, 3, 4)

3.3.1 PKC 412 induces mitotic catastrophe

The PKC 412-mediated sensitization of U1810 cells to DNA damage induced by etoposide or gamma radiation was dependent upon the order of treatment with DNA damage and addition of PKC 412 (paper 2). The best sensitization was seen when PKC 412 was added 24 hours after DNA damage inducing treatment, whereas a pre-treatment with PKC 412 decreased or did not change the cellular response to etoposide or gamma radiation, respectively. The observation of a long incubation time before cell death occurred, in combination with the dependency of the exposure order of DNA damage and PKC 412 (paper 2), suggested that PKC 412 induced death in a cell cycle-dependent manner.

Upon a 24 hours treatment with PKC 412 no cells were found in S-phase, and cells were instead accumulated predominantly in G2/M-phase (paper 3). Topoisomerase II
inhibitors are effective in S-phase (Kaufmann, 1998), which might explain why PKC 412 pre-treatment inhibited etoposide-induced apoptosis.

Since a 24 hours treatment of U1810 cells with etoposide accumulated cells in S and G2 phases, sensitization by PKC 412 added at this time point may involve inhibition of survival-related kinases activated by the DNA damage. The STS analog UCN-01 sensitizes S-phase arrested cells by driving them to the next phase of the cell cycle (Mack et al., 2003). It was not possible to show that etoposide-treated S-phase accumulated U1810 cells were driven to leave this phase by PKC 412 (described in study 2). Nevertheless, I still suspected that cell cycle regulation was important for sensitization mediated by PKC 412.

Formation of multinucleated cells is a sign of mitotic catastrophe (Rieder & Maiato, 2004). By using flow cytometry was shown that PKC 412 induces an increase of 4n cells (paper 3), which has also been shown in other lung cancer cells (Ikegami et al., 1996a; Ikegami et al., 1996b). PKC 412-induced death of glioblastoma cells is associated with formation of giant nuclei (Begemann et al., 1996). Depletion of survivin in oligodendroglioma cells leads to formation of large macronuclei and apoptosis (Shankar et al., 2001). In the same study was shown that inhibition of survivin depletion-induced apoptosis leads to accumulation of cells in metaphase. Single-treatment with PKC 412 induced an initial accumulation of cells in metaphase, followed by apoptosis and formation of 4n cells (paper 3), suggesting PKC 412-mediated events affecting mitosis.

STS has been reported to accumulate lymphocytic leukemic cells in G2, followed by additional rounds of DNA-replication without division (Bruno et al., 1992). Moreover, STS reduces anti-microtubule agent-induced apoptosis in spindle checkpoint proficient lung cancer cells, which is associated with a shortened mitotic arrest (Masuda et al., 2003). These studies indicate that STS may target kinases involved in regulation of mitosis. STS induced a much faster death in U1810 cells compared to PKC 412, indicating that the underlying reason for cell death was not completely the same (paper 2 and 4). Based on the available data it is impossible to speculate if STS-induced death of U1810 cells involves mitosis-related events.

### 3.3.2 PI3-kinase- and PARP- inhibitors induce mitotic catastrophe

Treatment of H157 and U1810 cells with wortmannin or LY-294002 accumulated cells in G1 (paper 3), which might be due to activation of Forkhead transcription factors (Martinez-Gac et al., 2004).

Upon LY-294002 treatment the DNA morphology of H157 cells indicated that these cells died in an apoptosis-like mode in anaphase, whereas the apoptosis-like death of U1810 cells upon the same treatment, by morphology, could not be related to a specific phase in mitosis (paper 3). However, in both cell lines PKC 412 decreased Histone H3 Ser10 phosphorylation, while LY-294002 increased this phosphorylation (paper 3), suggesting that the two cell lines responded in a similar way. Since Histone H3 Ser10 phosphorylation is believed to act as a signal for cells to leave metaphase (Hans & Dimitrov, 2001), the status of Ser10 phosphorylation correlated well with morphological data showing that PKC 412 induced accumulation of cells in metaphase while LY-294002 allowed, or forced, cells to leave metaphase (paper 3).
The effect on mitosis of LY-294002 and PKC 412 might represent two variants of mitotic catastrophe; the result of a forced anaphase or a metaphase arrest, respectively. In both cases the aberrant mitosis triggered activation of the apoptotic machinery. Wortmannin-induced death was apoptosis-like, and some of the cells also exhibited an anaphase-like morphology (paper 3), suggesting a similar apoptosis-inducing mechanism for wortmannin and LY-294002.

All three kinase inhibitors affected cell cycle regulation and increased cell death. However, since these inhibitors did not give identical experimental results, inhibition of other kinases than PI3-kinase probably influenced the cellular effect of each kinase inhibitor used.

PARP inhibitors induced mitotic catastrophe-like death in U1810 cells (paper 4). The same inhibitors could not inhibit and rather increased, PKC 412-induced death of U1810 cells (paper 4). Thus, as has been shown in other cell types (see introduction), PARP is important for proper mitosis.

### 3.3.3 Kinases possibly involved in PKC 412- and PI3-kinase inhibitor-induced mitotic catastrophe

Preliminary data of ours, obtained from confocal studies of NSCLC cells stained with an antibody targeting alpha-tubulin, indicate that LY-294002 induces formation of multiple mitotic spindles, whereas upon PKC 412 treatment there seems to be monopolar spindle formation (data not shown). These data suggests that LY-294002 might induce fragmentation or amplification of centrosomes, whereas PKC 412 inhibits separation of centrosomes. As mentioned, these two kinase inhibitors had different effect on Histone H3 phosphorylation; PKC 412 decreased, while LY-294002 increased, Ser\(^{10}\) phosphorylation (paper 3). Interestingly, Aurora A and B, which are both known to phosphorylate Histone H3, are essential for the maintenance of centrosome separation, and proper cytokinesis, respectively (Ke et al., 2003). Overexpression of Aurora A leads to centrosome amplification (Dutertre et al., 2002). Thus, Aurora kinases might be involved in the cellular response to PKC 412 and LY-294002. Interestingly, there is also a link between Aurora and PARP, since Aurora B is regulated in a negative manner by poly(ADP-ribosylation) (Monaco et al., 2005).

Polo-like kinases are known to be involved in centrosome maturation and spindle assembly (Li & Li, 2006), and might therefore also be involved in the response to PKC 412 and LY-294002. Of course, there is also a possibility that spindle check point proteins might be affected upon PKC 412 and LY-294002 treatment.

It has been shown that Akt protects glioma cells from temozolomide-induced mitotic catastrophe (Hirose et al., 2005), indicating that Akt might have a role also in mitotic catastrophe of NSCLC cells. Since mTOR in an Akt-dependent manner has been shown to suppress apoptosis and protect cells from microtubule-interacting drugs at the G2/M transition (Asnaghi et al., 2004), its involvement in PI3-kinase inhibition-induced mitotic catastrophe is possible. As mentioned, PI3-kinase inhibitors also inhibit PI3-kinase-like kinase inhibitors, such as ATM and ATR that are important for checkpoint regulation, and thus might play an important role for PKC 412 and LY-294002-induced mitotic catastrophe. In HeLa cells, DNA-PK activity is increased during S and G\(_2\) phase (Nilsson et al., 1999), which are the phases when PKC 412 gives
the best sensitization to etoposide (paper 2, 3), further suggesting that DNA-PK is an important target for PKC 412 in U1810 cells.

3.3.4 Etoposide changes the complexity of cellular signalling

There are numerous examples of repair protein involvement in cell cycle regulation and mitotic catastrophe (Kastan & Bartek, 2004; Löffler et al., 2006; Tutt et al., 1999; Xu et al., 1999). Activation of repair pathways (and inhibition of topoisomerase II which is an important enzyme in the process of replication) probably increases the complexity of signalling, leading to a different response upon kinase inhibition, compared to when kinase inhibitors are used alone. For example, as mentioned, etoposide increased Akt phosphorylation in U1810 cells (paper 4).

When etoposide and PKC 412 were combined, apoptosis was increased, and there was no formation of multinuclear cells (paper 3), suggesting that the DNA damage in an increased number of cells caused switching of the metaphase arrest to apoptosis. This theory was supported by the metaphase-like morphology of H157 cells dying in an apoptosis-like mode upon treatment with PKC 412 and etoposide (paper 3). However, LY-294002 or wortmannin in combination with etoposide increased apoptosis-like death, without morphological signs of mitotic catastrophe (paper 3). Thus, in general, there were fewer morphological signs of mitotic catastrophe seen when PKC 412 or PI3-kinase inhibitors were combined with etoposide, compared to when kinase inhibitors were used alone. As mentioned, PARP-inhibition also gave a completely different result in cells treated with etoposide in combination with PKC 412, compared to when PKC 412 was used alone (paper 4), further suggesting onset of a completely different signalling upon etoposide treatment. Thus, it could be that DNA damage, when combined with PI3-kinase inhibition, triggered checkpoint activation, directly leading to apoptosis, without involvement of mitosis activation. Alternatively, cells died at the same point in mitosis irrespectively if treated with kinase inhibitor alone, or in combination with etoposide. However, etoposide-induced DNA damage induced a stronger and faster stimulation of apoptosis, and thereby the morphological signs of mitotic catastrophe might not have been as easily observed. Another possibility is that PI3-kinase inhibition in combination with DNA damage might have provoked a different mechanism of induction of mitotic catastrophe, occurring at an earlier phase of the cell cycle, which is discussed below.

3.3.5 Why do U1810 and H157 cells respond in different manners to PKC 412 and PI3-kinase inhibitors?

As mentioned, in U1810 cells treatment with PKC 412 and LY-294002 induced a more “clear” apoptosis-like nuclear morphology, compared to the nuclear morphology observed in H157 cells upon the same type of treatments. This might reflect that the two cell lines have different types of aberrations in their cell cycle and apoptosis machineries. For example, when dividing, as visualized by flow-cytometry of PI-stained cells, a part of the U1810 population becomes aneuploid (discussed in paper 3). Aneuploid cells have been suggested to be more sensitive to cell cycle disturbances (Cahill et al., 1999), which might explain the more pronounced apoptotic cell death of
U1810 cells. Moreover, H157 cells are not sensitive to the microtubule-interacting drug nocodazole that stops cells at the G2/M transition, whereas U1810 cells die by apoptosis upon treatment with this drug (data not shown). This might indicate that U1810 cells in general are more sensitive to disturbances at mitosis, compared with H157 cells.

Interestingly, H157 cells have a lower basal level of survivin, compared to U1810 cells (data not shown). Moreover, upon etoposide treatment, the survivin level is decreased in H157 cells, whereas in U1810 cells, expression of survivin is increased (data not shown). Thus, the survival of U1810 cells might be dependent upon survivin expression, whereas H157 cell survival is determined by something else.

As mentioned, there might also be a difference between the two cell lines in MEK-dependency of death (paper 3), as well as a difference in PI3-kinase downstream regulation of MEK (paper 3). MEK is involved in cell cycle regulation (Chang et al., 2003), and its deregulation might therefore impact cell death. We tried in more detail to investigate the involvement of MEK in survival of NSCLC cells. Unfortunately, the ERK phosphorylation level varied between identical experiments, suggesting a complex regulation, and/or cell cycle dependent regulation, of this kinase (the problems associated with studies of cell cycle dependent proteins is discussed below).

Also in SCLC cell lines there are clear differences in the response to PKC 412; U1285 cells responded with apoptosis in a concentration-dependent manner (paper 2) whereas only a low amount of SCLC H69 cells showed morphological signs of mitotic catastrophe-like apoptosis upon PKC 412 treatment (data not shown).

### 3.3.6 Mitotic catastrophe is difficult to study

When trying to study mitotic catastrophe one obvious problem is the lack of good markers. Often mitotic catastrophe is described merely as a consequence of a failure in regulation of check point and cell cycle. Aberrations in cell cycle regulatory pathways that contribute to mitotic catastrophe varies, which makes it difficult finding markers for mitotic catastrophe. Moreover, there has been no thorough characterization of changes in protein levels and protein phosphorylations in lung-derived cells at the G2/M transition, making it difficult to know what is aberration and what is normal. Furthermore, morphological criteria, that are often used, might be mixed up with markers for other processes, such as different types of terminal differentiation, or focus of the studies might be solely directed towards the end point, which is often apoptosis. However, there might be good markers of mitotic catastrophe that we are not aware of.

One way of approaching the challenge of finding such markers could be to focus more on the underlying reasons for mitotic catastrophe. There is a possibility that mitotic catastrophe, except from being a consequence of cell cycle failure, may also be the result of one or several regulated processes. It has been proposed that abortive cell cycle re-entry is a fundamental mechanism for neuronal cells to undergo apoptosis (Bonni, 2003). As mentioned, there are also indications of a centrosomal check point which is activated upon DNA damage (Bonni, 2003; Loffler et al., 2006). When the centrosomal check point is activated there is induction of cell death through amplification or fragmentation of centrosomes, leading to mitotic catastrophe. This is interesting, since in those cases mitotic catastrophe is activated by a defined regulated process; it should be possible to find reliable markers.
One obvious problem when studying mitotic catastrophe is the variability between different experiments in the level of cells in different cell cycle phases. If, for example, many control cells are in mitosis at the time of harvest, the level of mitosis-related proteins and protein modifications is pronounced. If in the same experiment treatment gives an effect on cell cycle progression, possible treatment-induced increase in mitosis might thus not be detectable, when compared to control values. For example, we tried to characterize the status of Cdk1 Tyr15 phosphorylation upon treatment with PKC 412, wortmannin and LY-294002. Unfortunately we got variable results. To make data comprehensible, preferably, samples for western blot should be prepared in parallel with samples for flow cytometric analysis of the cell cycle.

Another problem encountered when studying mitotic catastrophe is that the time for transition between different phases of M-phase is short, and if not all cells are synchronized, it is difficult to find a good time point when changes can be detected.

3.3.7 Summary and future perspectives of “Inhibition of PI3-kinase signalling leads to mitotic catastrophe (paper 2, 3, 4)”

Taken together, we showed that PKC 412 induces apoptosis at metaphase in NSCLC cells while LY-294002-treated cells die at anaphase. When PKC 412 or PI3-kinase inhibitors are combined with etoposide, as compared to when used alone, in general death is increased and more apoptosis-like. However, the nuclear morphology of H157 cells indicated that the combination of PKC 412 with etoposide also induces death at metaphase. Moreover it was shown that PARP inhibition in the absence of etoposide stimulates mitotic catastrophe in U1810 cells.

It would be interesting to find out more about the contribution of mitosis-related kinases, such as Akt, Auroras and Polo-like kinases, in the regulation of mitotic catastrophe. Since there are differences in the cell line-specific response of U1810 and H157 cells to inducers of mitotic catastrophe, underlying reasons for the divergence in response needs further evaluation. For example, changes in protein levels and protein modifications at the G2/M transition in untreated cultures of the two cell lines require evaluation. Most interesting to me would be to understand the precise role of mitotic catastrophe in cell death induced by etoposide and PI3-kinase inhibition. In which manners is various check point proteins involved in the switch between cell cycle progression, apoptosis and mitotic catastrophe of lung cancer cells? Are there different “levels” of the G2/M progression where cell death may be promoted? What are the roles of PI3-kinase-like protein kinases and PARP isoforms in mitotic catastrophe/cell death that is induced by kinase inhibition alone, and in combination with etoposide?

Overall, there is a need for more studies on interactions between regulation of apoptosis, cell cycle, DNA repair and mitotic catastrophe. These systems are closely linked together, for example, through phosphorylation-based signalling systems, and are all involved in tumour progression. Since different modes of mitotic catastrophe might be “hidden” processes, more research in the “mitotic catastrophe field” is needed. I believe that more knowledge about mitotic catastrophe would enable better understanding on when to use different medicines. For example, Cdk1 inhibitors are tested for use in cancer therapy (Golsteyn, 2005). These agents are expected to stop cells in the G2 phase, since cells cannot enter mitosis without Cdk1 activation. In some cells this stop would probably lead to cell death. However, there are also reports of
cases when Cdk1-activation is necessary for cellular apoptosis induction. Thus, Cdk1 inhibition might in these latter cases inhibit cell death. Therefore, it is important to enable a characterization of the underlying mechanism of a drug, as well as to characterize the cellular context when the medicine is appropriate to use.

Many anti-cancer medicines rely on the induction of apoptosis. In a cancer cell exhibiting an aberrant regulation of the apoptotic machinery, a medicine relying on the onset of this cell death pathway might be useless, or even evolve the tumour, if not combined with some drug that reactivates the death pathway. For example, combination of DNA damage with drugs that drive the cells prematurely out of G2 may also increase the risk for formation of aneuploid cells (Rieder & Maiato, 2004). Thus, to avoid propagation of the disease, it is important to make sure that the machinery needed for execution is intact. One also has to take into consideration that some proteins, such as PARP-1, might be important for different cellular functions. For example, recently it was shown that PARP-1 inhibition specifically sensitizes cells deficient in BRCA-1 and -2, to death (Bryant et al., 2005; Farmer et al., 2005). Would this also be true for cells that induce apoptosis in an AIF- and PARP- dependent manner? In the future, to enable effective treatment of NSCLC patients, many different markers that are related to different processes and aberrations will probably be needed for evaluation of which medicines to be used.
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5 REFERENCES


