Chapter 1

General introduction

Lung cancer

Lung cancer is a devastating illness. In the 25 countries of the European Union, it is the third most common form of cancer and the most common cause of cancer-related death. In 2006, lung cancer caused an estimated 171.900 deaths in the EU and 9.426 deaths in the Netherlands, representing approximately a quarter of total cancer deaths [Ferlay et al., 2007;CBS, 2006]. As early stage lung cancer is frequently asymptomatic or ill recognized, over 80% of cases are diagnosed in an advanced, and therefore incurable, stage. Despite advances in development of new treatment modalities, the overall 5-year survival rate has only slightly increased over the last 25 years, remaining at approximately 16% [Brundage et al., 2002;Jemal et al., 2007;Spira and Ettinger, 2004]. Apart from some notorious environmental and work-related factors, a habit of tobacco smoking has been established as the main etiological factor, estimated to account for 85% of all lung cancer cases [Peto et al., 2000;Polder et al., 2002;Williams and Sandler, 2001;Alberg et al., 2005]. As only 10-15% of (heavy) smokers develop lung cancer, endogenous factors are thought to play an important role as well. Although lung cancers rarely result from inherited mutations of oncogenes or tumor suppressor genes, it has been related to a decreased capacity to detoxify certain types of cancer-causing chemicals or tobacco carcinogens [Clapper, 2000;Hecht, 2002]. Notably, decreased DNA repair capacity, increasing cellular susceptibility to accumulation of mutations, was found to be an independent risk factor for the development of non-small cell lung cancer (NSCLC) [Qingyi et al., 2000;Shen et al., 2003]. Gene-diet interactions may also be relevant to lung carcinogenesis [London et al., 2000].

Non-small cell lung cancer

NSCLC consists of epithelial tumors that represent approximately 80-85% of lung carcinomas [Ettinger, 2004]. The three major histological types are: squamous cell carcinoma, adenocarcinoma and large-cell carcinoma [Brambilla et al., 2001]. Other subtypes, such as bronchioloalveolar carcinoma (BAC), comprise only 3-4% of cases, with 10-15% of adenocarcinomas having BAC features [Read et al., 2004]. Small cell lung cancer (SCLC) represents about 15-20% of lung cancer cases and is characterized by distinct pathologic and clinical features. In recent years, the histopathology of NSCLC has changed,

adenocarcinoma now supplanting squamous cell carcinoma as the most prevalent subtype. The increased incidence of adenocarcinoma might be explained by advances in diagnostic technology (increased ability to perform biopsies on tumors in more distal airways) and changes in cigarette design [Thun et al., 1997;Hecht, 1999;Janssen-Heijnen and Coebergh, 2003].

NSCLC staging and survival

Lung cancer staging is based on the tumor-node-metastasis (TNM) system, as revised in 1997 by Mountain [Mountain, 1997]. The TNM system considers the characteristics of the local tumor (T), presence or absence of regional lymph node involvement (N), and presence or absence of distant metastases (M), see also Table 1. Based on the TNM staging system, NSCLC can be divided into different stages, ranging from local (I-IIA) to locally advanced (stages IIB-IIIA) and advanced disease (stages IIIB-IV). An update of the TNM classification for lung cancer is expected in 2009, proposed changes including additional cutoffs for tumor size (tumors >7 cm moving from T2 to T3), reclassifying pleural effusion as an M descriptor and re-allocation of certain cases to a neighboring higher or lower stage [Goldstraw et al., 2007]. Unfortunately, only a minority of NSCLC patients is diagnosed at stage I or IIA disease when there is still a fair chance for cure, median 5-year survival rates being equivalent to 50 to 60% and 40%, respectively. Approximately 40% of patients present with regional disease and 35 to 40% with distant metastases. Survival rates quickly plummet in higher stage disease and only 1-2% of patients with metastatic disease will survive 5 years from diagnosis, see also Table 1 [Stat Bite, 2005;Goldstraw et al., 2007;Jemal et al., 2007;Free et al., 2007;Mountain, 1997]. Recently, due to the incorporation of (up-front) [^{18}F] fluorodeoxyglucose positron emission tomography (FDG-PET) in the initial staging work-up for newly diagnosed NSCLC patients, the number of unnecessary invasive surgical (diagnostic) procedures has been reduced [Herder et al., 2006;van Tinteren et al., 2002;Ung et al., 2007]. Despite a vast effort made by the medical as well as scientific community, no effective screening methods for detection of early stage lung cancer have been established. Recently proposed low-dose computed tomography (LD-CT)-based screening strategies as well as assessment of serum tumor markers are still enveloped with controversy [Schnoll et al., 2007;Bach et al., 2007;Black and Baron, 2007;Patz, Jr. et al., 2007].

Table 1: TNM staging for lung cancer

Stage	TNM	General Description	OS*	5-year#
Local				
0	TisN0M0	Tis – carcinoma in situ N0 - no regional lymph node involvement M0 - distant metastasis absent		
IA	T1 N0 M0	T1 – tumor ≤ 3cm, without invasion more proximal than lobar bronchus	60	50-61%
IB	T2 N0 M0	T2 - tumor > 3 cm OR tumor of any size with any of the following: invades visceral pleura; atelectasis of less than entire lung; proximal extent at least 2 cm from carina	37	38-40%
IIA	T1 N1 M0	N1 - metastasis to ipsilateral hilar and/ or ipsilateral peribronchial nodes	38	24-34%
Locally advanced				
IIB	T2 N1 M0 T3 N0 M0	T3 - tumor of any size with any of the following:invasion of chest wall; involvement of the diaphragm,mediastinal pleura or pericardium; proximal extent within 2 cm of carina	18	24-25%
IIIA	T3 N1 M0 T1-3 N2 M0	N2 - metastasis to ipsilateral mediastinal and/or subcarinal nodes N3 - metastasis to contralateral mediastinal or hilar nodes OR ipsilateral or contralateral scalene or supraclavicular nodes	14	13-18%
IIIB	**Any T N3 M0**			
Advanced				
IIIB	T4 Any N M0	T4 - Tumor of any size with any of the following: invasion of mediastinum; invasion of heart or great vessels; invasion of trachea or esophagus; invasion of vertebral body or carina; presence of malignant pleural or pericardial effusion; satellite tumor nodule(s) within same lobe as primary tumor	10	5-8%
IV	Any T Any N M1	M1 - distant metastasis present (including metastatic tumor nodules in a different lobe from the primary tumor)	6	1-2%

* OS: median overall survival time, months; # 5-year survival rate; [Mountain, 1997;Goldstraw et al., 2007]

Treatment of NSCLC

Uncontrolled, invasive growth and a tendency to form distant metastatic disease sites summarize the deadly character of malignant disease. Combined treatment modalities are used to treat both the primary tumor and (micro) metastases, whilst ideally sparing healthy tissues. For resectable NSCLC, typically stage I, II and limited stage III (T3N1), surgery constitutes the basis of treatment. Administration of chemotherapy and radiotherapy are confined to the (neo)adjuvant setting [Scagliotti, 2007]. Stage III locally advanced patients with a good performance score (ECOG 0-1) can be candidates for (concurrent) chemoradiation, with curative intent [Glynne-Jones and Hoskin, 2007]. Symptomatic stage I-III patients with a poor performance score (ECOG 2 [Oken et al., 1982]) are usually treated with palliative radiotherapy only [Okawara et al., 2006].

Patients with unresectable and metastatic disease may benefit from (palliative) chemotherapy. According to current guidelines, first-line chemotherapeutic treatment consists of a platinum agent-based doublet, e.g. cisplatin or carboplatin in combination with a third generation cytotoxic drug, gemcitabine, a taxane (paclitaxel, docetaxel) or vinorelbine.

Meta-analyses of randomized clinical trials comparing cisplatin with carboplatin suggest that clinical outcome of cisplatin doublets is slightly superior to carboplatin-based chemotherapy, without being associated with an increase in severe toxic effects [Hotta et al., 2004;Ardizzoni et al., 2007]. Another meta-analysis showed a reduction in overall mortality in favor of gemcitabine-platinum regimens compared to platinum-based comparator regimens [Le Chevalier et al., 2005]. Late 2006, bevacizumab, a monoclonal antibody directed against vascular endothelial growth factor (VEGF), was approved in combination with paclitaxel and carboplatin chemotherapy for first-line treatment of patients with non-squamous NSCLC [Cohen et al., 2007;Sandler et al., 2006]. For patients with an adequate performance status, who have progressed on first-line therapy, docetaxel and pemetrexed can be considered as second-line therapy [VIKC, 2007;Pfister et al., 2004;Cohen et al., 2005]. Additionally, small molecule epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKIs), gefitinib and erlotinib, have been approved for second-line treatment [Johnson et al., 2005;Cohen et al., 2004].

First-line chemotherapeutic regimens typically result in partial response rates of 20-35% and stable disease rates of approximately 30%. Response is

hereby measured following the response evaluation criteria in solid tumors (RECIST) guidelines [Therasse et al., 2000]**.** Median time to progression is typically around 4 to 5 months, median survival around 8 to 11 months and 1-year survival 35 to 40% [Schiller et al., 2002;Shepherd, 2004]. Poor performance status (ECOG 1 or 2) is an important negative prognostic factor for survival [Hoang et al., 2005]. Median survival upon second-line docetaxel treatment is around 5-8 months [Hanna et al., 2004;Shepherd et al., 2000]. Overall, at the cost of potentially life-threatening and disabling toxicity, the average survival advantage of chemotherapy in comparison with best supportive care is modest, estimated at several months and new, more effective -and preferably less toxic- treatments are urgently needed [Marino et al., 1994;Brown et al., 2005;Stewart and Pignon, 1995;Baggstrom et al., 2007].

Resistance to cisplatin-gemcitabine chemotherapy

DNA damaging agents such as cisplatin and carboplatin exert their antitumor effect by formation of DNA adducts. Resistance mechanisms that limit the extent of DNA damage include reduced drug uptake and increased DNA adduct repair [Siddik, 2003]. Polymorphisms in DNA repair genes were shown to influence survival in cisplatin-gemcitabine-treated NSCLC patients [de las Penas et al., 2006;Ryu et al., 2004]. In a prospective randomized clinical study, assessment of excision repair cross-complementation group 1 (ERCC1) mRNA expression in patient tumor tissue was shown to predict response to docetaxel and cisplatin chemotherapy [Cobo et al., 2007]. Furthermore, downstream from inflicted DNA damage, alterations in the apoptotic response machinery are thought to account for cisplatin-resistance.

Gemcitabine, a deoxycytidine analogue, requires phosphorylation to mono-, di- and triphosphates (dFdCMP, dFdCDP, dFdCTP, respectively) to be active. The cytotoxic effects of gemcitabine are mediated through incorporation of dFdCTP into DNA, resulting in inhibition of DNA repair and synthesis as well as induction of apoptosis. The effect of gemcitabine on DNA repair has been suggested to account for the synergistic cytotoxicity observed when cisplatin and gemcitabine are combined [Moufarij et al., 2003]. Gemcitabine (acquired) resistance is associated with altered activities of enzymes involved in the metabolism of the drug and target enzymes. Comparable to cisplatin, pathological inhibition of apoptotic pathways is thought to contribute to gemcitabine-resistance. A key role in acquired gemcitabine resistance has

been attributed to the expression level of ribonucleotide reductase subunit M1 (RRM1), an enzyme catalyzing synthesis of deoxyribonucleoside diphosphates (dNDP). RRM1 mRNA expression was found to be a predictive marker of survival in cisplatin-gemcitabine-treated patients [Rosell et al., 2004;Bergman et al., 2005]. A close correlation has been shown between levels of ERCC1 and RRM1 and a recent study suggested therapeutic decision making based on RRM1 and ERCC1 gene expression for patients with advanced NSCLC may contribute to improve patient outcome [Simon et al., 2007].
In the clinical study described in this thesis we investigated whether modulation of the ubiquitin-proteasome pathway of protein degradation might enhance efficacy of cisplatin-gemcitabine chemotherapy and reverse chemoresistance.

Ubiquitin Proteasome Pathway

Regulated protein degradation is one of the cell's most important cyclical processes involved in the regulation of a broad variety of crucial cellular processes, such as cell cycle regulation, quality control, regulation of transcription factors and degradation of damaged or misfolded proteins. With the discovery of the lysosome by Christian de Duve in the 1950s it was long assumed that cellular proteins are degraded within this organelle [de Duve, 2005]. However, subsequent experimental evidence strongly suggested that proteolysis of intracellular proteins is largely non-lysosomal. The ultimate discovery of the ubiquitin-proteasome pathway (UPP) of protein degradation at the beginning of the 1980s yielded Aaron Ciechanover, Avram Hershko and Irwin Rose in 2004 the Nobel Prize in chemistry [Ciechanover, 2006;Ciechanover, 1994].

Ubiquitin

Ubiquitin is an evolutionarily highly conserved 9-kDa cellular protein, composed of 76 amino acids. Ubiquitin is involved in numerous cell processes and used as a covalent modifier of other proteins to activate their function or to target them for degradation, depending on the degree of ubiquitin ligation [Goldstein et al., 1975]. Ubiquitin ligation, ensuring selective targeting of proteins for degradation by the 26S proteasome, is executed through a multi-step process, involving the concerted interaction of three separate enzyme activities, E1, E2 and E3, see also Figure 1. In presence of ATP, ubiquitin

activating enzyme E1 catalyses formation of activated ubiquitin which is then stably bound to the enzyme [Haas et al., 1982]. Subsequently, activated ubiquitin is transferred from E1 to E2, the ubiquitin-conjugating enzyme. Finally, ubiquitin is transferred from E2 to the target protein, a step requiring the enzymatic activity of E2 as well as E3, ubiquitin-protein ligase. This process repeats itself until a poly-ubiquitin chain is formed which can be recognized by the 26S proteasome. Mammalian cells contain only one or a few E1s, several different E2s and hundreds of different E3s, each binding to specific substrates targeted for degradation, e.g. because of conformational change of misfolding [Pickart and Rose, 1985]. An additional regulatory mechanism for ubiquitin-proteasome mediated protein degradation is formed by the presence of deubiquitinating enzymes (DUBs), able to deubiquitinate poly-ubiquitinated proteins, thus preventing degradation [Love et al., 2007].

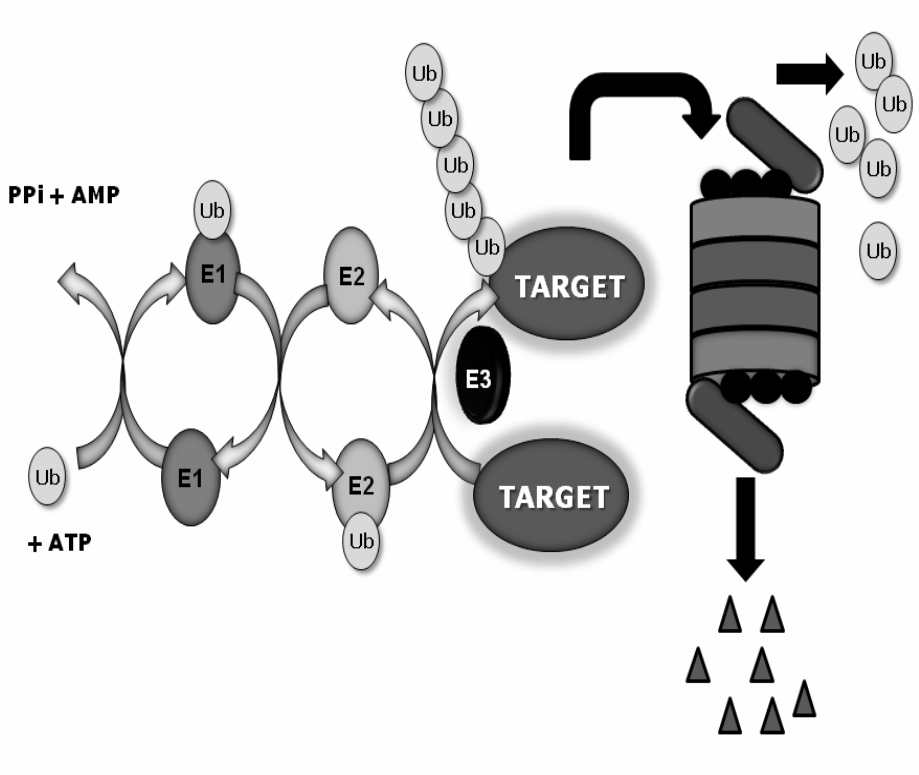

Figure 1. Conjugation of ubiquitin to the protein substrate
Ub: ubiquitin; E1: ubiquiting activating enzyme; E2: ubiquitin-conjugating enzyme; E3: ubiquitin-protein ligase; TARGET: protein targeted for proteasomal degradation;

The 26S proteasome

The 26S proteasome is an ATP-dependent proteolytic complex. It is widely expressed in the nucleus as well as in the cytosol, distribution patterns varying according to cell or tissue types [Brooks et al., 2000]. Furthermore, cell cycle-specific cytoplasmic-nuclear redistribution seems to occur [Reits et al., 1997]. The 26S proteasome consists of a proteolytic core, the 20S (720-kDa) proteasome, sandwiched between two 19S (890-kDa) regulatory complexes, "caps", see also Figure 2. The 20S proteasome forms a hollow cylinder composed of four stacked rings. Each outer ring contains 7 α-subunits, each inner ring 7 different β-subunits. The proteolytically active sites of the proteasome, post-glutamyl peptidyl hydrolytic-like (caspase-like), tryptic-like and chymotryptic-like, are harbored in the β_1, β_2 and β_5 subunits, respectively. Unusual feature of these β-subunits compared to other cellular proteinases is their catalytic nucleophile of amino-terminal threonine residues.

Access to the 20S nanocompartment is restricted to unfolded substrate polypeptides. The 19S complex is composed of six ATPases, a few polypeptides at its base and a lid [Glickman et al., 1998]. It acts as a substrate recognition and peptide unfolding machinery, assisting translocation of target proteins through the narrow gate into the 20S proteasome [Benaroudj et al., 2003]. Complex allosteric interactions determine the sequence of the different proteolytic activities, degradation being progressive until oligopeptides of 7-9 amino acids remain [Baumeister et al., 1997;Voges et al., 1999;Kisselev et al., 1999]. In this process ubiquitin is spared from destruction and recycled, see also Figure 1.

Another complex associated with the proteasome is the 11S regulator (PA28), consisting of a 28-kDa α-subunit and a 28-kDa β-subunit. Its expression is induced by interferon [Groettrup et al., 1996]. Interferon-γ equally induces replacement of the catalytic subunits with ß1i (LMP2), ß2i (MECL1), and ß5i (LMP7), forming what is termed the immunoproteasome. The 11S activator and the changes in catalytic activity play a role in immune surveillance by enhanced generation of antigenic peptides presented by class I MHC molecules [Kloetzel and Ossendorp, 2004]. Interestingly, the proteasome was shown to have non-proteolytic functions as well, e.g. by facilitating transcription events, adding to the complexity of this structure and its role [Baker and Grant, 2005].

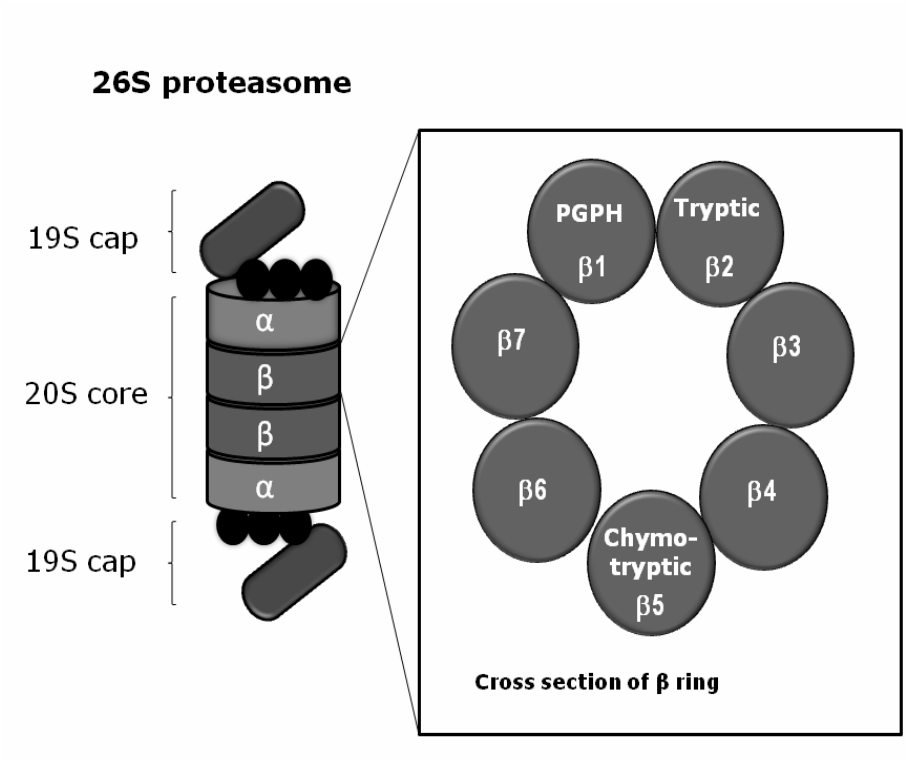

Figure 2. Structure of the 26S proteasome
α: α-ring; β: β-ring; PGPH: post-glutamyl peptidyl hydrolytic-like

Proteasome substrates implicated in cancer and NSCLC

Numerous 26S proteasome substrates are implicated in pathological conditions such as neurodegenerative (aggresome-mediated) disease, chronic inflammatory conditions and neoplastic disease [Ciechanover and Brundin, 2003;Reinstein and Ciechanover, 2006]. Among important physiological substrates of ubiquitin-mediated degradation are proteins involved in regulation of the cell cycle, DNA repair, apoptosis and gene transcription. See also Table 2. As for cell cycle regulation, rapid and timely ubiquitin-mediated degradation of cyclins and cyclin dependent kinase inhibitors such as p21 (CIP1) and p27 (KIP1), constitutes a crucial regulatory mechanism for cell cycle progression [Murray, 2004;King et al., 1996]. Previously, the E3 enzyme responsible for ubiquitin-mediated cyclin degradation was identified as the anaphase-promoting complex [Glotzer et al., 1991;Mishima et al., 2004;Holloway et al., 1993;Nasmyth, 2001]. Malignant tumors frequently have altered numbers of chromosomes due to repeated mis-segregation of chromosomes.

Table 2. Important rapidly 26S proteasome-degraded regulatory proteins

Oncogenic products	p53 and MDM2; c-fos; c-jun; c-Mos; E2A proteins
Cell cycle regulatory proteins	CDK inhibitors (including p21, p27) Cyclins (mitotic, G1, others)
Transcriptional regulators	IκB and NF-κB STAT proteins Activating transcription factor 2 (ATF2) Hypoxia inducible factor-1 (HIF-1) β-catenins
Enzymes	DNA polymerase Ornithine decarboxylase Receptor associated protein kinases RNA polymerase II large subunit Iron regulatory protein 2 (IRF2)

[Adams (ed.), 2005]

Loss or low levels of p27 have been associated with poor prognosis in NSCLC and tumors expressing low to undetectable levels of p27 were shown to display high p27 ubiquitin-mediated degradation activity [Hirabayashi et al., 2002;Esposito et al., 1997] Overexpression of p27 in NSCLC cells was shown to promote apoptosis [Katayose et al., 1997]. Oncogenesis is regarded as a process requiring typically four to six (epi)genetic changes [Hahn and Weinberg, 2002]. Cellular protective mechanisms to prevent accumulation of mutations and malignant transformation include stress-induced stabilization of tumor suppressor protein p53, known as the "guardian of the genome". Phosphorylation of p53 inhibits its interaction with E3 enzyme Mdm2, diminishing degradation [Honda et al., 1997]. By inducing cell cycle arrest, p53 allows time for repair, differentiation, senescence, or, when damage is too

extensive, stimulates an apoptotic response [Schmitt et al., 2000;Levine, 1997;Brown and Attardi, 2005;Vousden and Lu, 2002]. Not surprisingly, loss of p53 function has been found in many cancers, including NSCLC [Wallace-Brodeur and Lowe, 1999;Vousden and Lane, 2007;Hollstein et al., 1991;Rusch et al., 1995;Li et al., 1994]. An ingenuous strategy to inhibit p53 function is being employed by the human papilloma virus which is strongly associated with cervical cancer. A viral protein was shown to activate a specific E3 enzyme, E6-AP, mediating p53 ubiquitination and degradation [Scheffner et al., 1993].

Nuclear factor kappa B (NF-κB) is an important transcription factor regulating the transcription of many genes involved in inflammation, immune response and cell survival. NF-κB activation is regulated by the ubiquitin-mediated degradation of inhibitor protein I kappa B (IκB). IκB is degraded upon (stress-induced) phosphorylation, releasing NF-κB, which then translocates to the cell nucleus and binds to the promoter regions of target genes.

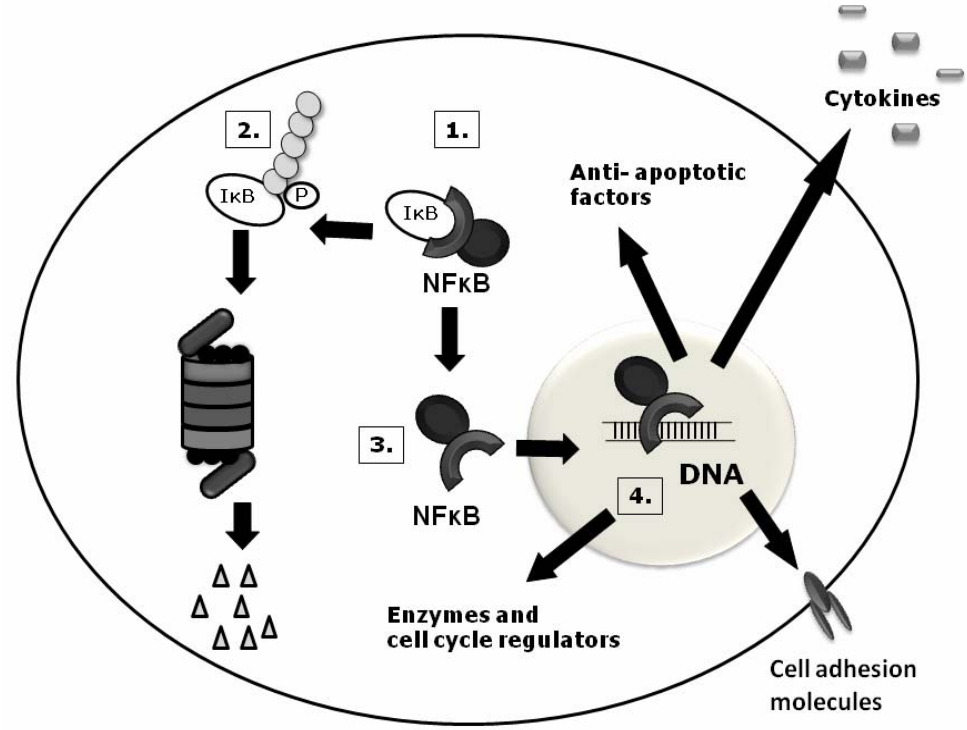

Figure 3. The NF-κB pathway
1. NF-κB bound to its inhibitory protein IκB; 2. Phosphorylated IκB tagged for degradation by the proteasome; 3. Unbound NF-κB translocates to the nucleus; 4. NF-κB activates transcription;

NF-κB has been recognized as one of the key players in tumor development and drug resistance [Karin et al., 2002]. Transcribed gene products include cell adhesion molecules, involved in angiogenesis and metastasis, anti-apoptotic factors such as B-cell leukemia/ lymphoma-2 (Bcl-2), Bcl-X_L, FLICE inhibitory protein (FLIP), cIAP, survivin, and growth factors such as interleukin-6, see also Figure 3 [Palombella et al., 1994;Zetter, 1993;Fahy et al., 2005]. Activation of NF-κB can suppress caspase-8 activation, e.g. in response to TNF stimulation, thus inhibiting apoptosis [Wang et al., 1998]. Additionally, NF-κB induces drug resistance, for example by decreasing the level of p53 stabilization and induction of P-glycoprotein expression, a plasma membrane transporter involved in the efflux of chemotherapeutic molecules [Bentires-Alj et al., 2003;Tergaonkar et al., 2002]. It was shown in NSCLC cells that a viable NF-κB pathway was an important factor for survival of cells following chemotherapy or tumor necrosis factor-alpha (TNF-α) stimulation [Jones et al., 2000;Berman et al., 2002;Cheng et al., 2000].

Targeting the proteasome in cancer

As exemplified in the previous section, ubiquitin-mediated proteasomal degradation is integral to the mechanisms underlying oncogenesis and is crucial for cancer cell survival. Boosting the levels of substrates such as p53, p27 and NF-κB inhibitory protein IκB by chemical inhibition of proteasome activity would seem therapeutically beneficial. However, modulation of protein homeostasis is very likely to (negatively) affect the functioning of normal cells as well. Therefore, at first sight, inhibition of proteasome activity did not look like an attractive candidate strategy for selective killing of cancer cells. Nonetheless, from the late 1990s onwards, evidence for a striking selective sensitivity of (proliferating) cancer cells to proteasome inhibition started to accumulate. Several *in vitro* studies demonstrated potent cytotoxic effects of drug-induced proteasome inhibition in malignant cells, at drug concentrations which left their untransformed, normal counterparts unaffected [An et al., 1998;Kudo et al., 2000;Hideshima et al., 2001;Guzman et al., 2002]. Many researchers have since then tried to provide an explanation for this remarkable phenomenon. Sensitivity of B-CLL cells to proteasome inhibition, as compared to normal lymphocytes, was associated with a highly up-regulated ubiquitin-proteasome system, characterized by a three-fold increase in chymotryptic-like activity, as well as a pathological disturbance in the regulation of p53 proteolysis, resulting in selective accumulation of nuclear

wild-type p53 in malignant cells. These results suggested an essential role of the ubiquitin system in apoptotic cell death control in CLL lymphocytes. Inhibition was hypothesized to result in a discriminatory apoptotic stimulus between normal versus malignant lymphocytes [Masdehors et al., 2000]. An important aspect may be that many malignant cells proliferate rapidly and, having one or more aberrant cell-cycle checkpoints, are more likely to accumulate damaged proteins for which they rely on the proteasome system to clear [Adams, 2004b]. However, as even quiescent malignant cells were shown to be more sensitive to proteasome inhibition than proliferating normal cells, more mechanisms must underlie this susceptibility [Guzman et al., 2002]. Differential regulation of pro-apoptotic protein Noxa (see also below) was proposed to account for the efficacy of proteasome inhibitor bortezomib to induce apoptotic cell death in melanoma cells and not in normal melanocytes [Fernandez et al., 2005]. Furthermore, it is now known certain malignancies are dependent on aberrant, constitutive activation of the NF-κB pathway for their survival [Feinman et al., 1999;Ni et al., 2001;Kordes et al., 2000]. Proteasome inhibition results in inhibition of NF-κB activation in these cells, preventing the expression of all types of pro-survival and drug resistance related factors. A good example of such a malignancy is multiple myeloma [Hideshima et al., 2002]. Finally, inhibition of proteasome activity might, by enhancing levels of ubiquitin-mediated cell-cycle and apoptosis regulatory proteins, counteract uncontrolled proliferation and apoptosis inhibition [Adams, 2004b]. Although the exact mechanisms of selectivity still have to be further investigated, (clinical) development of proteasome inhibitors as anti-cancer drugs became of huge interest.

Proteasome inhibitors

Initially sought after for fundamental research purposes, the first compounds shown to inhibit proteasomal activity were β-lacton lactacystin and synthetic peptide aldehydes. Later, other classes such as the peptide vinyl sulphones, peptide epoxiketones and peptide boronates were recognized. Compounds differ in reversibility of the inhibitory interaction and in selectivity for proteasomal enzyme activities as well as non-proteasomal enzymes. A (partial) list of proteasome inhibitors for laboratory use is provided in Table 2.

Table 2. Proteasome inhibitors in preclinical use

Compound	Properties
Aclacinomycin A	Natural inhibitor chymotrypsin-like activity [Figueiredo-Pereira et al., 1996]
Benzamide	Synthetic inhibitor chymotrypsin-like activity [Lum et al., 1998]
Calpain Inhibitors I and II	Synthetic catalytic β-subunit inhibitor [Donkor, 2000;Wilk et al., 1991]
Eponemycin	Natural catalytic β-subunit inhibitor [Meng et al., 1999]
Epoxomycin	Natural catalytic β-subunit inhibitor [Kim et al., 1999]
Lactacystin	Natural metabolite of *Streptomyces;* catalytic β-subunit and cathepsin A inhibitor [Fenteany and Schreiber, 1998]
MG132	Synthetic peptide aldehyde inhibitor; inhibits calpains and cathepsins, chymotryptic-like (and PGPH-like) activity [Crawford et al., 2006]

Bortezomib

Proteasome inhibitor bortezomib (previously known as PS-341, MLN341 or LPD-341) was synthesized in 1992 by medicinal chemistry. Julian Adams, whose lab pursued physiologic studies of the control of protein degradation in muscle in normal and disease states, was responsible for introducing the boron in the molecule and played a key role in the development of bortezomib. Peptide aldehydes were unattractive for clinical development as they are

unstable and quickly oxidized to an inactive acid. Additionally, they inhibit other cellular non-proteasomal proteases as well [Kisselev and Goldberg, 2001]. In comparison, bortezomib's favorable pharmacokinetic and pharmacodynamic properties have been responsible for igniting a tremendous surge in clinical (and preclinical) research on proteasome inhibition as a target for treating neoplastic disease. Bortezomib selectively and reversibly inhibits the chymotryptic-like (and caspase-like) activity of the 20S proteasome [Altun et al., 2005]. It was one compound of a group of dipeptidyl boronic-acid proteasome inhibitors, tested in the National Cancer Institute panel of 60 tumor cell lines for their potency as an anti-neoplastic agent. As *in vitro* cytotoxicity results with bortezomib were very promising, development was continued, bortezomib constituting the first proteasome inhibitor to be tested in clinical trials [Adams et al., 1999;Adams, 2004a;Papandreou et al., 2004]. Early clinical studies showed especially single-agent activity in multiple myeloma patients and it did not take long before bortezomib received accelerated FDA approval for treatment of relapsed refractory multiple myeloma patients in 2003 [Kane et al., 2003].

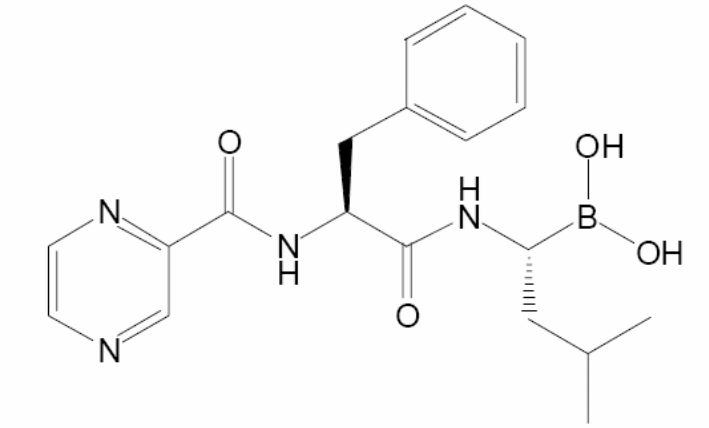

Figure 4. Chemical structure of bortezomib
B: boron atom

Preclinical experience with bortezomib in NSCLC

Early studies showed that bortezomib was effective, at nanomolar concentrations, in inducing apoptotic cell death in NSCLC cell lines, preceded by G_2M phase arrest, p53 stabilization and cleavage of anti-apoptotic protein Bcl-2 [Ling et al., 2002;Fahy et al., 2005]. Cell-cycle arrest was associated with inhibition of degradation of cell cycle regulators, including CDKI p21.

Furthermore, reactive oxygen species (ROS) generation as well as cytosolic release of pro-apoptotic factor cytochrome c were shown to initiate bortezomib-induced apoptosis [Ling et al., 2003a;Ling et al., 2003b]. Another study additionally showed induction of apoptosis in NSCLC cells by activation of the JNK/c-Jun/AP-1 pathway [Yang et al., 2004]. In a xenograft mouse model bortezomib showed activity against the Lewis lung carcinoma and was highly effective against lung metastatic disease [Teicher et al., 1999]. Another study in NSCLC cells demonstrated that up-regulation of BH3-only pro-apoptotic protein Bik correlated with apoptosis induction by bortezomib [Zhu et al., 2005].

In response to cellular insults, such as chemotherapy, NF-κB is often activated to circumvent cell death [Wang et al., 1999]. As proteasome inhibitors, including bortezomib, were shown to be effective inhibitors of NF-κB activation, studies were conducted to evaluate their potential as chemosensitizers [Denlinger et al., 2004a]. In the above mentioned *in vivo* study by Teicher *et al.* an additive anti-tumor effect was observed when cisplatin was combined with bortezomib. In NSCLC cells it was shown that bortezomib could act as a sensitizing agent for gemcitabine-induced apoptosis, preventing gemcitabine-induced activation of NF-κB [Denlinger et al., 2004b]. Similar findings of bortezomib-induced chemosensitization coinciding with inhibition of NF-κB activation were reported in other tumor models [Shah et al., 2001;Cusack, Jr. et al., 2001].

Finding the right administration sequence of bortezomib-chemotherapy combinations has been the focus of a number of studies. Administration of chemotherapy prior to bortezomib administration was found to be more cytotoxic than the reverse sequence, coinciding with increased p27 accumulation and Bcl-2 down-regulation [Mack et al., 2003]. These results led some to propose a model of therapy response based on the cell cycle effects of the bortezomib plus docetaxel combinations [Garfield and Cadranel, 2007]. Docetaxel induces M-phase arrest and apoptosis, which is enhanced when followed by bortezomib-induced proteasome inhibition. However, when bortezomib precedes docetaxel treatment, the M-phase activity of docetaxel is blocked by bortezomib-induced G_2M arrest. Interestingly, findings of *in vivo* studies, using a xenograft NSCLC model, showed similar tumor growth inhibition of around 40% in either sequence. No growth inhibition was observed when agents were simultaneously administered, suggesting an

antagonistic effect. These findings were contradictory to *in vivo* results from studies conducted by Millennium Pharmaceuticals, the producer of bortezomib, and Teicher *et al*. which did not show an antagonistic effect of co-administration or sequence dependency [Scagliotti, 2006;Teicher et al., 1999]. In combinations with other drugs, such as carboplatin and gemcitabine, a sequence dependent effect was also reported *in vitro*, favoring initial or concurrent administration of chemotherapy over initial bortezomib treatment. Findings were similar using a xenograft tumor model [Mortenson et al., 2004]. It was hypothesized that bortezomib-induced cell cycle arrest prevents cells from entering the S-phase, when a lot of chemotherapeutic drugs, such as gemcitabine, are most effective.

Clinical development of single agent bortezomib in NSCLC

Bortezomib is administered as a short intravenous injection. As bortezomib is rapidly cleared from the vascular compartment, quickly reaching limits of detection, an *ex vivo* assay to measure proteasome activity in peripheral blood mononuclear cells (PBMCs) was developed to complement pharmacokinetic studies [Lightcap et al., 2000]. Based on the pharmacodynamic profile in PBMCs a tolerable two-weekly dosing schedule was established, allowing enough time between two gifts of bortezomib for proteasome activity to restore [Orlowski et al., 2002]. Most common side-effects reported from clinical trials with single agent bortezomib administration were fatigue or asthenia, nausea, diarrhea, decreased appetite, constipation, thrombocytopenia, peripheral neuropathy, pyrexia, vomiting and anemia [Adams, 2004b].

Initial clinical studies with bortezomib in solid tumor patients showed promising activity in non-small cell lung cancer patients [Aghajanian et al., 2002]. Especially patients with bronchioloalveolar carcinoma (BAC) seemed to benefit from bortezomib treatment with two convincingly responding patients in initial small studies and one anecdotal response described [Subramanian et al., 2006;Stevenson et al., 2004]. Subsequent phase 2 studies were aimed at determining efficacy of bortezomib monotherapy in non-small cell lung cancer patients. One study was conducted in BAC patients only as it was thought to constitute a more bortezomib-sensitive NSCLC subtype [Garfield and Cadranel, 2007]. See Table 3 for a summary of single agent studies with bortezomib in NSCLC.

Table 3: clinical studies in NSCLC with single-agent bortezomib

Single-agent Dose mg/m^2	Patient population	Phase	Activity
Up to 1.56	Advanced solid tumors	1	Partial response 1/8 refractory NSCLC patients [Aghajanian et al., 2002]
1.3/1.5	Advanced NSCLC	2	PR 1/18, SD 8/18 patients (all at 1.5) [Stevenson et al., 2004]
1.5	Advanced NSCLC (second-line)	2	8% Partial response, 29% disease control rate; 75 patients [Fanucchi et al., 2006]

Adapted from Scagliotti [Scagliotti, 2006]

The phase 2 study by Fanucchi *et al.* showed a partial response rate of 8%, disease control rate of 29% and median survival of 7.4 months, comparable to clinical outcomes of current second-line treatments for (unselected) advanced NSCLC patients, such as pemetrexed, docetaxel and erlotinib [Fossella et al., 2000;Hanna et al., 2004;Shepherd et al., 2000;Braithwaite and Shepherd, 1981]. However, duration until progression upon bortezomib treatment was much shorter (median 1.5 months), suggesting its role as second-line treatment in unselected NSCLC patients is limited. The results of the phase 2 study in BAC patients are eagerly awaited. However in absence of early reports on significant activity, dramatic positive results are unlikely.

Combination regimens incorporating bortezomib

A logical next step in the development of bortezomib was to combine it with other (chemo)therapeutic drugs, especially because of the promising *in vitro* and *in vivo* findings in combination regimens. See Table 4 for a summary on combination studies with bortezomib in advanced NSCLC patients. In the phase 2 study by Fanucchi *et al.* docetaxel administration was completed one hour before injection of bortezomib [Fanucchi et al., 2006]. The combination regimen resulted in a 9% partial response rate, 45% stable disease rate and a median survival of 7.8 months. A follow-up study was initiated evaluating a schedule where docetaxel is administered one day prior to bortezomib [Davies et al., 2007b].

Table 4: clinical combination studies with bortezomib in NSCLC

Combination agent	Patient population	Phase	Activity
Docetaxel	Advanced solid tumors	1	Stable disease 2/4 NSCLC patients [Lara, Jr. et al., 2006]
Docetaxel	Advanced NSCLC	2	9% partial response, 45% stable disease in 80 patients [Fanucchi et al., 2006]
Gemcitabine	Advanced solid tumors	1	Partial response 1/5 patients relapsed advanced NSCLC [Appleman et al., 2003]
Gemcitabine + carboplatin	Untreated/ pretreated Advanced NSCLC	1	Partial response 9/26 Stable disease 8/26 [Davies et al., 2008]
Gemcitabine + carboplatin	Advanced NSCLC	2	Partial response 20% Stable disease 66% 114 patients [Davies et al., 2006]
Pemetrexed	Advanced solid tumors	1	Partial response 2/16 NSCLC patients (12.5%) [Davies et al., 2007a]

Adapted from Scagliotti [Scagliotti, 2006]

Results from this study are eagerly awaited. Promising results were obtained in initial phase 1 studies combining bortezomib with gemcitabine, alone or in combination with carboplatin. Davies *et al.* initiated, based on the *in vitro* findings of sequence dependency, a phase 2 study where chemotherapy was administered prior to bortezomib [Davies et al., 2006]. A study combining gemcitabine-cisplatin chemotherapy and bortezomib is described in this thesis. Additionally, a number of studies, also supported by preclinical findings, investigate the clinical potential of as a radiosensitizer, alone or in combination with chemotherapy [Davies et al., 2007b].

Other proteasome inhibitors in clinical development

Proven effective as treatment for patients with certain malignant diseases, clinical development of various other proteasome inhibitors is ongoing. The added value of these new agents has to be still established. In preclinical models sensitivity some of these new drugs were shown to overcome bortezomib-resistance or to induce synergistic cell death in combination with bortezomib [Chauhan et al., 2007;Williamson et al., 2006;Kuhn et al., 2007]. An overview of proteasome inhibitors in clinical development, including bortezomib, is provided in Table 5.

Table 5. Proteasome inhibitors in clinical development

Compound	Properties	Phase	
Bortezomib (PS-341)	Synthetic reversible peptide boronate inhibitor of chymotryptic-like (and PGPH-like) activity; i.v.	Phase 1-3, various indications; Approved for second line treatment multiple myeloma and mantle cell lymphoma	[Adams, 2004a; Kane et al., 2007; Bross et al., 2004; Crawford et al., 2006]
Carfilzomib (PR-171)	Synthetic irreversible epoxyketone peptidyl catalytic β-subunit inhibitor; i.v.	Phase 1, hematological malignancies	[Demo et al., 2007]
CEP-18770	Synthetic reversible peptide boronate inhibitor of chymotryptic-like activity; oral	To enter clinical development for multiple myeloma	[Piva et al., 2007]
MLN-519	Synthetic lactacystin derivative; catalytic β-subunit inhibitor; i.v.	Phase 1, planned development for stroke	[DiNapoli and Papa, 2003]
Salinosporamide A (NPI-0052)	Natural metabolite of marine bacterium *Salinispora tropica;* catalytic β-subunit inhibitor; oral and i.v.	Phase 1, solid tumors, hematological malignancies (i.v. only, phase 1 with oral administration planned to start in 2009)	[Ahn et al., 2007;Chauhan et al., 2005]

Apoptosis

It was frequently mentioned before that bortezomib induces apoptotic cell death in NSCLC cells. However, the exact mechanism of bortezomib-induced apoptosis remains largely unknown. Therefore, in our preclinical studies, we investigated further the mechanisms of bortezomib-induced apoptosis.

A regulated balance between cell proliferation and cell death is pivotal for the differentiation and maintenance of multicellular organisms. According to the circumstances, eukaryotic cells can perish in different ways. A division has been made based upon the involvement or not of a certain type of proteases, called caspases. Caspase-dependent cell death or apoptosis (from Greek "falling leaves"), constitutes an organized, genetically controlled, cellular collapse ("self-killing"; programmed cell death). Aberrant apoptosis has been implicated in many human diseases and evasion of apoptosis is being regarded as one of the primary hallmarks of cancer [Hanahan and Weinberg, 2000]. Necrosis and autophagy are examples of caspase-independent cell death. Autophagy is a genetically controlled process of auto-engulfment ("self-eating"). It can result in stress adaptation, preventing cell death and inhibiting apoptosis, whereas in other cellular settings, it can result in cell-death. Recent studies have shown that the apoptotic and autophagic response machineries share common pathways that either connect or polarize the cellular responses. The full scope and mechanisms of these interaction have yet to be fully appreciated [Maiuri et al., 2007].

Several morphological and molecular hallmarks characterize apoptotic death such as chromatin condensation, DNA fragmentation, membrane blebbing, cytoplasmic shrinkage, formation of apoptotic bodies and externalization of phosphatidylserine residues [Strasser et al., 2000;Kerr et al., 1972]. Caspases, enzymes belonging to the family of cystein proteases that cleave proteins at aspartic acid residues, play a central role in the execution of apoptosis [Nicholson and Thornberry, 1997]. Caspases are expressed as zymogens and their activation requires dimerization and stabilization. The so-called "caspase cascade" is triggered by activation of initiator caspases, caspase-8 and caspase-9, which, by proteolytic cleavage, subsequently activate downstream effector caspases, such as caspase-3 [Riedl and Shi, 2004]. The process is completed through effector caspase-mediated

proteolysis of over 70 cellular target substrates, culminating in apoptotic cell death [Nunez et al., 1998;Hengartner, 2000;Kaufmann and Hengartner, 2001]. Interestingly, target substrates for effector caspases include subunits of the 19S regulatory complex of the proteasome. Caspase-mediated cleavage of proteasome components results in inhibition of proteasome activity and is thought to result, due to the accumulation of pro-apoptotic proteasome substrates (e.g. Smac/DIABLO), in a feed-forward amplification loop, enhancing the apoptotic response [Sun et al., 2004].

Two pathways exist for the activation of apoptosis. The intrinsic apoptotic pathway, headed by caspase-9, is activated by cellular stress such as UV or ionizing radiation and chemotherapeutic drugs [Kuida et al., 1998]. Caspase-8 is the apical caspase of the extrinsic pathway, which is activated by binding of a ligand to cell surface receptors known as "death receptors".

Intrinsic apoptotic pathway

Equally known as the "mitochondrial" apoptotic pathway, the intrinsic apoptotic pathway is characterized by mitochondrial outer membrane permeabilization (MOMP) and release of cytochrome C, resulting in the assembly of the apoptosome, an activating complex between caspase-9 and Apaf-1, see also Figure 4 [Armstrong, 2006;Cory and Adams, 2002].

Bcl-2 family

The Bcl-2 family of proteins plays a central role in the regulation of mitochondrial permeabilization. Members are divided in pro-apoptotic and anti-apoptotic members. An additional classification has been made according to the conservation of Bcl-2 homology (BH) domains 1-4. Examples of multi-domain anti-apoptotic members are Bcl-2, Bcl-xl and Mcl-1. Pro-apoptotic BH3-only members include Noxa, Puma, Bid, Bim, Hrk, Bmf, and Bik. BH3-only proteins serve as sentinels for cellular stress, infection, or organelle specific damage [Huang and Strasser, 2000]. Intrinsic apoptosis activation can be regulated upstream and downstream from MOMP. Downstream regulation involves inhibition of caspase activity, e.g. by Inhibitor of Apoptosis Proteins (IAPs), such as XIAP. Upstream, MOMP is regulated by interactions between Bcl-2 family proteins.

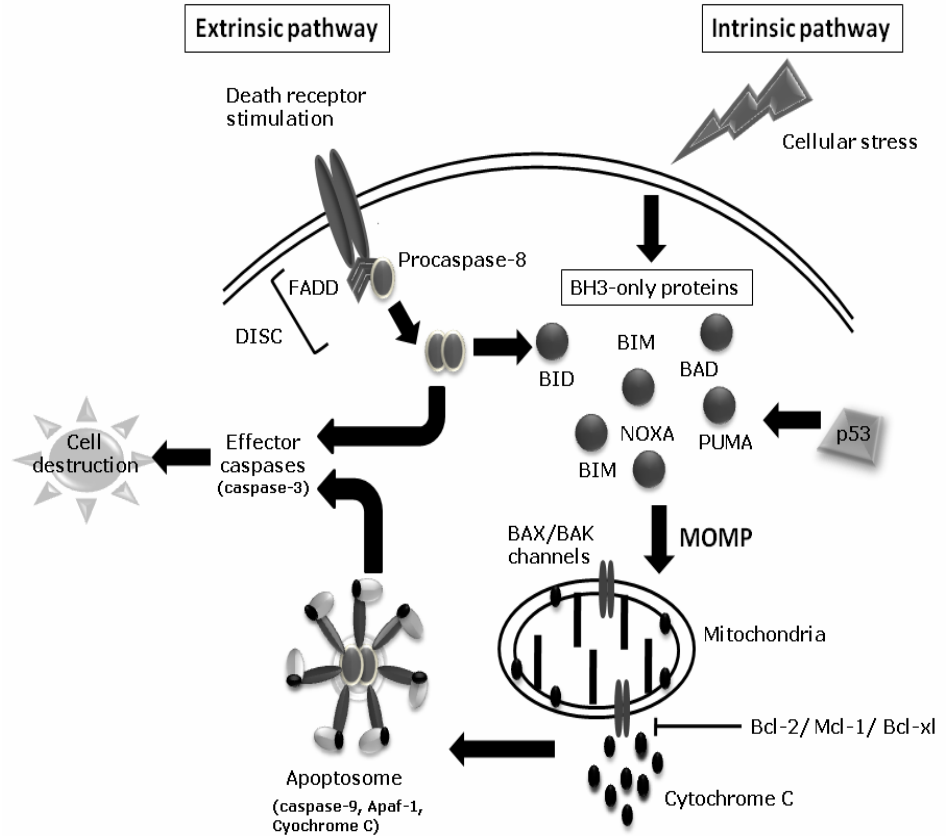

Figure 4. Apoptosis pathways

Transcription of BH3-only proteins is tightly regulated and members can be mobilized by post-translational modification (e.g. phosphorylation or proteolysis) or subcellular relocalization. BH3-only proteins regulate the activation of multidomain pro-apoptotic Bcl-2 family members Bax and Bak in the outer mitochondrial membrane by triggering their oligomerization, resulting in formation of channels permitting the cytosolic release of multiple factors, such as cytochrome C and Smac/DIABLO [Lucken-Ardjomande and Martinou, 2005;Willis and Adams, 2005]. BH3-only proteins Bid and Bim are able to directly interact with Bax and Bak and therefore known as direct activators. Noxa, Puma, Bad, Hrk, Bik and Bmf cannot directly interact with Bak and Bax but function as de-repressors or sensitizers of Bax and Bak. By occupation of the interaction pocket of anti-apoptotic Bcl-2 family members, such as Bcl-2 and Mcl-1, pro-apoptotic members such as Bim and Bid are

released from the inhibitory interaction [Letai et al., 2002;Kuwana et al., 2005].

Extrinsic apoptotic pathway

The extrinsic apoptotic pathway, also referred to as the death receptor pathway, is activated through members of the tumor necrosis factor (TNF) receptor superfamily. Extracellular binding of a ligand such as TNF-α, CD95L (FasL/Apo-1L) or TNF- related apoptosis-inducing ligand (TRAIL or Apo-2L) to their cognate receptors results in intracellular formation of a so-called death-inducing signalling complex (DISC), involving recruitment and activation of initiator caspase-8 in the presence of an adaptor molecule, FADD or TRADD [Krammer, 1998;Fulda and Debatin, 2006;Salvesen, 1999]. Downstream effector caspase-3 can be activated in two ways depending on the cell type. In type I cells activated caspase-8 directly activates procaspase-3. In type II cells the DISC formation is impaired, but sufficient caspase-8 is generated to cleave Bid into its truncated form, tBid, triggering the mitochondria-dependent apoptotic pathway, leading to caspase-9 activation. This cross-talk between the extrinsic and intrinsic apoptotic pathways serves to amplify the apoptotic response.

TNF- related apoptosis-inducing ligand (TRAIL)

Death receptor ligand TRAIL (Apo-2L) is expressed in a broad range of tissues and exerts great antitumor activity, selectively inducing apoptosis in tumor cells as opposed to normal, healthy cells [MacFarlane, 2003;Wang and el-Deiry, 2003;Pitti et al., 1996]. This feature triggered great interest in development of TRAIL as a potential anti-cancer agent, especially when TRAIL administration was shown to be exempt from the (hepato)toxic effects as observed with TNF-α therapy [Suliman et al., 2001;Daniel et al., 2001]. Nevertheless, many cancer cells are resistant to TRAIL-induced apoptosis as well. The mechanisms of this resistance are not fully understood. Initial preclinical studies in other tumor types than NSCLC showed a sensitization to TRAIL-induced apoptosis when TRAIL was combined with bortezomib [Khanbolooki et al., 2006;Georgakis et al., 2005;Johnson et al., 2003;Nikrad et al., 2005].

Proteomics

Proteomics deals with the large scale analysis of gene and cellular function directly at the protein level. Proteomics includes not only the identification and quantification of proteins but also determination of their localization, post-translational modifications, interaction activities and, ultimately, their function. The explosive growth of the proteomics field has been fueled by the sequencing of the genome, development of powerful new technologies, such as mass spectrometry approaches, as well as innovative computational tools and methods to process, analyze and interpret the enormous amounts of generated data. [Hanash, 2003;Aebersold and Mann, 2003]. In oncology, proteomics-based approaches are being employed in an attempt to understand the biology of cancer through the analysis of protein expression. Proteomics-based protein signatures might serve as diagnostic assays for early detection of cancer. Furthermore, as only a limited number of cancer patients benefit from often toxic treatments such as chemotherapy, protein-signatures are being investigated which might predict clinical outcome parameters, such as survival and tumor regression, with those treatments. This might help to prevent overtreatment, enabling personalized medicine.

Blood-related proteomics

As blood constitutes a readily accessible source of specimens, blood-based proteomics strategies are increasingly used. However, plasma contains over 10.000 commonly present proteins. Furthermore, their concentrations range over 15 orders of magnitude, albumin alone for example representing 50% of plasma protein content. The highly complex proteome of blood therefore imposes important analytical challenges, e.g. for detection of low abundant proteins and protein fragments. Recently, surface-enhanced laser desorption ionization (SELDI) and matrix-assisted laser desorption/ ionization time of flight (MALDI-TOF) mass spectrometry-based serum or plasma peptide profiling strategies have been applied to establish plasma or serum peptide patterns distinctive for NSCLC [Kikuchi and Carbone, 2007;Patz, Jr. et al., 2007]. Blood serum is the part of plasma that remains after fibrinogen, prothrombin, and other clotting factors have been removed by clot formation. The serum peptidome, that comprises peptides and proteins less than 10-kDa, contains not only fragments derived from high abundance circulating proteins

but also derived from (tumor) cells and tissues. Therefore, it is suggested to represent a dynamic image of biological events, containing disease-related peptide patterns [Liotta and Petricoin, 2006].

Interestingly, findings by Vilanueva *et al.* suggest serum contains relevant information that plasma does not harbor. They showed that differential exoprotease activities superimposed on the *ex vivo* coagulation and complement degradation pathways result in cancer-type specific serum peptide patterns, constituting an indirect "snapshot" of the enzyme activity of tumor cells [Villanueva et al., 2006]. Additionally, several attempts have been made to establish serum peptide signatures in NSCLC patients correlating to clinical outcome, e,g, survival or tumor shrinkage, upon different types of treatment [Taguchi et al., 2007;Kurup et al., 2006]

Outline of the thesis

The 26S proteasome is a cellular structure which enables a cell, in an intricately regulated way, to degrade intracellular proteins. The work described in this thesis focuses on the proteasome as a potential therapeutic target for the treatment of non-small cell lung cancer (NSCLC). For our studies we used bortezomib, a small molecule inhibitor of proteasome activity. Bortezomib has already been approved for treatment of patients with certain hematological malignancies.

Cisplatin and gemcitabine chemotherapy is considered as one of the standard treatments for patients with advanced, incurable NSCLC. Disappointingly, only a limited number of patients benefit from this toxic treatment in terms of prolonged survival and disease regression. Improvement of treatment outcome is urgently needed. The preclinical studies (Chapters 2-4) describe the mechanism of bortezomib-induced cell death in non-small cell lung cancer cell lines. A comparative analysis of molecular events underlying cell death in bortezomib-treated versus cisplatin-treated NSCLC cells was conducted. A promising synergistic combination of bortezomib and another new anti-cancer agent, death-receptor ligand TRAIL, was also investigated.

In the clinical study (Chapter 5) we investigated tolerability and efficacy of bortezomib in combination with gemcitabine and cisplatin chemotherapy in patients with solid tumors and provided preliminary clinical outcome results in NSCLC patients. A case report (Chapter 6) of a patient with congestive heart

failure and a review on bortezomib-induced neurotoxicity (Chapter 7) illustrate examples of toxicity associated with bortezomib (and chemotherapeutic) treatment. As pharmacokinetic studies in participating study patients showed an unexpected plasma concentration profile of gemcitabine, we further investigated a potential interaction between bortezomib and gemcitabine in peripheral blood mononuclear cells as well as in NSCLC cell lines (Chapter 8). Finally, serum samples of participating non-small cell lung cancer patients were used for proteomics analysis in order to establish serum peptide patterns characterizing (advanced) NSCLC patients and to develop predicting algorithms for clinical outcome and treatment-related effects (Chapter 9). Outcomes from such studies might help clinical decision making in the future, potentially preventing overtreatment and, importantly, aid in developing strategies for early detection of lung cancer patients when the disease is still in a curable stage.

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