Chapter 10

Summarizing discussion

In non-small cell lung cancer, a highly heterogeneous disease, the currently dominant "one size fits all" treatment approach has reached a survival plateau [Shepherd, 2004]. Histopathologic and radiographic staging do not sufficiently predict overall outcome and treatment response in individual patients. Identification of distinct molecular targets makes it increasingly possible to define clinically relevant subgroups. Targeted cancer therapy, pioneered in NSCLC by the approval of bevacizumab and EGFR-TKIs, may constitute a more effective treatment strategy in selected patients, interfering with specific molecular targets needed for carcinogenesis and tumor growth, rather than by simply interfering with rapidly dividing cells. Key issues in the development of novel molecularly targeted agents for NSCLC include: defining the target of an agent, demonstrating that the agent has a clinically meaningful impact on the target, and showing that the target is relevant in disease [Lynch et al., 2006].

As described in Chapter 1, numerous substrates of proteasome-mediated degradation play a crucial role in the process of oncogenesis. However, not only malignant but also normal cells heavily rely on proteasome-mediated degradation to function. Therefore, initially, the proteasome did not seem like a probable therapeutic target for the selective killing of tumor cells. Surprisingly, malignant cells were shown to be more sensitive to cell death induced by inhibition of proteasome activity than normal cells. This created a therapeutic window and provided a rationale for the development of proteasome inhibitors as anti-cancer drugs. We investigated whether the proteasome is a relevant therapeutic target for the treatment of advanced non-small cell lung cancer. For our studies we made use of bortezomib, a small molecule proteasome inhibitor. Bortezomib is a potent inhibitor of proteasomal activity. It reversibly and selectively targets the proteasomal proteolytic activity, harbored in the 20S proteasome. Bortezomib was the first-in-class proteasome inhibitor to enter the clinic and is currently approved for second-line treatment of multiple myeloma and mantle cell lymphoma patients.
Current treatment paradigms for advanced NSCLC include first-line platinum-based chemotherapy. Unfortunately, many NSCLC tumors are resistant to platinum-containing chemotherapy. Previous research showed the chemoresistant phenotype of NSCLC is partly founded on a pathologic inhibition of the apoptotic response machinery. For example, it was shown by

our group that NSCLC H460 cells are deficient in caspase-9 activation upon cisplatin treatment [Ferreira et al., 2000]. Subsequent findings suggested the presence of a deficiency upstream of apoptosome formation in NSCLC cells [Checinska et al., 2006].

In Chapter 2 we describe a comparative analysis of molecular events underlying cell death in bortezomib-treated versus cisplatin-treated H460 cells by characterization of apoptosis activation. We found that bortezomib, but not cisplatin, induced abundant release of pro-apoptotic factors cytochrome c and Smac/Diablo from mitochondria. These events were preceded by differential effects of both drugs on the level of Bcl-2 family proteins, which regulate mitochondrial outer membrane permeabilization (MOMP). Only bortezomib treatment induced a marked increase in the expression level of pro-apoptotic BH3-only protein Noxa. We showed that Noxa and anti-apoptotic protein Mcl-1 strongly interact in bortezomib treated cells, hypothesizing that up-regulated Noxa is able to antagonize the anti-apoptotic effect of Mcl-1, disrupting the balance of Bcl-2 proteins in favor of MOMP. Overall, bortezomib more potently induced apoptotic cell death in H460 cells compared to cisplatin.

Our observation of bortezomib-induced Noxa up-regulation was in agreement with reported findings in other tumor models [Perez-Galan et al., 2006]. Soengas *et al.* showed, comparing melanocytes and melanoma cells, that bortezomib dramatically induced Noxa in a tumor-restricted manner. [Fernandez et al., 2005]. Recently, researchers from the same group demonstrated that induction of Noxa is directly dependent on the oncogene c-myc, a proteasome substrate, which was demonstrated to interact with the Noxa promoter [Nikiforov et al., 2007].

In Chapter 3 we characterized NSCLC cells for sensitivity to bortezomib. We found that the apoptosis-inducing potential of bortezomib was not only dependent on the apoptotic phenotype but also on the proteasomal phenotype of NSCLC cells. The threshold dose of bortezomib necessary to inhibit the proteasome activity differed among cell lines, which was associated with different expression patterns of proteasome components.

Recently, an increasing number of reports is emerging on the mechanism of (acquired) resistance to bortezomib, or other proteasome inhibitors. Adaptation of malignant cells to continuous exposure of bortezomib was shown to result from increased expression and altered subunit composition of the proteasome, indicating the tremendous plasticity of this cell structure [Fuchs

et al., 2007;Kraus et al., 2007]. Successful proteasome inhibitor-based treatment strategies face the challenge of having to overcome apoptosis resistance as well as (acquired) proteasomal resistance of individual lung cancer cells.

Activation of the TRAIL-receptor pathway is a promising therapeutic strategy as TRAIL was shown to selectively eradicate tumor cells. However, many tumors are resistant to TRAIL induced apoptosis. We aimed to better understand the role of the TRAIL receptors in TRAIL-mediated apoptosis resistance. To achieve this, we studied susceptibility to TRAIL therapy and analysed TRAIL-receptor expression in a panel of NSCLC cells, as described in Chapter 4. We found that (surface) expression of TRAIL-receptors could not be related to TRAIL therapy sensitivity in NSCLC cells. Next, we combined bortezomib and several forms of TRAIL therapy in NSCLC cells. We observed synergistic apoptosis induction by the combination of these drugs. In the combination with bortezomib, enhancement of TRAIL-induced apoptosis was caspase-dependent, implicating both extrinsic as well as intrinsic apoptosis activation. Furthermore, increased surface expression of TRAIL-R2 (DR5) and TRAIL-R1 (DR4) was associated with bortezomib treatment. Finally, TRAIL-induced activation of NF-κB was shown to be abrogated by bortezomib co-incubation.

Sensitization to TRAIL-induced apoptosis by bortezomib was demonstrated in other tumor types as well. Several additional mechanisms of synergy have been proposed, such as bortezomib-induced down-regulation of FLIP or stabilization of Bax [Sayers and Murphy, 2005;Liu et al., 2007;Johnson et al., 2003]. The role of DR4/5 upregulation in TRAIL-sensitization by chemotherapeutic drugs has been highly debated [Koschny et al., 2007c]. It was reported in glioma cells that TRAIL sensitivity did not rely on DR5 expression levels [Puduvalli et al., 2005]. In agreement with these findings, Inoue *et al.* showed that up-regulation of the DR5 receptor upon histone deacetylase (HDAC) inhibitor treatment was dispensable for TRAIL sensitization by HDAC inhibitors of CLL cells [Inoue et al., 2006]. Another report claimed that bortezomib co-administration releases a decisive blockade of TRAIL-signaling in astrocytoma cells, which is independent of DR4/DR5 up-regulation as well [Koschny et al., 2007b].

Chapter 5 describes a phase 1B study in advanced solid tumor patients, mostly NSCLC patients, evaluating a treatment regimen consisting of cisplatin-

gemcitabine chemotherapy in combination with a once or twice weekly co-administration of bortezomib. We hypothesized that co-administration of bortezomib might sensitize tumor cells to cisplatin-gemcitabine chemotherapy. We chose for a once weekly schedule next to the typical twice weekly schedule as it constitutes a more patient friendly regimen. Patients were assigned to increasing doses of bortezomib days 1 and 8 (weekly schedule) or days 1,4,8, and 11 (twice-weekly schedule), in addition to gemcitabine 1,000 mg/m^2 days 1 and 8 and cisplatin 70mg/m^2 day 1, every 21 days. We found that bortezomib and cisplatin-gemcitabine chemotherapy can be safely combined, all drugs at clinically relevant doses. The maximum tolerated dose (MTD) of bortezomib was equal in both schedules at 1.0 mg/m^2. Dose-limiting toxicities in the weekly schedule were diarrhea, neutropenia and thrombocytopenia. In the twice-weekly schedule they were febrile neutropenia and thrombocytopenia with bleeding. Most common ≥ grade 3 (severe) events (according to the Common Terminology Criteria for Adverse Events (CTC-AE) version 3.0) were thrombocytopenia and neutropenia. Notably, neither treatment-emergent neurotoxicity nor ototoxicity was observed, which was initially feared as both cisplatin and bortezomib are considered neurotoxic drugs. In this regard, Chapter 7 provides an overview of bortezomib-associated neurotoxicity and possible underlying mechanisms.

In our study population, the incidence of (severe) neutropenia and especially thrombocytopenia was superior to incidences reported in patients treated with cisplatin-gemcitabine chemotherapy only [Scagliotti et al., 2002;Zatloukal et al., 2003]. Interestingly, cause and kinetics of bortezomib-induced thrombocytopenia differ from chemotherapy-induced thrombocytopenia, being due to a reversible effect on megakaryocyte function rather than resulting from a direct myelotoxic effect [Lonial et al., 2005]. Consequently, bortezomib-induced thrombocytopenia is characterized by rapid recovery during the wash-out period and is rarely associated with bleeding, which was also our experience in the combination with cisplatin and gemcitabine. Chapter 6 describes the case of a participating patient who experienced severe, but reversible, congestive cardiac failure upon treatment with bortezomib and cisplatin-gemcitabine treatment. As cisplatin and gemcitabine have not clearly been associated with cardiac failure, we attributed the adverse event to the bortezomib co-administration. We hypothesized that baseline presence of subclinical cardiomyopathy, characterized by a dysregulation of the ubiquitin-

proteasome system, predisposed this patient to a cardiac side effect induced by proteasome inhibition.

In 12 patients treated at the MTD of the combination treatment plasma pharmacokinetic (PK) variables were measured to investigate a potential effect of bortezomib on the plasma PK profile of cisplatin and gemcitabine. In general, variables were not affected. However endogenous deoxycytidine levels did show an unexpected, transient drop. This finding prompted us, in an additional correlative study, to investigate bortezomib-induced effects on gemcitabine pharmacokinetics and pharmacodynamics in NSCLC cells as well as in peripheral blood mononuclear cells (PBMCs), as described in Chapter 8. Our preliminary results demonstrate that bortezomib affects differently gemcitabine pharmacokinetics and pharmacodynamics in PBMCs and NSCLC cells. For the combination of gemcitabine and bortezomib in NSCLC cells, our results indicate pre-incubation with bortezomib as the most cytotoxic schedule in H460 cells. Sequence dependency in bortezomib containing combination regimens is a highly debated issue, as discussed in Chapter 1. Most preclinical studies point towards a schedule favoring chemotherapy administration prior to bortezomib. However, most of those studies were performed using different drug concentrations with respect to those used in the clinical setting. Typically, cells were treated for longer exposure times, i.e. 24, 48 and 72 hours, as well. For our *in vitro* studies we used a schedule which may be more comparable to the clinical setting. Cells were exposed for 4h to gemcitabine 50 µM, which is the peak concentration in NSCLC patients administered a dose of 1000 mg/m^2 over 30 minutes, with or without bortezomib (2h exposure) at a concentration of 100 nM, which is the peak concentration in patients receiving 1.6 mg/m^2.

In a recent phase 2 study, evaluating efficacy of carboplatin, gemcitabine and bortezomib combination treatment in advanced NSCLC patients, a schedule was used in which chemotherapy was administered prior to bortezomib [Davies et al., 2006]. In our clinical study, bortezomib was administered right before administration of chemotherapy. Our preliminary efficacy data show comparable progression-free survival and median overall survival duration to those observed in the study by Davies *et al.*

Nonetheless, as bortezomib induces G_2M cell cycle arrest, administration sequence is likely to play a role in the activity of bortezomib-containing regimens, depending on the mechanism of action of the combination agent(s). Recently an *in vitro* study was published on the combination of EGFR-TKI

erlotinib and bortezomib in NSCLC cells. In this combination, a schedule-dependent antagonistic effect of erlotinib pre-exposure was observed. It was suggested that erlotinib pre-exposure, by causing G_1 cell cycle arrest, abrogated the activity of bortezomib [Piperdi et al., 2007]. Optimization of the administration sequence led in bronchioloalveolar carcinoma cells to increased activity compared to the single agent drugs. An ensuing clinical study, employing a schedule of erlotinib pretreatment followed by bortezomib, is currently ongoing in BAC patients [Davies et al., 2007].

Treatment outcomes may be improved by better selection of patients likely to respond to platinum-based therapy. Additionally, effective screening strategies for early detection of NSCLC are urgently needed. In Chapter 9, preliminary results are provided of another correlative study accompanying the clinical study with cisplatin, gemcitabine and bortezomib. Serum samples were collected from treated patients at baseline, during, and at end of treatment. Using automated magnetic C18 bead-assisted serum peptide capture coupled to matrix-assisted laser desorption/ ionization time of flight mass spectrometry (MALDI-TOF MS), serum peptide profiling of the 27 participating NSCLC patients was conducted and peptide mass profiles (spectra) obtained. Spectra were compared to those generated from 13 healthy volunteers. Data analyses of pretreatment serum peptide profiles, as well as dynamic changes in peptide abundance during treatment, were performed to establish algorithms that can 1. discriminate healthy subjects from (advanced) NSCLC patients 2. predict clinical outcome for treatment with cisplatin, gemcitabine and bortezomib 3.classify for treatment specific effects.

Analysis, including MALDI-TOF/TOF-based MS/MS identification of selected peptides, is ongoing. Interestingly, we found, based on peak value, several peptides to match those reported by Villanueva *et al.*. In their landmark study Villanueva *et al.* claimed that the serum peptidome is largely a product of a small number of resident substrates and their proteolytic breakdown products [Villanueva et al., 2006]. None of the cancer-type specific signature peptides they found appeared to be derived from cancer tissues. Their data suggested that cancer cells may contribute tumor-derived (exo)proteases which result in subtle but signature alterations of the complex serum peptidome, constituting a surrogate marker for detection and classification of cancer. Cancer-type specific exopeptidase activities were superimposed on the proteolytic events of the *ex vivo* coagulation and complement degradation pathways, requiring

serum, as opposed to plasma, for detection of the classifying features. In a study by Taguchi *et al.* an algorithm based on 8 distinct *m/z* features was developed which could classify patients for "good" or "poor" clinical outcome prior to treatment with EGFR-TKIs. Interestingly, plasma as well as serum analysis both gave similar values for all eight MALDI MS features used in their classification algorithm. Unfortunately they did not identify the proteins yet that make up the MALDI MS features [Taguchi et al., 2007]. Typically, only baseline serum samples are analyzed for signature generation. In our study we also included the analysis of serum samples during treatment as the dynamic changes in the serum peptidome upon treatment may also harbor clinically relevant information.
Before signature patterns generated by proteomics-analysis can be routinely applied as a tool for patient management decisions, significant hurdles are still to overcome. These include the prerequisites of rigorous standardized sample handling in the clinic and long-term reproducibility of disease signatures in independent cohorts and in multi-center studies. The first reports on the ability of serum MS proteomics to diagnose ovarian and prostate cancers with impressive sensitivity and accuracy generated great initial excitement and promise [Petricoin et al., 2002;Petricoin, III et al., 2002;Powell, 2003]. However, the reproducibility of the data and diagnostic profiles was questioned subsequently, leading to a general skepticism about the reliability of MS-based serum proteomics as cancer biomarkers [Diamandis, 2004].

By the research described in this thesis it was aimed to address the question whether or not targeting the proteasome holds promise for the treatment of advanced non-small cell lung cancer patients. NSCLC cells depend on sustained proteasomal function for survival. Studies in NSCLC cells demonstrated the potent cytotoxic effects of bortezomib. However, efficacy of bortezomib monotherapy in advanced NSCLC patients was limited. Pharmacodynamic studies in NSCLC patients were based on the surrogate marker of *ex vivo* measurement of proteasome activity in PBMCs. However, PBMCs might not serve as an adequate model to demonstrate that the agent has a clinically meaningful impact on the target. Comparative analysis of the level of proteasome inhibition in PBMCs and simultaneously obtained tumor samples upon bortezomib treatment were only conducted in a handful of patients. The proteasomal phenotype varies greatly between separate cells

and tissues, such as NSCLC tumors and PBMCs. Therefore, the impact of bortezomib on the target, i.e. effective proteasome inhibition in NSCLC cells, may not be accurately reflected by PBMC derived assays. Finally, NSCLC tumors, in contrast to bortezomib-sensitive multiple myeloma, are likely to be less dependent on (constitutive) NF-κB activation and dispose of several additional pathologic survival mechanisms.

In vitro studies suggest cytotoxicity of bortezomib is greatly enhanced when it is rationally combined with an "apoptotic trigger" or some sort of "second hit". An effective strategy in melanoma cells has been the combination of bortezomib and gossypol, an Mcl-1 inhibitor [Wolter et al., 2007]. Enhanced anti-tumor activity was observed in breast cancer cells when bortezomib was combined with a heat shock protein (Hsp) 90 inhibitor [Mimnaugh et al., 2004]. In NSCLC cells an example of such an additional apoptotic trigger is TRAIL, as described in Chapter 4. Clinical studies combining rhTRAIL as well as TRAIL-receptor directed monoclonal antibodies and bortezomib are currently in development. Caution is warranted, as TRAIL resistance of normal cells is still ill understood and, hypothetically, DR4 and DR5 targeted antibodies could have a different effect in normal and tumor cells compared to rhTRAIL as they do not bind to decoy receptors. Furthermore, TRAIL and bortezomib co-treatment at clinically relevant concentrations caused toxicity in primary human hepatocytes which potentially limits the clinical applicability of bortezomib-TRAIL combination therapy. However, at lower concentrations of bortezomib, TRAIL-resistant cancer cell lines but not hepatocytes were efficiently sensitized for TRAIL-induced apoptosis [Koschny et al., 2007a].

Concluding, despite disappointing results achieved with bortezomib monotherapy in clinical studies, viability of the proteasome as a relevant target for NSCLC treatment is not excluded. Proteasome inhibition-based therapy is likely to have its greatest clinical benefit when rationally combined with other (targeted) agents, aiming to effectively exploit the aberrant tumor circuitry for selective killing of tumor cells. Optimal dosing regimens are crucial in that regard. Furthermore, inclusion of valid pharmacodynamic as well as pharmacokinetic endpoints in clinical study design is pivotal. Finally, future directions of proteasome-directed therapies may also constitute more subtle ways of interfering with proteolysis, e.g. agents targeting regulatory mechanisms upstream from proteasome such as deubiquitinating enzymes and phosphorylation or ubiquitination of target substrates.

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