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CHARACTERIZATION OF MALIGNANT PLEURAL MESOTHELIOMA: POSSIBILITIES FOR AN INDIVIDUALIZED THERAPEUTIC ARSENAL

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Front cover: chemical structure of pemetrexed, cisplatin and carboplatin adapted from Wikimedia Commons.

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ABSTRACT

Less than 50% of malignant mesothelioma patients respond to standard chemotherapy treatment and there is a great need to identify these patients, and find their best treatment options. The aim of this thesis was to increase the understanding of drug sensitivity in malignant pleural mesothelioma.

We studied six mesothelioma cell lines with different phenotype and growth characteristics. The apoptosis signaling mechanism after treatment with selenite was evaluated in two of the cell lines. The drug sensitivity to selenite, bortezomib and four conventional drugs, together with their expression of potential predictive markers was evaluated in all six cell lines with WST-1 and immunocytochemistry. We followed the development of resistance to pemetrexed and carboplatin treatment in a patient with a genome-wide analysis as well as studying specific proteins through silencing, immunohistochemistry and measuring serum levels in the patient. Pleural effusions containing primary malignant mesothelioma cells were received from 18 patients and we characterized and tested their sensitivity to 32 different drugs in a robotized *ex vivo* assay. Primary cells were further characterized by immunocytochemistry to evaluate the amount of malignant cells and to study the RRM1 and ERCC1 reactivity.

The apoptosis and loss of mitochondrial membrane potential induced by selenite treatment was described and presents a complex signaling pattern. In samples from the drug resistant patient we observed that genes involved in the metabolic processes of pyrimidine and purine were upregulated and immunorectivity of EMA and cytokeratin 7 was increased at resistance. Silencing of NT5C gene did not induce pemetrexed sensitivity in cell lines and levels of serum mesothelin related protein and carcinoma antigen 125 in serum correlated to the tumor burden.

Selenite affected four out of six mesothelioma cell lines, and was in combination with bortezomib cytotoxic to all six. Epithelioid cells were more sensitive to the different drug and drug combinations than the sarcomatoid cells. Pemetrexed induced an extensive S-phase arrest in affected cell lines. The MRP-1 immunoreactivity of cell lines predicted carboplatin sensitivity and xCT predicted pemetrexed effect.

Large individual variability was observed in the drug sensitivity of the primary cells. The cell isolates were affected by between one and ten drugs and actinomycin D and daunorubicin were the most potent drugs. When adjusting the drug efficiency for theoretical effect on benign cell isolates and for the varying proportion of tumor cells we observed better correlations with pemetrexed, cisplatin and survival time. Proportion of malignant cells, reactivity to RRM1 and general drug sensitivity correlated to each other and to survival of the patients.

The drug sensitivity in malignant mesotheliomas is highly variable. These results indicate that *in vitro* testing of drug sensitivity may provide a tool for personalized treatment options.

LIST OF PUBLICATIONS

This thesis is based on the following publications, which will be referred to by their Roman numerals:

I. **Phenotype-dependent apoptosis signalling in mesothelioma cells after selenite exposure.** *Journal of Experimental and Clinical Cancer Research* 2009 28:92 doi: 10.1186/1756-9966-28-92.

Gustav Nilsonne, Eric Olm, **Adam Szulkin**, Filip Mundt, Agnes Stein, Branka Kocic, Anna-Klara Rundlöf, Aristi P. Fernandes, Mikael Björnstedt and Katalin Dobra.

II. **Molecular resistance fingerprint of pemetrexed and platinum in a long-term survivor of mesothelioma.** *PLOS ONE* 2012 7(8):e40521 doi: 10.1371/journal.pone.0040521.

Oluf Dimitri Røe, **Adam Szulkin**, Endre Anderssen, Arnar Flatberg, Helmut Sandeck, Tore Amundsen, Sten Even Erlandsen, Katalin Dobra and Stein Harald Sundstrøm.

III. **Variation in drug sensitivity of malignant mesothelioma cell lines with substantial effects of selenite and bortezomib, highlights need for individualized therapy.** *PLOS ONE* 2013 8(6):e65903 doi: 10.1371/journal.pone.0065903.

Adam Szulkin, Gustav Nilsonne, Filip Mundt, Agata M. Wasik, Pega Souri, Anders Hjerpe and Katalin Dobra.

IV. **Characterization and drug sensitivity profiling of primary malignant mesothelioma cells from pleural effusions.** *Manuscript.*

Adam Szulkin, Rita Ötvös, Carl-Olof Hillerdal, Aytekin Celep, Orsolya Muhari, Henriette Skribek, Anders Hjerpe, László Székely and Katalin Dobra.

CONTENTS

LIST OF ABBREVIATIONS

20S P	20S proteasome
BAP1	Breast cancer 1, early onset-associated protein 1
CDKN2A	Cyclin-dependent kinase inhibitor 2A
CEA	Carcinoembryonic antigen
DNA	Deoxyribonucleic acid
ELISA	Enzyme linked immunosorbent assay
EMA	Epithelial membrane antigen
EMT	Epithelial-mesenchymal transition
EPP	Extrapleural pneumonectomy
ERCC1	Excision repair cross-complementing rodent repair deficiency, complementation group 1
FISH	Fluorescence *in situ* hybridization
JNK	c-Jun N-terminal kinase
Mdm2	Mouse double minute 2 homolog
Merlin	Moesin-ezrin-radixin-like protein
MET	Mesenchymal-epithelial transition
MRP-1	Multidrug resistance-associated protein 1
P/D	Pleurectomy or decortication
Pgp	P-glycoprotein
PI	Propidium Iodide
RNA	Ribonucleic acid
RRM1	The ribonucleotide reductase large subunit M1
TS	Thymidylate synthase
TTF-1	Thyroid transcription factor-1
WST-1	Water soluble tetrazolium-1
xCT	X_c^- cystine transporter

1 BACKGROUND

1.1 BIOLOGY OF THE PLEURAL CAVITY

1.1.1 General

The pleural cavity is the sealed space between the two layers of the pleura, the visceral and parietal pleurae, which surround the lung. The two layers arise from one continuous serous membrane connected at the hilum of the lung. The pleura consist of a flat and thin monolayer of cobblestone like mesothelial cells on a basement membrane, with a connective tissue underneath[1]. These cells, covering the pleural surface, originate from the mesoderm, grow in a pattern resembling epithelial cells and the tissue is therefore called the mesothelium. The pleura covers both lungs and anatomically they are separated by the mediastinum with no connection between the two pleural cavities[2].

The visceral pleura is the inner layer covering the lung, while the parietal pleura is the outer layer, covering the inside of thoracic wall, mediastinum and diaphragm. The visceral pleura is poorly innervated and shares blood supply with the lung through the bronchial arteries. Blood and lymph vessels are connected to the visceral pleurae through the connective tissue, which also protects the lung by restricting the inflation volume and contributes to elastic recoil of the lung after inhalation. The intercostal arteries supply the parietal pleura with blood and the tissue is innervated by intercostal and phrenic nerves[3]. In the parietal pleura, the mesothelial stomata are found. These are channels between mesothelial cells connecting the pleural cavity to the lymphatic system[4].

The mesothelial cells have microvilli on the apical side and cells are attached to each other by tight junctions and reside on the basement membrane underneath. In the inactive form, mesothelial cells are flat with a polygonal shape and few organelles. When activated, mesothelial cells round up, their nuclei enlarge, show a prominent nucleolus, increased numbers of pinocytic vesicles and an increased number of organelles involved in protein synthesis[5].

1.1.2 Function

The pleural cavity normally contains an evenly distributed thin layer of pleural fluid that functions as a protective barrier and as lubricate to minimize friction between the two pleural layers while breathing. This lubricant contains glycosaminoglycans such as hyaluronan, which is synthesized and secreted by mesothelial cells[6]. The volume of this fluid is regulated by the mesothelial cells together with the movement of proteins and fluids across the serous membrane. Small molecules and water can pass between mesothelial cells by passive diffusion while larger molecules and proteins are actively transported by pinocytic and cytoplasmic vesicles[7,8].

By expressing intercellular adhesion molecule-1 and by activating T-cells through antigen presentation, the mesothelial cells initiate some of the immune response in the serous membranes[9]. The cells further participate in the response to foreign agents, such as asbestos, by secreting cytokines, chemokines, growth factors and extracellular matrix molecules[5,10]. When this occurs, a massive leukocyte migration into the serosal space is initiated. Some of the mesothelial cells are stimulated and become reactive with more microvilli expression and an altered morphology[11]. Mesothelial cells can

participate in coagulation by secreting tissue factors and have a fibrinolytic activity by secreting tissue plasminogen activator[12].

The mesothelial layer is delicate and quite sensitive to tissue damage. When disrupted, cells secrete a variety of growth factors and extracellular matrix molecules. This initiates mesothelial cell division and migration at the wound edge. Attachment and incorporation of free-floating mesothelial cells from the pleural fluid, stimulated by macrophages, is also a central part of healing the injury[5,13,14].

1.1.3 Pleural fluid

Pleural fluid is the small amount of fluid produced by the normal pleurae. When this fluid is accumulated due to a pathological process it is called a pleural effusion. Under normal conditions the pleural fluid is continuously produced, secreted into the pleural cavity and reabsorbed by caveolar endocytosis in mesothelial cells. The reabsorption is adapted to an increased secretion and can be greatly increased before fluid accumulates in the pleural space. Therefore, a substantial increase in pleural fluid production or an inhibition of reabsorption has to occur to yield an increased amount of pleural fluid. Events causing this are lymphatic obstructions, increased microvascular permeability, decreased colloid osmotic or intrapleural pressure, and increased capillary venous pressure[15].

1.1.3.1 Pathological features

Pleural effusion hampers the expansion of the lung, and thereby the ability of the patient to breathe properly. It is therefore drained by pleurocentesis in order to ease respiration. This fluid is also studied to establish the etiology of the effusion. The first step is to determine if the effusion is transudative or exudative. This can be established by measuring the levels of proteins and lactate dehydrogenase. Exudates have a higher ratio of the two measurements in the pleural fluid compared to the serum[16]. Transudative pleural effusions are usually a consequence of pressure changes in the cardiovascular system caused by heart failure, cirrhosis and nephrotic syndrome, diseases not originating in the pleura[17]. Exudative pleural effusions are usually caused by infections, malignancies or granulomatous disease in the pleura or lung. An infectious cause of the effusion can be established by culturing for bacteria and fungi, testing for tuberculosis and measuring glucose levels[18].

The possible presence of an alien cell population in the effusion is established by a cytopathological evaluation. The cellular morphology is observed in smears, stained according to Papanicolaou and/or May-Grünewald-Giemsa. This provides useful information for the choice of further analyses. By immunocytochemistry it is often possible to diagnose malignancy by the demonstration of reactivity to epithelial markers such as epitopes related to epithelial cell adhesion molecules, thyroid transcription factor-1 (TTF-1), carcinoembryonic antigen (CEA) and epithelial membrane antigen (EMA) in combination with mesothelial markers such as calretinin and desmin[19]. The issue of distinguishing malignant cells from reactive mesothelial cells can preferably be addressed by double staining for EpCam and calretinin to distinguish an alien epithelial cell population from reactive mesothelium, and desmin together with EMA to indicate malignancy of mesothelial cells by loss of desmin reactivity and cell membrane accentuation of the EMA stain. To establish a malignant diagnosis, ultrastructure analysis is performed with electron microscopy and cell ploidy can be studied with fluorescence *in situ* hybridization (FISH)[20]. Further information, particularly when suspecting malignant mesothelioma, can also be obtained from

measuring levels of the soluble biomarkers, hyaluronan and mesothelin by enzyme linked immunosorbent assay (ELISA)[21].

Using these adjuvant analyses, clinical cytology can often provide information concerning the origin of the tumor and in some cases predict possible therapy effects. In some cases epidermal growth factor receptor mutation or deletion and echinoderm microtubule-associated protein-like 4-anaplastic lymphoma kinase fusion protein are observed, tumor driving mechanisms for which targeted therapies are available[22,23]. In patients with malignant pleural effusion a pleurodesis is often performed to prevent recurrence of a pleural exudate[24].

1.2 MALIGNANT MESOTHELIOMA

1.2.1 General

Malignant mesothelioma is a rare and fatal tumor originating from the mesothelial tissue. The site of the neoplasm is principally in the pleura, but it also occurs in the peritoneum, pericardium or tunica vaginalis testis. Exposure to asbestos is a risk factor independent of tumor localization[25-28]. Due to the association with asbestos exposure this tumor is often the result of industrial practices. The development of a mesothelioma is a lengthy process, the tumor appearing 25-60 years after asbestos exposure[29,30]. Therefore the typical mesothelioma patient is a male over 60 years[31,32].

1.2.2 Incidence

The incidence of malignant mesothelioma is still increasing due to the long latency time and mainly affects industrialized countries where asbestos exposure has previously occurred. Australia has the highest reported incidence of malignant mesothelioma with annual rates of 40 cases per million[33]. This is because of the extensive and long term mining and import of asbestos and asbestos containing goods. Today the awareness of the neoplasm is widespread in Australia, with advanced diagnostic practice and well developed national surveillance systems[34]. In the United States the incidence is 15 cases per million with statistically significant increases of incidence in areas with a history of shipbuilding[33,35].

In Europe the average incidence is 18 cases per million, with the highest incidence in Great Britain and the Netherlands, with 33 and 30 cases per million, respectively[33]. Europe has long been a big producer and consumer of asbestos. The use started to decrease in the 1970s through different prohibitions whereas in some European countries the complete ban did not occur until recently. The incidence is especially high in areas with large shipyards[36,37]. In Sweden the use of asbestos was banned in the mid-1970s but still around 100 cases of malignant mesothelioma are diagnosed every year and the incidence is related to previous use of asbestos in shipyards and construction industry. Apart from shipyard workers many persons affected by the neoplasm have been plumbers or people working with insulation, but is also common among concrete workers, painters and electricians. Nowadays, asbestos exposure in Sweden mainly occurs through different materials remaining in constructions from 1970 or older[38].

The incidence of mesothelioma in most affected countries is expected to peak during the next decade and might already have been reached in some countries[35,39-41]. Because of the long latency time and differences in when asbestos use was prohibited, the incidence between these countries varies. However, the use of asbestos is still not banned in several countries and is increasing in parts of Eastern Europe and Asia.

Russia and China are the main consumers and producers and the global incidence is expected to increase for many decades to come[42,43].

1.2.3 Localization of malignant mesothelioma

1.2.3.1 Malignant pleural mesothelioma

Malignant pleural mesothelioma is a serosal tumor arising from the mesothelium of the pleural cavity, with a predisposal for the parietal pleura[30,44]. As mentioned above, most cases are caused by exposure to asbestos, reported for the first time in 1960[45]. This causality has been shown through; heterogeneous accumulation of asbestos fibers in the parietal pleura, correlation of intensity and frequency of exposure to the risk of developing the disease and the consumption levels of the mineral as a predictor of malignant pleural mesothelioma rate in different populations[46-48].

There are two main classes of asbestos, the serpentines and the amphiboles. Chrysotile is the most common serpentine accounting for 95% of all asbestos that has been mined in the world and the only type still being used. The carcinogenicity of chrysotile is lower than other types of asbestos but its extensive use will make it responsible for a large proportion of the malignant mesothelioma cases during the coming decades[49]. The amphiboles mainly consist of crocidolite, amosite, tremolite, anthophyllite and actinolite. The first three are more carcinogenic in pleural mesothelioma than the chrysotile fibers[49]. The properties of asbestos that makes it useful are its tensile strength, resistance to different external influences and ability to insulate. This affordable mineral has therefore been used in different types of electrical and building installations[50].

The mesotheliomas caused by asbestos can be divided into two different groups, environmental and occupational. The environmental mesotheliomas are caused by natural exposure to asbestos in areas where the mineral is present. These mesotheliomas are mainly caused by amphiboles in areas where houses have been painted white with products containing the mineral or in areas near to an asbestos mine or factory[50,51]. In the Cappadocia region of Turkey there is an asbestos-like mineral called erionite present in rocks. These rocks have been used to build houses in several villages and the mineral itself creates a white powder that has been used to paint walls. The tumorigenesis of erionite has been shown to be strong, responsible for an extremely high incidence of mesotheliomas in these Turkish villages[52].

Occupational mesotheliomas are caused by exposure directly in asbestos mines but also through exposure when handling asbestos containing material. Professions like shipbuilders, construction workers, electricians and plumbers are heavily affected by malignant pleural mesothelioma. Nowadays, the amount of people occupationally exposed to asbestos is larger than the amount environmentally exposed[53,54].

Mining of different minerals is important in Sweden but asbestos has never been mined. However, when other minerals are extracted, some asbestos exposure occurs, which previously has caused a few cases of malignant pleural mesothelioma. The remaining mines are today carefully controlled to avoid asbestos exposure. Asbestos was instead imported to Sweden, starting in the 1930s and lasting until the prohibition in the mid-1970s. Today there are strict rules for handling the remaining material containing asbestos[55].

1.2.3.2 Other malignant mesotheliomas

Malignant peritoneal mesothelioma accounts for a smaller proportion of the mesothelioma cases. In Sweden some 10% are peritoneal, while other studies have reported figures around 25%[32]. The median survival time is less than one year, first symptoms are abdominal distress and the tumor is often diagnosed at a late stage[56]. When possible, radical cytoreductive surgery followed by intraperitoneal hyperthermic chemoperfusion with a combination of cisplatin, doxorubicin, paclitaxel, 5-flourouracil or mitomycin C is a therapeutic alternative[57]. When surgery is not possible, patients receive palliative treatment by systemic or local chemotherapy with cisplatin in combination with pemetrexed[58].

Although quite rare, malignant mesotheliomas can also develop in the pericardium and in the tunica vaginalis testis. The mean survival time in pericardial mesothelioma is less than eight months and surgery is the main treatment modality even though good response to pemetrexed and carboplatin has been reported[59]. Testicular mesothelioma can be treated with radical orchiectomy and chemotherapy if metastases are present, with a mean survival time of up to three years[60-63]. In this thesis we focus on studying malignant pleural mesothelioma.

1.2.4 Pathogenesis

The exact mechanism of asbestos tumorigenesis in malignant mesothelioma is not completely understood. Asbestos fibers are thin and sharp and can perforate cellular membranes without killing the cells. This can disturb cell division and cause aneuploidy and structural abnormalities in the chromosomes of daughter cells[64]. The asbestos fibers also generate reactive oxygen species, causing oxidative stress and cellular damage, triggering DNA alterations. This occurs through catalysis of radical reactions by iron in the asbestos fibers and the release of reactive oxygen species by phagocytizing macrophages and neutrophils, activated by asbestos[65-67]. Altogether, asbestos causes a permanent state of cellular damage, chronic inflammation and regeneration in the lung and pleura, seen as a pleuritis in some patients, and these are thought to be the main causes of malignant mesothelioma development[68].

Several common cytogenetic changes in malignant pleural mesothelioma are consequences of these events. These include deletions of the short arms on chromosome 1, 3 and 9 and the long arms on chromosome 6 and 22[54,69-71]. Several important loci are located in these chromosome arms such as; cyclin-dependent kinase inhibitor 2A (CDKN2A, 9p21), neurofibromin 2 (22q12) and breast cancer 1, early onset-associated protein 1 (BAP1, 3p21). These are all tumor suppressor genes frequently inactivated in malignant mesothelioma cells[72,73].

The CDKN2A locus encodes for the $p16^{INK4A}$ and $p14^{ARF}$ protein. $p16^{INK4A}$ inhibits cyclin D1 from binding cyclin D-dependent kinases 4 and 6, a complex that phosphorylates and inactivates the retinoblastoma protein, which is a central step for initiating entry into S-phase in the cell cycle[74]. p53 is not mutated in malignant pleural mesothelioma but is inactivated by mouse double minute 2 homolog (Mdm2)[75-77]. In normal cells the Mdm2 inactivation is regulated by $p14^{ARF}$ and when this protein is mutated, Mdm2 is active and binds p53[78,79]. This blocks important function of p53 in the control of DNA repair, cell cycle and apoptosis[80]. The CDKN2A locus is reported to be disrupted in a majority of the malignant pleural mesothelioma cases[72,81].

Moesin-ezrin-radixin-like protein (merlin) is encoded by the neurofibromin 2 locus. Merlin is a membrane scaffolding protein in the cytoskeleton with the function to process extracellular signals and transmit them inside the cell. These signals mainly concern contact-dependence of cells and when cell-cell contact is lost, the protein function as a tumor suppressor by signaling for growth inhibition, decreased proliferation and increased apoptosis[82]. Around 50% of malignant pleural mesotheliomas have a disrupted neurofibromin 2 gene but it remains unclear if the remaining patients express functional merlin[70,83,84].

The BAP1 gene produces a deubiquitinating enzyme that removes ubiquitin from proteins and other molecules to prevent degradation. By doing this, the protein affects several processes in the cell such as DNA repair, cell cycle progression, differentiation and apoptosis. All these functions are central in cancer development and the BAP1 locus is mutated in approximately 30–60% of mesotheliomas[73,85].

Besides these genes and proteins, several others have been studied to understand the apoptosis resistance in malignant mesothelioma cells[86]. No dominant DNA alterations have been found but increased levels of the inhibitor of apoptosis protein family, with the X-linked inhibitor of apoptosis protein and survivin have been reported[87,88]. Mitochondria are involved in the apoptosis resistance through the Bcl-2 family but the role of these proteins in mesothelioma is unknown[86,89]. The expression of c-fos and c-jun, together forming activator protein 1, is induced by asbestos and might have a role in mesothelioma but the pathogenesis of malignant pleural mesothelioma needs to be further elucidated[90].

1.2.5 Diagnosis

The diagnosis of malignant pleural mesothelioma is often complicated, involving several different examinations. The first symptoms displayed by patients are usually chest pains, shortness of breath, tiredness and poor physical functioning with difficulties to perform even a short walk, causing a reduced quality of life[91]. These symptoms arise as a result of decreased ability of the patients to expand the lungs during inhalation and are mainly caused by a pleural effusion. To alleviate these symptoms and make a correct diagnosis, the fluid is withdrawn by pleurocentesis.

1.2.5.1 Imaging

Many malignant pleural mesotheliomas are initially detected by chest radiography as a pleural mass or diffuse pleural thickening and with a simultaneous pleural effusion. When these changes are seen, the tumor is in most cases already advanced; earlier stages are not possible to demonstrate with chest radiography. An x-ray computed tomography gives additional information concerning pleural plaques associated with asbestos exposure, general pleural thickening and occurrence of a pleural effusion. Particularly in combination with positron emission tomography, which after uptake of fluorodeoxyglucose demonstrates metabolically active cells, many malignant pleural diseases can be detected. This examination can provide additional information concerning the spread of the neoplasm as well as evaluate treatment response in patients with malignant pleural mesothelioma[92-94].

Together, these visualizing methods might indicate a malignant disease but for the origin to be established and to give a conclusive diagnosis, cytopathology or histopathology is needed. The majority of the tumors in the pleura have metastasized from other locations rather than being a malignant mesothelioma. The pleural fluid is

often the first material available for such morphological evaluation, aiming at establishing of precise diagnosis.

1.2.5.2 Cytology

The pleural effusions are separated into transudates and exudates as described in section 1.1.3. The transudates are caused by non-malignant diseases not originating in the pleura, while a malignancy is one cause of an exudate. Exudative pleural effusions are cytologically evaluated after staining of smears according to Papanicolaou and/or May-Grünewald-Giemsa. The cytomorphological features indicating malignant mesothelioma cells are high cellularity, papillary cell aggregates, cell membrane protrusions, acidophilic cytoplasmic vacuoles, background extracellular material and nucleolar prominence[95].

Immunocytochemical staining is performed to show the presence of several epitopes in malignant mesothelioma cells. Immunological markers of mesothelial lineage include calretinin, HBME-1, mesothelin, Wilms tumor-1and D2-40. Strong positive membrane EMA reactivity is seen in a majority of mesotheliomas[95,96]. Combining this reactivity with negative staining for epithelial markers CEA and EpCam is fully predictive for mesotheliomas, however with a sensitivity of only around 50 %[96]. One marker distinguishing between malignant and reactive mesothelial cells is desmin, which is often lost early during oncogenesis. The use of double staining techniques helps in the evaluation of cases with atypical or non-specific staining, which is often weaker. Thus, double staining for EpCam and calretinin clarifies if a cell population is metastatic or mesothelial, while in most cases staining including EMA and desmin will reveal if the mesothelial cell is malignant or reactive[96].

1.2.5.3 Fluorescence in situ hybridization

To strengthen a malignant diagnosis, the chromosomal ploidy of the cells can be studied by FISH. Using a commercial regent kit (Urovysion®) this FISH is performed using four directly labeled DNA-probes that hybridize to the centromere region on chromosomes 3, 7 and 17 and to the CDKN2A gene. A cell is considered to be malignant if the reaction to centromeric probes is increased in number in at least two of the chromosomes or if the CDKN2A gene is missing[20].

1.2.5.4 Biomarkers

A malignant mesothelioma diagnosis is supported by measuring levels of soluble biomarkers. High levels of hyaluronan (above 75 µg uronic acid/mL) is a strong indicator for malignant pleural mesothelioma and combined with the N-ERC/mesothelin the diagnostic accuracy is further increased[21,96-98].

1.2.5.5 Electron microscopy

Electron microscopy can be used to differentiate between mesotheliomas and other malignant diseases that have metastasized to the pleural cavity. By looking at the ultrastructure of the malignant cells, mesothelial cells have several features that differ from other neoplasms such as; thin and very long apical microvilli without glycocalyx, large desmosomes, prominent anchoring junctions, basal lamina and perinuclear distribution of tonofilaments[96,99,100].

In summary, effusion cytology in combination with FISH, biomarkers and electron microscopy is adequate in diagnosing malignant pleural mesothelioma[95,96]. This is,

however, still debated. The latest guidelines for diagnosing malignant mesothelioma state that the diagnoses should be based on histological samples and staining, although complementary guidelines are currently being formulated[99].

1.2.6 Phenotypes

There are two different phenotypes of malignant mesothelioma cells, one with epithelial cobblestone-shaped cells and one with sarcomatoid fibroblast-like cells. Several studies have demonstrated that there is a difference in the gene-expression between the two phenotypes[101-103]. This difference results in a phenotype-dependent sensitivity to experimental and conventional drugs seen in our previous studies[104,105]. Similar differences are seen in patients where these two phenotypes give three different histological patterns: one dominated by the epithelioid cells, one dominated by sarcomatoid cells and one biphasic type showing a mixture of the other two phenotypes[32]. A presence of non-epithelial cells in the tumor is associated with shorter survival time and increased therapy resistance[106-108].

Epithelial-mesenchymal transition (EMT) is the process when epithelial cells transform into mesenchymal cells. During this transition, E-cadherin is downregulated and N-cadherin is upregulated. Increased levels of stress fibers and focal adhesions can also been seen while cell-cell junctions are decreased and disrupted. Morphologically this transformation also causes actin cytoskeleton reorganization and increased migratory characteristics[109]. EMT is essential in developmental processes including mesoderm formation and occurs in wound healing. In the malignant tumor such conversion is seen as the tumor cells infiltrate and disseminates and when forming the metastatic lesions the cells regain their epitheliolid characteristics by mesenchymal-epithelial transition (MET). In malignant mesothelioma cells, with their dual phenotype, EMT and MET seem to be frequent, epithelioid and sarcomatoid cells being transformed into each other. These conversion reactions seem to be controlled by signals from microenvironment influencing the malignant potential of the tumor[110,111].

1.2.7 Treatment

The malignant pleural mesothelioma is in most cases comparatively resistant to therapy, and attempts to cure the disease are often associated with significant co-morbidity. The main treatment option for most cases is chemotherapy, described in further details in section 1.5. In early stages of the disease, surgery might be an option for patients. The most radical attempt is extrapleural pneumonectomy (EPP) where the entire affected pleura is removed together with the lung, sometimes also including partial or complete removal of diaphragm. An alternative is the less radical pleurectomy or decortication (P/D), with local resection of tumor tissue in pleura, lung and chest wall.

An EPP decreases the quality of life, has a high perioperative mortality rate and the procedure does not seem to be beneficial compared to other treatment options[112,113]. Video-assisted thoracoscopic surgery pleurectomy or decortication has a better outcome, with lower patient mortality rate, a better quality of life and improved survival time for patients[114-116]. Patients with a tumor dominated by epithelioid cells and without weight loss benefits most from the procedure with a longer survival time[117].

Radiotherapy is mainly used to decrease pain from local tumor masses, thereby increasing patient quality of life. Treating patients with radiotherapy seem to improve

survival time but has not been compared to other treatments in randomized studies[118,119]. Adjuvant radiotherapy to surgery may reduce local recurrence and prolong survival[120]. The combination of surgery, chemotherapy and extensive radiotherapy in trimodality protocols has shown to improve local tumor control[121-125]. This procedure is, however, associated with substantial co-morbidity, preventing more general use.

1.2.8 Prognosis

Generally the prognosis for patients with malignant pleural mesothelioma is poor with a mean survival time from diagnosis of 12 months[33,126]. Patients with neoplasm dominated by epithelial cells have a better prognosis and a few are long time survivors, surviving several years after their diagnosis. Treatment with standard chemotherapy increases the average survival by three months and longer survival is seen in patients responding to chemotherapy[127]. Factors associated with a poor prognosis and shorter survival time are male sex, higher age, dyspnea, tumor stage, involvement of the visceral pleura, low hemoglobin level, high lactose dehydrogenase, a high thrombocyte and white blood cell count, pain and loss of appetite and weight[108,128-130]. The obtained survival times depend on the limited rate of tumor response, which motivates the search for tests that may optimize the choice of drugs and drug combinations.

1.3 CHEMOTHERAPY

1.3.1 General

The first clinical trial using chemotherapy was carried out in 1943 when patients with different hematological malignancies demonstrated remarkable improvement after intravenous injections of mustine hydrochloride (nitrogen mustard)[131,132]. The malignancy soon recurred in these patients and a few years later, children with acute leukemia treated with aminopterin achieved longer temporary remission[133]. The first curative effect of chemotherapy was seen on gestational choriocarcinoma patients treated with methotrexate and 6-mercaptopurine[134].

Although chemotherapy was effective in hematological cancers, results in solid tumors were disappointing. This was changed when fluorouracil was discovered and when chemotherapy was used adjuvant to surgery[135-137]. Chemotherapy is also used neoadjuvant to radiotherapy or surgery, mainly to reduce tumor burden and decrease risk of metastases[138,139].

Most drugs target dividing cells, a characteristic for many malignant cells. However, normal dividing cells are also affected, causing cell death in bone marrow, digestive tract and hair follicles. This is the reason for the most common side effects; gastrointestinal distress, alopecia, myelosuppression, leukopenia, anemia and thrombocytopenia, increasing the susceptibility for infections[140].

1.3.2 Classification of anticancer drugs

Chemotherapeutical drugs are divided into different groups, according to their mechanisms of action, as follows. Table 1 summarizes the chemotherapeutical agents included in this thesis.

Alkylating Agents	Nitrogen mustard	Chlorambucil
	Platinum	Cisplatin
		Carboplatin
		Oxaliplatin
	Aziridine	Mitomycin C
	Tetrazine	Dacarbazine
Antimetabolites	Pyrimidine analogues	Fluorouracil
		Cytarabine
		Gemcitabine
	Purine analogues	Mercaptopurine
		Fludarabine
		Cladribine
	Antifolates	Methotrexate
		Pemetrexed
	Other	Hydroxyurea
Antimicrotubule agents	Taxanes	Paclitaxel
		Docetaxel
	Vinca alkaloids	Vinblastine
		Vincristine
		Vinorelbine
Topoisomerase inhibitors	Type I	Topotecan
		Irinotecan
	Type II	Etoposide
		Amsacrine
Proteasome inhibitor		Bortezomib
Multifunctional drugs	Anthracyclines	Daunorubicin
		Doxorubicin
		Epirubicin
	Other	Actinomycin D
		Bleomycin
Experimental drug		Selenite

Table 1. The 31 chemotherapeutical drugs included in this thesis, divided according to their mechanism of action.

1.3.2.1 Alkylating agents

This group of drugs covalently binds macromolecules, including proteins, RNA and DNA. There is no specificity with these chemotherapeutical agents but the main cytotoxic effect is random binding and forming adducts in DNA[141-143]. A majority of the agents bind alkyl groups to DNA but this group of drugs also includes other compounds, binding in a similar fashion. This binding hampers DNA synthesis and stops cell cycle progression, causing apoptosis.

Nitrogen mustards are the oldest group of alkylating agents and mustine hydrochloride was the first nitrogen mustard used, as described above. The nitrogen mustard was developed by adding other chemical moieties and alkylators such as melphalan and chlorambucil were developed[144-148].

Drugs containing a platinum atom are widely used because of their potency. The platinum atom binds different molecules in the cell and creates adducts when binding to DNA, causing inter- and intrastrand cross linking and hence apoptosis in a similar fashion to alkyl groups[149,150]. Cisplatin (figure 1A) is the most common platinum analogue, inactive in its original form but positively charged and reactive after a stepwise aquation inside cells[151]. The compound is used in several different malignancies and cures nearly 100% of testicular cancer. Together with pemetrexed it is the standard treatment for malignant pleural mesothelioma[152-155]. Carboplatin (figure 1B) is the second generation of platinum compounds and is an analogue to cisplatin with a bidentate cyclobutanedicarboxylate ligand in its molecular structure, adding stability and causing a slower aquation[150]. The compound was discovered in a mass screening of compounds effective on *in vitro* cultured tumor cell lines[156]. The effects of carboplatin were studied in six mesothelioma cell lines in **paper III**. Carboplatin has been shown to be less toxic than cisplatin and is used in treatment of patients with non-small cell lung cancer and ovarian cancer[157-159]. Resistance to cisplatin and carboplatin is a major problem. Therefore, oxaliplatin was developed as the third generation and was shown to be effective in cisplatin resistant cell lines[160-162]. Oxaliplatin (figure 1C) seems to act in the same way as cisplatin and carboplatin and is used in treatment of patients with ovarian cancer[163].

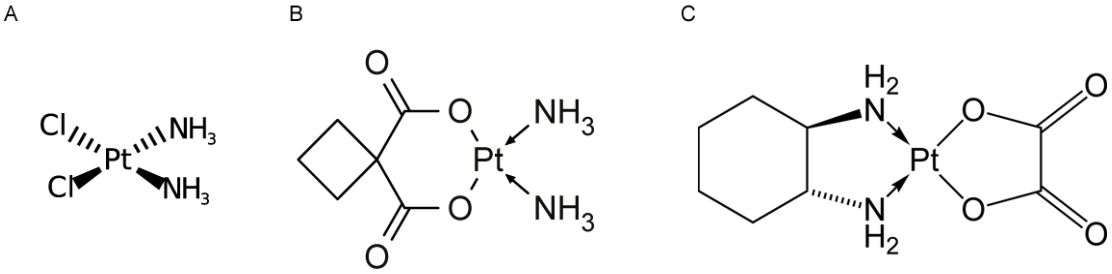

Figure 1. The chemical structure of the three platinum drugs included in this thesis. A. Cisplatin. B. Carboplatin. C. Oxaliplatin. Pictures adapted from Wikimedia Commons.

Mitomycin C, isolated from *Streptomyces caespitosus*, is a member of the aziridines (all contain an aziridine ring) and needs to be reduced before binding and creating adducts in DNA[164,165]. Today, mitomycin C is used in several different drug combinations in lung and colorectal cancer[166,167]. Dacarbazine is a tetrazine, which means that it consists of an aromatic ring with four nitrogen atoms. The compound is a purine analogue but it seems like it exerts its toxic effect by binding DNA as an alkylator[168,169]. Dacarbazine is the main treatment of malignant metastatic melanoma[170].

1.3.2.2 Antimetabolites

This group of chemotherapeutical agents is structurally similar to the basic substances in DNA and RNA and therefore interferes with their synthesis. DNA and RNA are assembled from nucleotides that consist of a nucleobase, a phosphate and a sugar group[171,172].

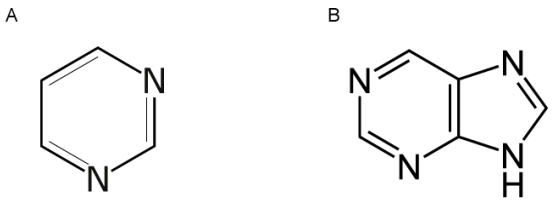

Figure 2. The chemical structure of pyrimidine (A) and purine (B). Pictures adapted from Wikimedia Commons.

The pyrimidine and purines analogues are two groups of antimetabolites that resemble the respective nucleobases or nucleosides (nucleobases with a sugar group, figure 2). Their mechanism of action is similar; they are converted inside the cells to analogues of nucleobases or nucleosides. The compounds then inhibit enzymes central in DNA and RNA synthesis or becomes incorporated into DNA or RNA, inducing apoptosis[173].

Fluorouracil is the nucleobase uracil with the substitution of a hydrogen atom with a fluorine atom[135]. The compound blocks thymidylate synthetase, an enzyme important in the formation of building blocks for DNA[174]. Fluorouracil is similar in structure to, but does not perform the same chemistry as uracil; the drug inhibits RNA replication enzymes, thereby eliminating RNA synthesis. The metabolites also incorporate into DNA and RNA, and all together this inhibits DNA synthesis, causing apoptosis[175-177]. Fluorouracil was used to treat patients with both hematological and solid tumors but the severe side-effects limit the use of the drug today[178,179].

Cytarabine is the nucleobase cytosine with an additional hydroxyl and a sugar group. In the cell the drug acts as the nucleotide analogue deoxycytidine and incorporates into the DNA. This stops the DNA and RNA polymerases and DNA replication, causing apoptosis[180-182]. Cytarabine is used in patients with different types of leukemia[183]. The structure of gemcitabine is similar to cytarabine but with two fluorine atoms instead of the extra hydroxyl. The function is also similar but the converted gemcitabine in the cell also inhibits ribonucleotide reductase, an enzyme important for synthesis of nucleotides[184,185]. This causes apoptosis and the long half-life of gemcitabine metabolites improves the effect in several different solid tumors and hematological malignancies[186-191]. The cytotoxic effects of gemcitabine in six mesothelioma cell lines were studied in **paper III**.

Mercaptopurine is an analogue to the purine nucleobase guanine without an amino group and the oxygen in guanine has been replaced by a sulfur atom. The agent inhibits proteins involved in *de novo* synthesis of purines and also incorporates into DNA causing DNA damage and altogether apoptosis[192,193]. Mercaptopurine was included in the first curative chemotherapy when treating gestational choriocarcinoma patients[134]. Today the drug is used in patients with acute leukemia[194].

Fludarabine is an adenine analogue with an extra fluorine atom. The compound is incorporated into DNA and inhibits the ribonucleotide reductase[195]. Fludarabine is used in different drug combinations and as single agent to treat chronic lymphocytic leukemia and follicular lymphoma[148,196]. Cladribine is structurally similar to

fludarabine but with a chlorine atom instead of the fluorine. The compound has the same mechanism of action as fludarabine and is the standard treatment for patients with hairy cell leukemia[195,197,198].

The antifolates constitute another group of antimetabolites that target enzymes essential for the synthesis of nucleotides. Methotrexate inhibits the enzyme dihydrofolate reductase, responsible for producing tetrahydrofolic acid, which is required for thymidylate production[199]. This causes cell cycle arrest and apoptosis. Methotrexate and the previously described agent mercaptopurine were used to cure choriocarcinoma patients in 1958[134]. Low dose methotrexate is still used to treat childhood acute lymphoblastic leukemia[200]. Pemetrexed is an antifolate that enters cells via the reduced folate carrier[201]. The agent mainly inhibits the enzyme thymidylate synthase, but also effects dihydrofolate reductase, aminoimidazole carboxamide ribonucleotide formyltransferase and glycinamide ribonucleotide formyltransferase, enzymes involved in purine and pyrimidine synthesis[202,203]. This causes S-phase arrest and apoptosis[204-207]. Pemetrexed in combination with a platinum drug is the standard treatment of patients with malignant pleural mesothelioma and is also used in non-small-cell lung cancer[155,208,209]. Drug resistance development in a patients treated with pemetrexed was studied in **paper II**.

Hydroxyurea is an anticancer drug first synthesized in 1869 that inhibits the enzyme ribonucleotide reductase by eliminating radicals needed for the enzyme to produce nucleotides[210,211]. Hydroxyurea is used to treat patients with chronic myeloid leukemia and polycythemia vera[212].

1.3.2.3 Antimicrotubule agents

A vital part of cell division is the assembly of tubulin molecules to form microtubules. Microtubules form the base of the mitotic spindles, which pull the chromosomes apart during cell division[213,214]. The tubulin molecules are composed of two proteins; α-tubulin and β-tubulin. The antimicrotubule agents are a group of plant-derived drugs that bind these molecules, preventing cell division and inducing apoptosis. There are two main groups of antimicrotubule agents, the taxanes and the vinca alkaloids, with the opposing mechanisms of action. Taxanes promote microtubule stability, preventing their disassembly, while the vinca alkaloids prevent the formation by disturbing the stability of the microtubules.

The taxanes bind β-tubulin, which promotes polymerization of tubulin, stabilizing the microtubules and thereby inhibiting mitosis[215,216]. Paclitaxel is a taxane extracted from the yew tree, *Taxus brevifolia* or *Taxus baccata*. Pacitaxel has been used in treatment of patients with several different types of malignancies[217-220]. The docetaxel molecule differs from paclitaxel at two positions, making the compound more water-soluble. Docetaxel has a higher affinity for binding β-tubulin than paclitaxel, and is used to treat ovarian, breast, prostate and lung cancers[218,221-225].

Vinblastine is a vinca alkaloid that was discovered and extracted from the plant *Catharanthus roseus* (formerly known as *Vinca rosea*) and is today synthetically produced[226]. Vinblastine binds β-tubulin at low concentrations inhibiting mitosis by stabilizing the microtubules and at higher concentrations by depolymerizing the

microtubules[227-229]. After the discovery of the drug, it was used in several different malignancies but it has since then been exchanged for more effective drugs in different treatment regimens[230]. Vincristine is another vinca alkaloid, also extracted from *Catharanthus roseus* and it is structurally very similar to vinblastine. The drug has a similar mechanism of action to vinblastine[231]. Vincristine is mainly used in combinations with other drugs to treat patients with different malignancies[232-234]. After the success of these two agents, vinorelbine was synthesized from the same plant[235]. Vinorelbine acts in the same way as the other vinca alkaloids and is used in treatment of breast cancer and non-small-cell lung cancer[236,237].

1.3.2.4 Topoisomerase inhibitors

This group of drugs inhibits the actions of two groups of enzymes: the topoisomerase I and topoisomerase II. When processing DNA, during normal replication or translation, the double stranded DNA helix is unwound at the part that is being processed. The unprocessed parts are at the same time wound tighter and this creates a tension. The topoisomerases decreased this tension by producing temporary breaks in the DNA. The drug topotecan is metabolized in cells, inhibiting the function of topoisomerase I and causing permanent DNA strand breaks. The drug has been shown to be effective in treatment of ovarian and lung cancer[238,239]. Irinotecan is also metabolized in the cell, inhibiting topoisomerase I and is used in treatment of colon cancer[240]. The topoisomerase II is essential in cell division by removing links between chromosomes when segregated into the two daughter cells. Etoposide is a plant derivative that binds the topoisomerase II-DNA complex, inhibiting the enzyme and the drug is used in treatment of lung cancer and lymphomas[241-243]. Amsacrine increases the ability of topoisomerase II to break DNA, causing DNA damage and cell death[244]. The drug is used to treat tumors in urinary bladder and pharynx[245,246].

1.3.2.5 Proteasome inhibitors

The proteasome is a protein complex in cells with the function of degrading proteins that have been damaged, are defective or not needed in the cell[247]. This protein complex is composed of one 20S subunit and two 19S subunits[248]. Many malignant cells have been shown to have a higher proteasome activity and expression than normal cells, making the proteasome a suitable target for chemotherapy[249,250]. Several different proteasome inhibitors have been proposed but so far bortezomib is the only one approved for treatment of cancer[251]. Bortezomib targets the 20S subunit of the proteasome and inhibits the function, causing accumulation of proteins in the cells and apoptosis[252,253]. The combination of bortezomib and doxorubicin is the standard treatment for recurrent multiple myeloma[254]. Mesothelioma cell lines were affected by bortezomib in **paper III** but in clinical trial the effect was disappointing[104,255,256].

1.3.2.6 Multifunctional drugs

The anthracyclines constitute a group of drugs originating from the same chemical structure, a four-ringed organic quinone. This group is also known as antitumor antibiotics, due to their antibiotic effect. The mechanism of action of these drugs is multiple; the agents intercalate DNA, inhibit topoisomerase II, form DNA adducts, crosslink DNA, interfere with synthesis of macromolecular and generate reactive free radicals in cells damaging DNA and proteins[257]. The anthracycline daunorubicin was originally isolated from the bacteria *Streptomyces peucetius* and is used to treat patients

with leukemia[258-260]. Doxorubicin was isolated from the same bacteria and is structurally very similar to daunorubicin. Doxorubicin effects in six mesothelioma cell lines were evaluated in **paper III** and the drug is widely used in different drug combinations to treat patients with leukemias and solid tumors[261-263]. Epirubicin was developed from daunorubicin and doxorubicin. The cytotoxic actions of this drug are similar to the other anthracyclines but with less side-effects[264]. The compound is used to treat breast and gastric cancer[265,266]. Doxorubicin and daunorubicin are well known for their cardiotoxicity, which is the dose limiting factor in several treatments.

Actinomycin D is a drug isolated from the bacteria *Streptomyces parvulus*[267]. The drug's mechanism of action is by intercalating the DNA and interfering with the RNA polymerase, thereby preventing transcription and affecting the activity of topoisomerase I and II[268-270]. The compound is used to treat patients with some less common tumors such as gestational trophoblastic neoplasia, Wilms' tumor and rhabdomyosarcoma[271-273]. Bleomycin is a family of compounds isolated from the bacteria *Streptomyces verticillus*. Bleomycin A_2 and B_2 are the two forms of the compound that are effective and included in drug combinations when treating Hodgkin's and non-Hodgkin lymphoma patients[274-276]. Cell death by bleomycin mainly seems to be induced by bleomycin through inhibition of DNA synthesis and generation of free radicals, causing DNA strand breaks[277].

1.3.2.7 Selenite, a potent experimental drugs

The apoptotic mechanisms and cytotoxic effects of the experimental drug selenite have been studied in **paper I** and **III**. Selenium is essential for cells, where low concentrations are vital for the cell survival, while high concentrations induce cell death[278]. Selenite is an oxidized form of selenium (SeO_3^{2-}) and the compound is easily reduced by cysteine to selenide (HSe^-). The mechanism of cellular uptake of selenium is unknown but a reducing extra-cellular environment with cysteine increases the proportion of selenide and this increases the cellular uptake of the drug[279].

Inside the cell selenium reacts with thiols, forming reactive oxygen species and inducing oxidative stress, which causes apoptosis[105]. The mechanism of this is unknown and the essential part of this reactivity is demonstrated as decreased drug effect in cells with high expression of antioxidant proteins[280]. The selenite effect is hampered by the addition of antioxidant compounds, resulting in decreased formation of reactive oxygen species[105,281,282]. Malignant cells, including drug resistant ones, have been shown to be more sensitive to selenite treatment than benign cells[105,283-285]. Primary malignant cells collected from patients with leukemia are sensitive to the drug, and in patients selenite has been used in combination with other drugs, surgery and radiotherapy[286-289]. There are, however, still no large clinical trials evaluating the clinical usefulness of selenite. The effect of selenite on six mesothelioma cell lines, as a single agent or in different combinations with conventional drugs, was evaluated in **paper III**.

1.4 OTHER DRUGS

1.4.1 Prednisone

Prednisone is a glucocorticoid hormone metabolized in the liver to its active form prednisolone[290]. Glucocorticoids function by binding to their receptor in the cell, this complex enters the nucleus, interacts with DNA, changes the transcription and alters which proteins that are synthesized[291]. Prednisolone binds to the glucocorticoid receptors, changing protein expression, which reduces the side effects of drugs used in combination with prednisone and induces apoptosis[292,293]. Prednisone is used in treatment of lymphomas, multiple myeloma and prostate cancer in combination with other anticancer drugs[294-297].

1.4.2 L-asparaginase

Some malignant cells are not able to synthesize asparagine and obtain this amino acid from an external source. L-asparaginase is an enzyme that catalyzes the hydrolysis of asparagine, thereby decreasing the amount of circulating asparagine in the body when administered to patients, causing inhibited protein synthesis in malignant cells and apoptosis[298]. L-asparaginase has limited effect when used as a single agent and therefore the enzyme is primarily used in combination with other anticancer drugs to treat patients with leukemia[299-301].

1.5 CHEMOTHERAPY FOR MALIGNANT PLEURAL MESOTHELIOMA

1.5.1 Different combinations

The standard chemotherapy used for patients with malignant pleural mesothelioma is the combination of cisplatin and pemetrexed[302]. In the original study, this treatment increased the survival time compared to patients treated with only cisplatin by 2.8 months, yielding an average survival time of 12.1 months after diagnosis. The response rate was 41%. The increased side effects were suppressed by supplementation of folic acid and vitamin B_{12}, which also increased the survival time.

Several drug combinations have been evaluated but none of them have been superior to the standard treatment[303,304]. Exchanging cisplatin with carboplatin gives similar treatment outcome in patients[305,306]. Replacing pemetrexed with another antifolate, raltitrexed, showed lower response rates and shorter average survival time[307]. Combining cisplatin with gemcitabine instead of pemetrexed gave both higher and lower responses rates, but the average survival time was shorter[308,309]. Combining cisplatin with vinorelbine in a small phase II study presented interesting results with an average overall survival time of 16.8 months but these results need to be verified in a larger patient group[310]. Another small phase II study, treating malignant pleural mesothelioma patients with gemcitabine in combination with oxaliplatin, produced results comparable with the standard treatment[311]. Combining carboplatin, liposomized doxorubicin and gemcitabine resulted in a response rate of 32% and a survival time of 13 months[312].

Together these clinical trials demonstrate the need for more effective treatment of malignant pleural mesothelioma patients. This is highlighted by results showing that patients responding to treatment have the longest survival time[127]. Recent studies have also indicated that treatment outcome of malignant pleural mesothelioma patients might be predicted by immunohistochemical demonstration of biomarkers[313,314].

1.5.2 Predictive markers

There are a great number of different potential markers for predicting the treatment effects of different chemotherapeutical agents. One goal is to find markers that predict the outcome of different treatment regimens before starting the treatment and thereby choose an individualized therapy for each patient from the start. This would be central in future treatment of malignant pleural mesothelioma since only 40% of patients respond to standard treatment, as described above. The approach of tailoring treatment according to expression of predictive markers has been used in non-small-cell lung cancer patients with promising results[315]. In **paper III** we have chosen to study seven markers that might be related to the sensitivity or resistance to doxorubicin, gemcitabine, carboplatin, pemetrexed, bortezomib or selenite. In **paper IV** we have studied two markers and their relation to general drug sensitivity and patient survival time. The potential predictive markers that were evaluated in this thesis are described below.

P-glycoprotein (Pgp) is a membrane protein responsible for the transport of different substrates across cellular membranes. The protein is an effective drug efflux pump, decreasing cellular accumulation of several drugs[316]. Increasing levels of Pgp are often involved in development of drug resistance to several different anticancer drugs. This protein is often expressed in mesothelioma cells and Pgp expression correlates to paclitaxel and doxorubicin resistance in breast cancer[317-319]. Multidrug resistance-associated protein 1 (MRP-1) is a membrane protein from the same transport protein family as Pgp and with a similar function. MRP-1 secretes cysteine, a central mechanism for cellular uptake of selenite, as described above[279]. The protein also has the ability to efflux a large amount of conventional drugs, is frequently expressed in cells from patients with malignant pleural mesothelioma and is significantly associated with poor outcome in patients with neuroblastoma treated with different conventional drugs[318-320].

Excision repair cross-complementing rodent repair deficiency, complementation group 1 (ERCC1) is a protein in the nucleotide excision repair system, a system that repairs damaged DNA[321]. The protein is involved in resistance to platinum drugs and non-small-cell lung cancer patients with low ERCC1 expression have been shown to respond better to cisplatin treatment[322-324]. In malignant pleural mesothelioma patients treated with pemetrexed and cisplatin or cisplatin and vinorelbine the overall survival is correlated to ERCC1 levels[314,325]. The ribonucleotide reductase large subunit M1 (RRM1) is an enzyme and a subunit of the ribonucleotide reductase, a protein central for deoxyribonucleotide synthesis[321]. RRM1 is a predictive factor in several malignancies and is inactivated by gemcitabine[326,327]. Expressions of ERCC1 and RRM1 have been widely studied in patients with non-small-cell lung cancer and correlate to treatment effects of gemcitabine or carboplatin[328-332].

Thymidylate synthase (TS) is an important enzyme in folate synthesis, catalyzing a reaction central for the supply of building blocks for DNA. TS is the main target of pemetrexed and in non-small cell lung cancer patients treated with the drug the expression of TS predicts the clinical outcome[333,334]. In mesothelioma patients treated with pemetrexed the predictive value of TS has been widely studied and is still uncertain[335,336]. The X_c^- cystine transporter (xCT) is a cystine/glutamate antiport protein that transports extracellular cystine into the cells and glutamate out from the cell. The protein is vital for cellular regulation of cystine/cysteine levels and is expressed in

many different human cells[337]. Increased xCT expression induces resistance to gemcitabine and is central in selenite toxicity, as described above[279,338]. 20S proteasome (20S P) is a subunit of the proteasome, thus involved in protein degradation and the main target of bortezomib[247,253]. A higher proteasome expression and activity has been observed in some malignancies and in acute myeloid leukemia cells, high expression of 20S P has been correlated to bortezomib sensitivity[250,339].

2 AIMS

There is a great need to improve and develop chemotherapy for malignant pleural mesothelioma. Less than 50% of patients respond to standard chemotherapy and treatment response yields an increased survival time[127,302]. This highlights the needed to identify which patients that fails in responding to treatment and find suitable chemotherapeutical treatment options for them.

The overall aim of this thesis was to increase the understanding of drug sensitivity in malignant pleural mesothelioma and bring this knowledge from the bench closer to the bedside. These aims are achieved through dividing the thesis into four different studies with the following specific aims:

Paper I: To examine the phenotype dependent differences in apoptosis signaling of malignant pleural mesothelioma cell line after exposure to selenite, a potent experimental drug.

Paper II: To study the molecular mechanisms of pemetrexed resistance in malignant pleural mesothelioma.

Paper III: To evaluate the drug sensitivity of six phenotypically different malignant pleural mesothelioma cell lines treated with conventional and experimental cytostatic drugs as single agents or in combinations of two. To correlate the phenotype and drug sensitivity of cell lines to their immunocytological reactivity of potential predictive markers.

Paper IV: To characterize primary cells from patients with malignant mesothelioma, evaluate their *ex vivo* sensitivity to a broad panel of anticancer drugs, their cytological staining of RRM1 and ERRC1, and correlate to clinical outcome of these patients.

3 REMARKS ON METHODOLOGY

3.1 CELL LINES

Cell lines used in these studies included six malignant pleural mesothelioma cell lines and one T-cell lymphoma cell line. STAV-AB and STAV-FCS cell lines were previously established from a single pleural effusion and differentiated into different phenotypes by growing STAV-AB cells in 10% human AB-serum and STAV-FCS cells in a 10% mixture of bovine serum and fetal bovine serum. These two cell lines have previously been characterized by us as a model system to study phenotype dependent differences[340].

ZL-34 and M-14-K are grown in the same way as STAV-FCS cells and the length/width ratio and morphology of these three cell lines and STAV-AB cells have been previously studied[341]. DM-3 and JL-1 cell lines were grown in 20% fetal bovine serum and these two cell lines had the largest length/width ratios when all six cell lines were characterized in **paper III**. The phenotypes of the cell lines are consistent with previous finding that signals from the microenvironment effects the phenotype of the tumor[111].

We used the STAV-AB and STAV-FCS cell lines in **paper I** and **III**[342]. The ZL-34 and M-14-K cell lines were used in **paper II** and **III**. In **paper III** we also included the DM-3 and JL-1 cell lines[343-345]. In these projects, the STAV-FCS, ZL-34 and M-14-K cells were appointed with different phenotypes which was due to a more detailed characterization and strict definition in **paper III**. However, length/width ratios and growth characteristics of the cell lines were consistent in the different projects. The Jurkat T-cell lymphoma cell line was used as a control in **paper I** and **III**[346].

3.2 PRIMARY CELLS

In **paper IV** we used eighteen malignant cell isolates from twelve patients diagnosed with malignant mesothelioma and four benign mesothelial cell isolates obtained from pleural effusions in patients with no malignant diagnosis. Twelve malignant and four benign cell isolates were seeded and grown to confluence before they were studied. Two of the effusions were also studied for several passages, two were repeated effusion from the same patient and in two of the effusions with papillary groups, these groups were separated and compared to simultaneously occurring disassociated cells at confluence.

A pleural effusion is often the first diagnostic material received from a patient and this provides a more clinically relevant model to study. The pleural effusions that we receive and culture consist of the exudates with a high cellular content. The tumor cells in these fluids are mainly epithelioid, since sarcomatoid cells are usually not exfoliated. Furthermore, the microenvironment in the pleural cavity may trigger a differentiation of exfoliated tumor cells towards an epithelioid morphology.

Culturing cells *in vitro* causes a certain amount of selection and we try to limit this by minimizing the culturing time. The cell isolates always contain a mixture of malignant mesothelioma cells, reactive mesothelial cells and inflammatory cells in unknown proportions. Attempts to evaluate the proportions of these different cell types were done by immunocytochemical demonstration of desmin, EMA and leukocyte common antigen. These stainings were examined by two cytopathologists, establishing the

proportion of malignant cells in all isolates except two cases, which were further evaluated with ploidy analysis.

3.3 IMMUNOCYTOCHEMICAL CHARACTERIZATION

The pretreatment of cell lines in **paper III** was optimized with control cells known to express the target protein. We used 5 minutes citrate buffer pH 6.0 as pretreatment for all targets except for Pgp, where an EDTA buffer pH 9.0 was used. The specificity of the primary antibody was demonstrated by using relevant isotype controls.

The staining of primary cells in **paper IV** was optimized in the same way as for the cell lines. This resulted in the use of EDTA buffer pH 9.0 for 20 minutes to get a sufficient exposure of the epitope and thus good binding of the primary antibody. The staining pattern of different markers in the cell lines was evenly distributed within the cell population. In the more heterogeneous primary cell isolates, the reactivity was diverse but the staining intensity of only the malignant cells was evaluated by two experienced cytopathologists.

The access to pleural effusions makes immunocytochemistry a suitable approach to evaluate levels of different predictive markers, compared to immunohistochemistry depending on a tissue sample from the tumor.

3.4 VIABILITY OF CELLS

In these projects we have used three different methods to measure the viability of cells after treatment with different anticancer drugs. The viability is obviously dependent on drug concentrations and in **paper I** and **III** we used concentrations corresponding to the IC_{30} values of STAV-AB and STAV-FCS cells. In **paper IV** we used four different dilutions of the drug concentrations, chosen to cover clinically relevant concentrations. Some of the concentrations were further adjusted to their clinical use, without affecting the outcome.

Water Soluble Tetrazolium-1 (WST-1) is a tetrazolium dye cleaved by mitochondrial enzymes when added to cells. The product can be measured by spectrophotometry and is strictly correlated to the metabolic activity of the cell population and the amount of live cells. By comparing treated cells to control cells, it is possible to calculate the viability of cells after treatment. The dye is easily used and the method gives quick results. The disadvantage of the method mainly concerns the uncertainty of the readout. Since the dye measures metabolic activity, a general increase or decrease of this activity can be incorrectly interpreted as proliferation or cell death. These limitations were observed in **paper III**, when cells were recognized as unaffected using WST-1, while many of them were actually affected and arrested in s-phase, as shown with propidium iodide (PI) staining.

In **paper II** and **III** we used PI to stain DNA in ethanol fixed cells and analyzed the staining by flow cytometry. Live cells in untreated controls were gated according to their forward/side scatter and when applying this gating on treated samples, live cells after treatment were gated and compared to the control cells. Gate settings are a limitation of this method and an advantage is that the cell cycle distribution of live cells is studied at the same time.

When PI was used together with Annexin V in **paper I**, cells were unfixed. Consequently, PI only stained the DNA of cells in late apoptosis or necrosis with a

disrupted cell membrane. Annexin V has a high affinity for phosphatidylserine, a phospholipid flipped from the inside to the outside of the plasma membrane in the early stages of apoptosis[347]. Annexin V was bound to fluorescein isothiocyanate, a fluorophore, evaluated using a flow cytometer and when combining with PI staining, cells in early apoptosis can be identified. This method is useful when studying the apoptotic response in cell populations but not all cells express phosphatidylserine in the early stages of apoptosis[348].

In **paper IV** we used a recently established method measuring the *ex vivo* viability of cells after exposure to a broad panel of anticancer drugs[349-351]. The VitalDye measures cell viability and the number of living and dead cells is automatically counted and compared to control cells. This method is more effective than the WST-1 and PI method when it comes to measuring drug sensitivity but the method is sophisticated and complex to setup.

These three methods give information concerning the state of cells at the measured time point but the effect of the drug before and after the readout is not studied. This is a limitation that might be overcome by doing measurements at different time points as was partially done in **paper III**.

3.5 SIGNS OF APOPTOSIS

Before apoptosis, several events occur in a cell and in this thesis we have studied a few of them. In **paper I** we inhibited different proteins in the apoptotic process and studied how this affected the apoptosis induced by selenite. This is a delicate issue and therefore inhibitor concentrations were carefully titrated, several negative controls were used and the outcome measured with different methods. The JC-1 probe was used to measure changes in mitochondrial membrane potential with positive controls and results were verified with the fluorescent 3,3-dihexyloxacarbocyanine iodide. Together this gives a picture of the apoptotic process in cells but unfortunately only at a specific time point and for the chosen apoptotic pathways.

The PI staining intensity in cells, discussed in section 3.4, corresponds to the amount of DNA, enabling the evaluation of cell cycle distribution of live cells. By doing this and comparing to untreated control cells, an S-phase arrest in pemetrexed treated cells was seen in **paper II** and **III**. Defining different phases of the cell cycle through DNA content might be problematic and is partly avoided using the FlowJo mathematical algorithms.

4 RESULTS

4.1 PAPER I

The apoptosis signaling in the epithelioid STAV-AB and sarcomatoid STAV-FCS cells was evaluated after 24 hours of 10 μM selenite treatment. At this time point around 15% of cells from both phenotypes were in late apoptosis or undergoing necrosis. The proportion of early apoptotic cells was also increased, yielding a decrease of the total amount of viable cells. The sarcomatoid cells were especially affected by selenite treatment. A 3-fold loss in mitochondrial membrane potential was induced in both cell lines measured with the JC-1 probe and verified with 3,3-dihexyloxacarbocyanine iodide staining.

Inhibiting p38 did not affect the ability of selenite to induce apoptosis in the epithelioid cells. In the sarcomatoid cells a small decrease in the apoptotic response was seen and in both cell types, inhibiting p38 caused a decrease in the mitochondrial membrane potential after selenite treatment. Inhibiting c-Jun N-terminal kinase (JNK) in selenite treated cells increased the amount of early apoptotic epithelial cells while no change in the effect was seen in sarcomatoid cells. Both cell lines responded to selenite with a time dependent increase of nuclear p53 immunoreactivity but the binding of p53 to DNA was decreased. The p21 expression is regulated by p53 and the small fraction of p21 positive cells in untreated cells was approximately doubled by selenite treatment. Inhibition of p53 did not result in a decrease of apoptosis frequency or loss of mitochondrial membrane potential.

Selenite induced Bax expression in the sarcomatoid cells but not in the epithelioid cells. The Bcl-XL expression decreased in epithelioid cells after selenite treatment and in a subpopulation of sarcomatoid cells. Both cell lines showed a large increase in caspase-mediated cleavage of cytokeratin 18 after selenite treatment. Baseline expression of procaspase-3 was similar in both phenotypes and after selenite treatment a loss of procaspase-3 was seen in subpopulations of both cell lines. Pretreatment with cathepsin B inhibitor caused slight changes in apoptotic rate and viability after selenite exposure and an increased loss of mitochondrial membrane potential. Inhibiting cathepsin D and E did not affect the mitochondrial membrane potential nor the apoptosis induced by selenite. Autophagy was not seen in cell lines after selenite treatment.

4.2 PAPER II

We compared the gene expression in tumor cells from a patient with malignant mesothelioma before and after developing resistance to pemetrexed and carboplatin treatment. This revealed 241 overexpressed and 289 down-regulated genes in the post resistant samples. The group of genes involved in metabolic process of nucleobase, nucleoside, nucleotide and nucleic acid were the most overexpressed genes. Negative regulators of the same process were down-regulated together with genes involved in cell communication.

The cell membrane EMA staining increased from 40% to 70% in the acquired resistance tumor cells compared to the original tumor. Reactivity of checkpoint kinase 1 and TS increased from no detectable staining to 50% and 25% of the tumor cells, respectively. Both the gene and the protein of cytokeratin 7 and thymidine phosphorylase were significantly overexpressed at resistance compared to the original tumor.

The NT5C gene codes for a protein that was indicated as a central protein in the development of resistance but when partly silencing the expression in M-14-K and ZL-34 cells, no significant differences were seen in response to pemetrexed treatment. However, we could see some minor differences in pemetrexed treated ZL-34 cells, with more live cells after NT5C silencing and changes in the cell cycle distribution, compared to pemetrexed treated control cells.

Serum CYFRA 21-1 was below cut-off levels at diagnosis and the value then increased 2-fold above cut-off at resistance. Serum mesothelin related protein in the original sample was increased 12-fold compared to the normal value, then decreased and increased again after resistance to treatment. Carcinoma antigen 125 followed a similar course, with high levels at diagnosis and at resistance. This indicates a potential of the two proteins to monitor the treatment progress in patients with malignant pleural mesothelioma.

4.3 PAPER III

We characterized six mesothelioma cell lines and observed considerable variations in their doubling time. Based on this and the displayed morphological heterogeneity, one cell line was considered as epithelioid, one as sarcomatoid and four cell lines as biphasic.

When evaluating the cytotoxicity of single drugs, selenite affected the epithelioid STAV-AB cells and the biphasic M-14-K, ZL-34 and STAV-FCS cells. Bortezomib affected two cell lines, while doxorubicin only affected one. Gemcitabine and carboplatin showed effects on two different cell lines. Pemetrexed was the least effective single drug when measured using WST-1. When staining treated cells with PI, we could see statistically significant effects on the viability in three cell lines and observe an early S-phase arrest. This arrest was also seen in the ZL-34 cells but the two cell lines with the highest length/width ratios, JL-1 and DM-3 cells, remained unaffected.

Selenite combined with bortezomib was the most potent combination in this study, affecting all six cell lines. The remaining combinations with selenite also had strong effects but the JL-1 and DM-3 remained unaffected. Drug combinations with bortezomib strongly affected some of the cell lines, especially in combination with carboplatin when effects were seen on all cell lines besides STAV-FCS. No general synergistic or antagonistic patterns could be demonstrated, even though doxorubicin showed statistically significant synergism in combination with other drugs. When combinations the conventional drugs doxorubicin and carboplatin was the most effective combination with effects on STAV-AB, M-14-K and ZL-34 cells. MRP-1 immunoreactivity predicted the sensitivity of the cell lines to treatment with carboplatin and presence of xCT predicted pemetrexed effect.

4.4 PAPER IV

Primary cells from patients with malignant mesothelioma display a large variability in their *ex vivo* chemosensitivity, comparing the different cell cultures. Actinomycin D and daunorubicin affected more than ten of the 18 malignant isolates, and taxanes, vinca alkaloids and anthracyclines were the most potent groups of drugs. The four most resistant cell isolates were affected by one drug and the four most sensitive by ten drugs.

The proportion of malignant cells in the primary cell isolates inversely correlated to the proportion of effective drugs and a trend towards correlation with overall survival of the patients was observed. The survival time correlated to the drug sensitivity estimated from the number of effective drugs but not to the drug efficiency. Separating samples into two groups according to proportion of malignant cells in a Kaplan-Meier analysis showed a longer survival time for patients with less than 45% malignant cells. Adjusting drug efficiency for pemetrexed, cisplatin and doxorubicin treatment increased the correlation with survival.

Immunocytochemical reactivity of RRM1 was correlated to the number of effective drugs and primary cell cultures were more resistant to antimicrotubule agents with a higher reactivity for RRM1. Cytoplasmatic staining for this epitope was stronger and number of effective drugs was lower in the group with the more than 45% malignant cells. Dividing the cell isolates into two groups according to their drug sensitivity showed that the proportion of malignant cells and RRM1 staining was lower and weaker in the group with the sensitivity samples. The number of malignant cells and RRM1 staining increased over time when looking at four isolates from the same patient.

5 DISCUSSION

Chemotherapy is the main therapeutic option in malignant pleural mesothelioma, however with limited benefits. The standard treatment, combining pemetrexed and cisplatin, only yields a response rate of around 40%[302]. This response rate correlates to the overall survival and tumor phenotype[106-108,127]. Since such treatment is associated with both considerable side effects and cost, it would be beneficial to identify responders and non-responders before treatment is initiated. For this purpose the development of methods and markers predicting the response becomes increasingly important. A greater and more detailed understanding of tumor characteristics, new drugs and drug combinations based on individual tumor features, can also hopefully improve outcome for malignant pleural mesothelioma patients.

In this thesis cell lines and primary cells from patients with malignant mesothelioma were used. Their drug sensitivity and the expression of different potential predictive markers were studied. The mechanisms for selenite and pemetrexed induced apoptosis and resistance development to pemetrexed treatment in patients were further evaluated.

The drug sensitivity of six malignant mesothelioma cell lines was presented in **paper III**. Selenite was the most potent drug but the two cell lines with the most sarcomatoid phenotypes, measured as highest length/width ratio, were unaffected. Drug combinations with selenite had powerful cytotoxic effects in all tested cell lines. The cytotoxic effect of selenite is mainly through oxidizing free thiols, generating reactive oxygen species and inducing oxidative stress[105]. This is controlled by the cellular uptake of selenite and an increased expression of xCT and MRP-1 increases the effect of selenite[279]. Immunocytochemical demonstrations of xCT and MRP-1 did however not predict the sensitivity of the six cell lines in **paper III**.

Results in **paper I** demonstrate that apoptosis observed in malignant mesothelioma cell lines after selenite treatment are the result of a complex mechanism. Selenite treatment induces p38 and JNK activation and decreases the mitochondrial membrane potential. At the same time p53 accumulation in the nucleus but does not bind to DNA. This might be due to changes in the redox system with more reactive oxygen species and suppressed activity of the thioredoxin system. A tumor dominated by sarcomatoid mesothelioma cells has a poor prognosis and this is also reflected by the chemoresistance of the STAV-FCS cell line, which seem to be biphasic with a dominating sarcomatoid component. Selenite has however, a stronger effect on the STAV-FCS than the epithelioid STAV-AB cells and this might partly be due to higher levels of proapoptotic mediator Bax in the STAV-FCS cells, while the lower sensitivity of the epithelioid cells might be due to down-regulation of Bcl-XL. As seen in **paper III**, this sensitivity to selenite is not general among the sarcomatoid mesothelioma cell lines.

Bortezomib treatment affected three of the cell lines in **paper III** and this effect was increased when combining with the conventional drugs. Bortezomib targets the 20S P subunit of the proteasome and is used in treatment of multiple myeloma but has not been effective in clinical trials with malignant mesothelioma patients[254,256]. The six cell lines were generally unaffected by treatment with doxorubicin, gemcitabine or carboplatin, but in different combinations, however, significant synergistic effects were induced by doxorubicin. This mirrors the clinical situation were treatment with a single drug is ineffective and drug combinations yield better results.

The limitations of using the WST-1 reaction to monitor drug effects were obvious when treating cell lines with pemetrexed (**paper III**). The WST-1 analysis could not detect any response, while staining cells with PI presented extensive effects with s-phase arrest in the three cell lines with the lowest length/width ratios. When using VitalDye to identifying live and dead cells (**paper IV**), pemetrexed did not show any effect. The variability between the outcomes of pemetrexed treatment when using the three different experimental settings can be explained by measurement of different cellular responses. Pemetrexed seems to induce cell cycle arrest and increased metabolic activity in the cell population, highlighting the need to study several aspects of cell viability.

To our knowledge, **paper II** is the first published where the gene expression at the time of diagnosis of a malignant mesothelioma is compared to that after developing resistance towards pemetrexed and carboplatin treatment. Our results indicate that changes in the metabolism of pyrimidine and purine are central in this development of resistance. The value of measuring TS protein expression and not mRNA in response to treatment was seen in these experiments. The NT5C gene was overexpressed, encoding a protein involved in the metabolism of purines. Silencing the gene has previously been shown to induce drug sensitivity but this was not the case in our experiments[352]. This might be because the protein coded for by the NT5C gene was still active; effect of silencing was not measured on protein level.

Checkpoint kinase 1 is important in the G2/M checkpoint as well as DNA repair and has been identified as a target for sensitizing cells to treatment with different drugs[353-355]. The importance of the protein was verified in our study with higher immunoreactivity in the resistant tumor. The increase in EMA and cytokeratin 7 at resistance might serve as a resistance marker, while levels of the remaining routinely used immunohistochemical diagnostic markers such as calretinin and EpCam were not altered and do not seem to be involved in the changed biology of the tumor. The levels of genes encoding for serum mesothelin related protein and carcinoma antigen 125 were not increased in the resistant samples, indicating that the increased amount of these proteins in serum would rather reflect an increased tumor burden. Therefore, the levels of the two proteins function as markers for disease progression but are not involved in the resistance.

Large variability in the sensitivity to different drugs and a broad resistance was observed in **paper IV**, reflecting the clinical situation with limited effect of chemotherapy and a scattered response rate. The proportion of malignant cells in the pleural effusions is crucial for the ability to estimate sensitivity in this experimental set-up, correlating to the proportion of effective drugs. There was also a trend towards correlations of this parameter to the survival time of patient. When the drug effect was adjusted according to proportion of malignant cells and theoretical effect on benign cells, the cell isolates with lower proportions were still affected by a higher proportion of drugs. This indicates that malignant mesothelioma patients with higher proportions of malignant cells have a more advanced disease, with drug resistance and shorter survival time.

RRM1 reactivity in the primary cell isolate seems to be important for drug sensitivity and survival of the patient. The ability of RRM1 reactivity to predict the effects of drugs has previously been shown in non-small cell lung cancer patients treated with cisplatin and vinorelbine[356]. In repeated pleural effusions from the same patient we

observed a progressed disease with an increased proportion of malignant cells, RRM1 reactivity and drug resistance.

Adjusting the drug effect to admixture of benign cells and the theoretical effect on the benign cells increased the correlation to survival time, especially for pemetrexed and cisplatin. Interestingly, after this adjustment, cell isolates not affected by pemetrexed and cisplatin were affected by drugs such as carboplatin, doxorubicin and gemcitabine, used in other drug combinations recently described for patients with malignant mesothelioma[312]. This indicates that these patients might have responded better to alternative drug combinations as first line treatment. This motivates a further development of *ex vivo* testing of drug sensitivity as basis for personalized choice of treatment.

5.1 CONCLUSIONS

In this thesis it is concluded that there is a need for individualized treatment of malignant mesothelioma patients. This can to a certain extent be based on results from studying tumor cells from their pleural effusion, optimizing the choice of drug combinations.

Selenite is a potent cytotoxic agent in malignant mesothelioma cell lines by inducing apoptosis in a complex and phenotype dependent manner. The apoptosis signaling caused by selenite is described and partly explained.

Several different genes coding for TS and proteins involved in the metabolic processes of pyrimidine and purine, are involved in resistance development to pemetrexed and carboplatin treatment in a patient. We suggest that EMA and cytokeratin 7 levels can be used as potential resistance markers and that tumor progression might be followed by levels of serum mesothelin related protein and carcinoma antigen 125.

We observed heterogeneity in the six mesothelioma cell lines with different morphology, variable drug sensitivity and different expression of predictive markers. Selenite and bortezomib were more effective than the conventional drugs and arrest in S-phase was seen in cell lines treated with pemetrexed.

When studying primary cell isolates from patients with malignant mesothelioma we observed a large variability in their drug sensitivity which seems to correlate to proportion of malignant cells and RRM1 reactivity. The observed sensitivity should be corrected for the theoretical drug effect on benign cells and admixture of benign cells.

5.2 FUTURE PERSPECTIVES

The results in this thesis indicate the need and possible approaches to individualized treatment of malignant mesothelioma patients. We hypothesize that by studying the drug sensitivity of primary tumor cells the optimal treatment strategy for each individual patient might be predicted, measuring the tumor specific cell death. Focus should then, preferably, be directed towards the *in vitro* effects of drugs that previously have been evaluated in treatment of malignant mesothelioma and therefore are accepted as alternative treatment options. The use of predictive markers can also be of help, although the arsenal can be improved. By performing such more detailed studies we might be able to suggest both the best first line treatment and the best alternative in patients that are not responding.

Difficulties in evaluating primary cultures due to admixture of variable amounts and types of benign cells should also be addressed. One possibility is to use flow cytometry. Such an analysis can be developed to include not only markers for apoptosis and cell cycle arrest, but also different proteins essential to drug resistance. Over time such studies may also help to better explain and understand resistance development in patients.

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