universität wien

# MASTERARBEIT

Titel der Masterarbeit

Targeting mTOR as novel therapy for
Human Malignant Mesothelioma

Verfasser

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angestrebter akademischer Grad

Master of Science (MSc)

Wien, 2011

| | |
|---|---|
| Studienkennzahl lt. Studienblatt | A 066 877 |
| Studienrichtung lt. Studienblatt: | Genetik und Entwicklungsbiologie |
| Betreuerin / Betreuer: | Univ. – Prof. Mag. Dr. Renée Schröder |

This master thesis was conducted at the

Medical University of Vienna

Department of Medicine I

Institute of Cancer Research

And was supervised by

Univ. – Prof. Mag. Dr. Renée Schröder

Ao. Univ. Prof. Dr. Walter Berger

# Danksagung

An dieser Stelle möchte ich all jenen danken, die mich während meines Studiums und bei der Durchführung dieser Arbeit unterstützt haben. Im Einzelnen gilt dieser Dank ganz besonders:

Prof. Dr. Walter Berger, der immer ein offenes Ohr für mich hatte, auch wenn sein Zeitplan noch so begrenzt war. Dank seiner fortwährenden Förderung und Betreuung, hat er mir nicht nur die Möglichkeit gegeben reichlich zu lernen, sondern auch selbstständig zu arbeiten und persönlich zu wachsen. Danke für dein Vertrauen und für die schöne Zeit.

Prof. Dr. Renée Schröder, vom Institut für Biochemie und Zellbiologie, für die Betreuung dieser Arbeit.

Dr. Ali Hoda, durch dessen enormes Engagement und Hingabe für dieses Projekt aus einem Einzelkämpfer ein ganzes "Meso"-Team entstanden ist. An dieser Stelle möchte ich auch Bahil herzlichst danken, der mit seiner Leidenschaft für IHC und SPSS mir stets eine große Hilfe gewesen ist. Mein Dank gilt auch Prof. Dr. Walter Klepetko, dem Leiter der Thoraxchirurgie des AKH Wien, für die Administration dieser Studie.

Meinen Kolleginnen Petra Heffeter, Christine Pirker, Daniela Lötsch, Ute Jungwirth und Bihter Atil die mich von Anfang an herzlichst in ihr Team aufgenommen haben. Eure Geduld und andauernde Unterstützung war maßgeblich für diese Arbeit und meine Entwicklung. DANKE Ladies.

Vera Bachinger und Christian Balcarek, für deren großartige Unterstützung und Hilfe im Labor, nicht nur in meiner Anfangszeit sondern während der gesamten Studie. Arbeitskollegen wie euch kann man sich nur wünschen.

Dr. Michael Grusch und seiner gesamten Crew, für deren außerordentliche Hilfsbereitschaft. Eure Geduld mit mir hätte ich selbst gar nicht gehabt ☺.

Dr. Balazs Hegedus und Judit Berta, für deren Unterstützung bei so manchen Experimenten und die wirklich nette Zeit im Labor. Köszönöm!

Meiner lieben Freundin, meinem Bruder und natürlich meinen Eltern. Vor allem meiner Mutter möchte ich dafür danken, dass sie mich immer Unterstützt und zu mir gehalten hat. Dank dir stehe ich jetzt hier und werde so wie du für mich, immer für dich da sein…

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# 1. ABSTRACT

Das humane maligne Mesotheliom (HMM) ist eine asbest-assoziierte neoplastische Erkrankung, die durch eine hohe Resistenz gegenüber den herkömmlichen zytostatischen Therapien gekennzeichnet werden kann. Vor allem in den westlichen Industrieländern wird eine Zunahme der Inzidenz verzeichnet, was vermutlich auf den im vergangenen Jahrhundert starken Gebrauch von Asbest zurückzuführen ist. Die Prognose der Erkrankung ist schlecht mit einer medianen Überlebensrate von 9 bis 12 Monate ab dem Zeitpunkt der Diagnose. Der Tumor neigt vor allem zu einer lokalen Ausbreitung, während eine Fernmetastasierung selten zu beobachten ist. Radiotherapie und Chemotherapie als Monotherapie sind weitgehend ineffektiv. Der chirurgischen Therapie kommt demnach eine wesentliche Bedeutung bei kurativer Intention zu. Aufgrund der häufig weit fortgeschrittenen Erkrankung bei Diagnose ist allerdings die Entwicklung neuer systemischer Therapiekonzepte mit erhöhter Wirksamkeit für Mesotheliom-patienten von außerordentlicher Bedeutung.

Innerhalb des karzinogenen Prozesses, nimmt der mTOR (mammalian target of rapamycin) Signalweg allgemein eine zentrale Stellung ein. In vielen soliden Tumoren, als auch hämatologischen Neoplasien des Menschen ist diese Signalkaskade durch verschiedenste molekulare Mechanismen überaktiviert. Dies führt unter anderem zu verstärkter Proliferation, erhöhtem Überlebenspotential und Resistenz gegenüber Apoptose und somit gegebenen falls zum Versagen der Chemotherapie. Daher stellt die Hemmung des mTOR Pathways einen zentralen Angriffspunkt der modernen, gezielten Krebstherapie dar. Der ursprüngliche und namensgebende mTOR-Inhibitor Rapamycin, ist ein bekanntes Immunsuppressivum mit antineoplastischer Wirkung. Folglich wurden weitere Rapamycin-Derivate entwickelt, darunter Temsirolimus, das bereits erfolgreich klinische Anwendung findet und untern anderem das Überleben von Patienten mit fortgeschrittenem Nierenzellkarzinom verbessert. Auch zur Behandlung des seltenen Mantelzell-Lymphoms wurde Temsirolimus seit kurzem zugelassen. In Bezug auf das maligne Mesotheliom, gibt es Hinweise auf eine häufige Hyperaktivierung des mTOR Pathways bzw. des „upstream“ gelegenen PI3K (Phosphoinositid-3-Kinasen) Signalwegs, der eine bedeutende mTOR-regulierende Funktion beinhaltet. Mögliche Ursachen die zu solch einer Hyperaktivierung beitragen könnten, wären zum Beispiel als

der Verlust des PI3K negativen Regulators PTEN (Phosphatase and Tensin homolog) als auch Mutationen einiger wichtiger Wachstumsfaktor-Rezeptoren wie IGF1R (Insulin-like Growth Factor 1 Receptor) oder HGFR (Hepatocyte Growth Factor Receptor).
In der hier präsentierten Arbeit wurden immunhistochemische Präparate von HMM Tumorproben (n=70) hergestellt und auf dessen mTOR-Phosphorylierungsstatus hin untersucht. Die Präparate als auch die hier verwendeten HMM-Zellmodelle (n=6), zeigten eine signifikante konstitutive mTOR-Aktvierung. Weiteres wurden die HMM Zelllinien mittels array comparative genomic hybridization (aCGH) auf genetische Aberrationen der wichtigsten in den mTOR Pathway involvierten Akteure, als auch auf allgemeine, häufig mit Krebs assoziierte genetische Mutationen untersucht. Als Hauptziel dieser Studie galt es zu evaluieren, inwieweit eine Blockade des mTOR Pathways mittels Temsirolimus einen vielversprechenden neuen Ansatz in der Therapie des malignen Mesothelioms darstellt. Dabei wurde die Substanz sowohl als Monotherapie als auch in Kombination mit der bei dieser Erkrankung verwendeten Standard-Chemotherapie (Cisplatin und Pemetrexed) angewandt. Temsirolimus bewirkte eine maßgebliche Inhibierung mTOR-vermittelnder Signale und zeigte zytostatische Wirkung, sowohl in als adhärenten einschichtigen in vitro kultivierten HMM-Zelllinien als auch in nicht-adhärenten Sphäroiden. Mesotheliom-Zelllinien die über eine intrinsische Cisplatin-Resistenz verfügten, zeigten hierbei das beste Ansprechverhalten. Auch durch in vitro Selektion erworbene Cisplatin-Resistenz, hatte eine deutliche Sensibilisierung gegenüber Temsirolimus zur Folge. Interessanterweise konnte gleichzeitig demonstriert werden, dass im Gegensatz dazu, die zytotoxische Wirkung des zweiten Standard-Chemotherapeutikums Pemetrexed in Cisplatin resistenten Zellen signifikant reduziert ist. Weiteres zeigte die Kombination von Temsirolimus und Cisplatin einen deutlich synergistischen Effekt in der Blockierung der mTOR „downstream“-Signaltransduktion und verstärkte die Wachstumsinhibition als auch die Indizierung von Apoptose in vitro. Schließlich demonstrierte Temsirolimus eine hohe antineoplastische Aktivität gegen HMM xenograft SCID-Mausmodelle als Einzelsubstanz als auch in Kombination mit Cisplatin.
Zusammengefasst legen die hier dargelegten Daten nahe, dass die Inhibierung von mTOR im humanen malignen Mesotheliom eine vielversprechende neue Strategie im Kampf gegen diese äußerst schwerwiegende Krebserkrankung darstellen könnte.

Human malignant mesothelioma is an asbestos-related malignancy characterised by frequent resistance against chemo- and radiotherapy. Because of the extensive asbestos usage in the last century primarily in western industrial countries an increasing incidence is prognosticated for the next decades. Generally, HMM shows a very poor prognosis with a median survival of nine to twelve month. Primarily the tumor tends to expand into the local areas, while metastasis is only rarely observed. Both, radiation therapy and chemotherapy are often intrinsically ineffective or result in acquired resistance during the course of disease. Consequently, extensive surgery is still of great importance in case of therapy with a curative intention. Due to the fact that in the majority of cases HMM is diagnosed only in late stage, an improved understanding of the complex molecular changes underlying this neoplasia is urgently needed to develop new and probably targeted treatment strategies.

The oncogenic signalling cascade of mTOR (mammalian target of rapamycin) is known to occupy a central position within carcinogenesis. In both, solid and hematopoietic tumors, this pathway has been reported to be hyper-activated, not only causing increased proliferation and survival, but also seems to confer apoptosis-resistance consequently leading to chemotherapy failure. For this reason, the inhibition of mTOR is suggested to be a central point of application for novel targeted cancer therapy. The primary discovered and name given inhibitor of mTOR, rapamycin, is known to exhibit immunosuppressive and anti-neoplastic effects. Its derivate temsirolimus, which is also already in clinical use, effectively prolongs survival of patients with mantel cell lymphoma and advanced renal cell carcinoma. With regard to human malignant mesothelioma, there is evidence that mTOR and also its important upstream regulator PI3K (phosphoinositid-3-kinase) are frequently hyper-activated. It is suggested, that deletion of PTEN, as negative regulator of PI3K, may be responsible for an aberrant mTOR signaling. Also mutations of several growth factor receptors such as IGF1R (insulin-like growth factor 1 receptor) or HGFR (hepatocyte growth factor receptor) are thought to be at least in part responsible for the genesis of an oncogenic mTOR pathway.

In this study, HMM tumor specimen (n=70) were analyzed for mTOR phosphorylation status using immunohistochemical standard procedure. The tumor samples and also investigated HMM cell models (n=6) showed significant constitutively mTOR hyper-activation. Further, we performed array comparative genomic hybridization (aCGH)

analyses of all HMM cell lines to detect both, possible genomic aberrations of key players involved in mTOR signaling and generally frequently reported mutations highly associated with carcinogenesis. A further main aspect of this study was to clarify whether mTOR inhibition by temsirolimus may be a feasible anti-mesothelioma strategy. Therefore cell lines were treated with temsirolimus as single agent or in combination with components of the standard chemotherapy regimen (cisplatin and pemetrexed). Temsirolimus potently blocked mTOR-mediated signals and exerted a cytostatic effect against mesothelioma cell lines in vitro cultured both as adherent monolayers and as non-adherent spheroids. Mesothelioma cells intrinsically resistant against the standard treatment component cisplatin tended to be hypersensitive against temsirolimus. Additionally, acquired cisplatin resistance by in vitro selection was accompanied by distinct upregulation of sensitivity against the mTOR inhibitor. In contrast, we observed that cytotoxic effects of pemetrexed were significantly reduced in cisplatin resistant cell models. Furthermore, cisplatin and temsirolimus exerted synergistic inhibition of the mTOR downstream signals and enhanced growth-inhibition and/or apoptosis-induction in mesothelioma cell lines in vitro. Finally, temsirolimus was highly active against human mesothelioma xenograft models in SCID mice both as single agent and in combination with cisplatin.

Taken together, the here presented data suggest mTOR inhibition as promising novel therapeutic strategy against malignant pleural mesothelioma.

# 2. INTRODUCTION

## 2.1 Epidemiology of cancer

Every multi-cellular organism's base of existence underlies a controlled equilibrium between cell growth, differentiation and death. Maintaining this balance requires specific cellular responses which are processed by complex extra- and intracellular signaling pathways. A consistent dysfunction of this routine may results in an uncontrolled growth of cells and further in their spreading into diverse tissues which describes the main characteristics of the second most common deadly disease in industrial countries: Cancer.

In Austria over 36.000 people [1] develop malignant neoplasms every year and approximately every third European will suffer from cancer during his lifetime [2]. Although the understanding of tumor development has made significant progress in the last decades, cancer remains a key public health concern and an enormous burden on society.

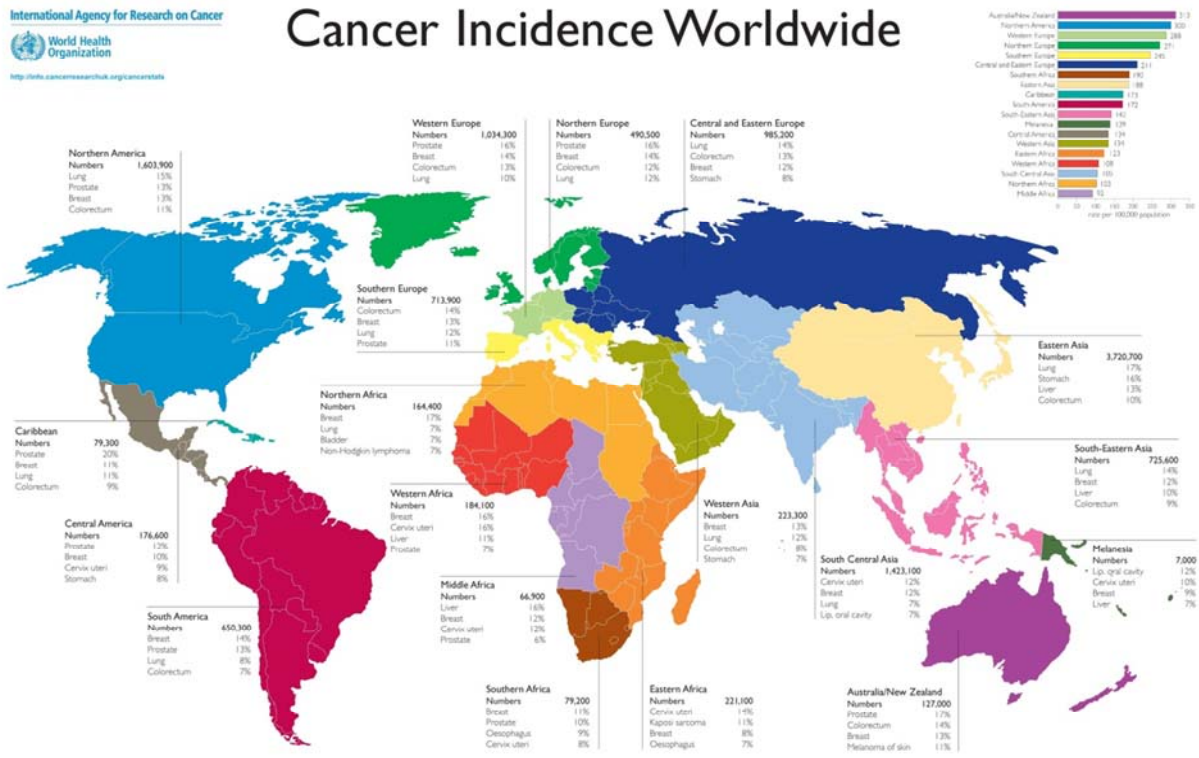



Figure 1. *Breakdown of estimated 12.7 million new cases, age standardized incidence rates and the most commonly diagnosed cancers by different regions of the world, 2008.*

The human organism features a multitude of autonomic mechanisms to secure that aberrant cells are stopped in their deviated progress and do not harm the organism. Today it has become basic knowledge that changes in the cellular genome are responsible for the malfunction of these protective mechanisms and the origin of cancer. Basically these mutations can occur spontaneously or due to environmental influences such as carcinogenic substances, physical or chemical mutagens and certain viruses. In fact, normally more than one somatic mutation is required to trigger all changes necessary for a fully developed tumor cell. Thus, a prominent factor determining susceptibility to neoplasia is age [3]. Because mutations occur a whole life long, age provides the time necessary for the accumulation of events needed for the development of cancer.

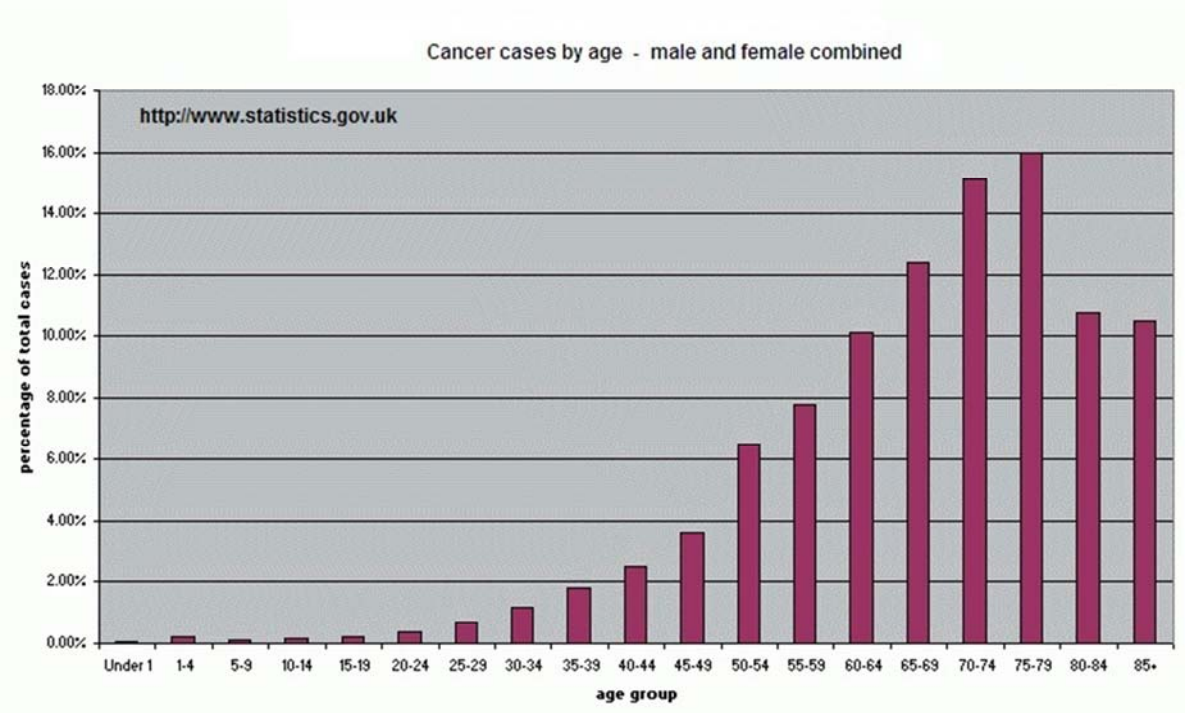



Figure 2. *Correlation between total cancer incidences and age in the UK indicative for industrial countries.*

## 2.2 Principal mechanisms of cancer development

Specific genes are responsible for the regulation of cell growth and division – the cell cycle. Mutations that interfere with the expression of these key players may induce cancer. Thus, genes with the property of initiating neoplastic growth are termed proto-oncogenes and consequently their expression products proto-oncoproteins [4]. Generally aberrant genomic changes that alter proto-oncogenes into carcinogenic oncogenes can be classified in three main groups: Migration of DNA within the genome (translocation), gene amplification and point mutations. Malignant cells often harbor broken chromosomes rearranged in an incorrect manner. It is possible that this exchange of DNA material, called chromosomal translocation, affects a proto-oncogene which may be relocated behind an active promoter over stimulating its transcription and hence turning it into an active oncogene. The second main type of genetic aberrations, gene amplification, prevalently appears during repair of harmful DNA double-strand breaks by homologous recombination. Failures during this complex repair mechanism or more frequently due to dysfunction of involved mutated enzymes

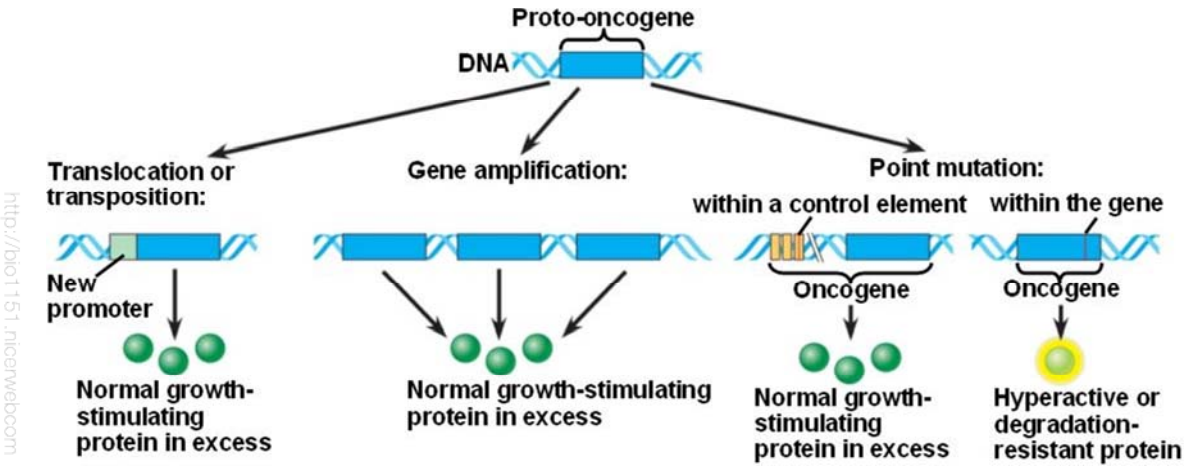



Figure 3. *Different types of mutations leading to proto-oncogene activation.*

may cause the duplication of a proto-oncogene increasing its expression products. The third way how a normal gene can become carcinogenic is a spontaneous single substitution, deletion or insertion of one or a few base pairs during DNA replication. The rate of these point mutations can be strongly increased by diverse mutagens. If this

damage is not repaired and hits a proto-oncogene, its product may be modified in a way that makes the protein more active (gain of function) or more resistant against degradation. All these mechanisms can lead to an aberrant stimulation of the cell-cycle and consequently to the genesis of malignancies.

Besides mutations that cause a stimulation of cell growth, also aberrations of control genes that are normally involved in cell-cycle inhibition, DNA-repair or cell-adhesion can be responsible for the development of neoplasia by a loss of function. These genes are called tumor suppressor genes because the code for proteins which normally prevent and/or inhibit cells from uncontrolled growth especially through apoptosis. Every mutation that decreases their activity generally leads to permitted stimulation of cell growth due to the lack of suppressive factors, a major driving force on the path to cancer.

A third, more specialized, class of genes referred to as 'caretaker' genes are also often linked to diverse malignancies. They normally protect the integrity of the genome and are involved in identification or repair of DNA damages and elimination of aberrant cells. When caretaker genes are inactivated, cells may acquire additional mutations based on the lost control mechanisms.

The change from a normal cell to a cancer cell commonly requires multiple steps, each one adding properties that makes cells more likely to become malignant. The process of malignant transformation is often described by a simplified three-stage model [5, 6]: Initiation is the first step and involves a change of the cells genome due to a mutation of a proto-oncogene, tumor suppressor- or caretaker gene (see above). In this case, intricate repair mechanisms may fail to correct the damaged DNA and the cell becomes capable of avoiding apoptosis. During the next step, called promotion, the initiated cell is

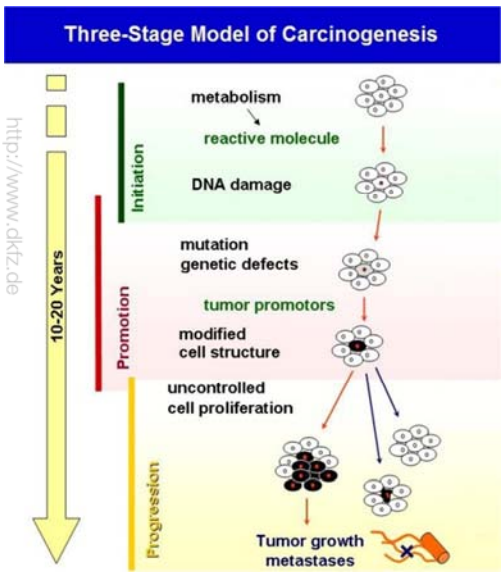



Figure 4. *Three-stage model of carcinogenesis.*

stimulated to grow and increases proliferation. The basic cause of this reaction are not mutagens like in the first step, it is primary the own body who releases growth factors and hormones. This so called none-genotoxic agents are particularly frequent in fast regenerating tissue like in the intestine, lung, breast and liver. Also during inflammatory events a mutated cell can be stimulated to grow by these none-genotoxic agents, e.g. wound healing hormones which normally initiate the regeneration of the affected tissue. Thus, especially chronic inflammation is frequently associated with cancer development. Through growth and proliferation, accumulated mutated cells form a pre-neoplastic mass which represents a pre-stage of cancer. This so called benign tumor is not capable to invade the surrounding tissue by now, although DNA instability increases the risk of further mutations with every cell division. New accumulated changes in the genome can now lead to the final step termed 'progression'. In this stage, multitude onco- and tumor suppressor genes are affected and after numerous divisions the cells lose their tissue identity and dedifferentiate. This modified cell structure and further mutations are responsible for the transformation of a benign tumor into an invasive neoplasia. The malign cells have now the potential to infiltrate the surrounding tissue and to metastasize to other parts of the body using either the lymphatic or hematogenic system.

With regard to the changes in the population of neoplastic cells and their microenvironment through selection of an initiated cell in the first stage up to their competition of space and resources within the malignant tumor, cancer can be described as a disease of clonal evolution within the body [7]. This Darwinian mechanism may be a contribution to apoptosis resistance of a mutated cell because evolution generally selects for increased proliferation and survival. Further the appearance of invasive property can be explained through the presence of selective pressure within a neoplasm [8]. Uncontrolled growth and over-consumption of resources consequently leads to limited space and nutrients which might be a selective force for dispersal.

Although the three-stage hypothesis illustrates a convincible path to cancer, further theories are more and more accepted. A new way of looking at carcinogenesis, but not contradicting earlier concepts, is the cancer stem cell hypothesis. It proposes that a

heterogenic malignant tumor mass arises originally from a unique transformed stem cell or a dedifferentiated somatic cell that recovered stem cell characteristics [9]. This cell represents the source of continuous proliferation and self-renewal capability what compensates the fact that differentiated cells cannot indefinitely divide because of telomere-shortening (Hayflick limit). Cancer stem cells may also be an important factor for tumor regression after therapy. While the differentiated part of the tumor degrades, the cancer stem cell survives and causes the relapse. Further tumor metastases commonly retain heterogeneity, implying that the origin of a spread tumor had the capacity to generate multiple cell types which is a typical attribute of a stem cell.

## 2.3 Hallmarks of cancer

Although cancer displays enormous complexity, a relatively simplified view of carcinogenesis suggested by D. Hanahan and R. Weinberg [10] about ten years ago has become a well-accepted model. It comprises six capabilities that are shared by most and perhaps all types of human tumors: insensitivity to growth-inhibitory signals, self-sufficiency in growth signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis. Recent years have seen that this model should be revised to include a probable seventh hallmark: cancer-related inflammation.

### 2.3.1 Self-sufficiency in growth signals

Normal cells require specific stimulatory signals from their tissue microenvironment to end their quiescent status and to move into a proliferative state. In contrast tumor cells cultured in vitro show a greatly reduced dependence on these exogenous factors suggesting that neoplastic cells are capable of generating autocrine growth stimulation [10]. Consequently critical mechanisms that are normally in order to ensure the homeostasis of various cell types within the tissue are disrupted. In addition to mutated oncogenes further factors can be responsible for this attribute. Numerous types of cancer feature deregulated growth signal transducing cell surface receptors carrying a tyrosine kinase activity which represents an important mechanism in signal transduction for regulation of cellular activity. An overexpression of these receptors may enable a

cancer cell to become hyper responsive to levels of growth factors that would normally be insufficient to trigger proliferation [10]. Further overexpression and/or structural alteration of receptors may lead to ligand-independent signaling allowing growth even without an appropriate stimulation.

### 2.3.2 Insensitivity to growth-inhibitory signals

The normal tissue is kept in homeostasis by specific signals that inhibits growth/proliferation or induce entrance into a postmitotic, differentiated state that is usually associated with acquisition of quiescent traits. These signals are received by transmembrane cell surface receptors and further coupled to intracellular pathways. An aberrant cell has generally two opportunities to evade this circuit: On the one hand the deficiency of signal transducing receptors blocks the initiation of growth-inhibitory pathways strait from the beginning by lock out of sensed antigrowth factors, a characteristic phenomenon of many malignant cells. On the other hand typical mutations of tumor suppressor genes consequently lead to the dysfunction of intracellular pathways, resulting in the inability of preventing cells from uncontrolled growth [10, 11].

### 2.3.3 Evading apoptosis

If a cell that accumulated mutant onco- and tumor suppressor genes becomes aberrant and does not respond to any repair mechanisms, the organism normally starts to defend himself by inducing apoptosis. This programmed cell death is a series of biochemical events that lead to disruption of cellular membranes, break down of cytoplasmic and nuclear skeletons, chromosomal degradation and fragmentation of the nucleus within a very short period. Finally phagocytes start to engulf the remaining cell parts (apoptotic bodies) until their complete disappearance [12]. For this reason an aberrant cell primarily has to circumvent this major barrier to cancer. This becomes possible if the accurate cohort of particularly pro-apoptotic genes is affected by irreversible mutations or anti-apoptotic proteins are overexpressed [10].

### 2.3.4 Limitless replicative potential

All previously mentioned acquired capabilities may be the beginning path to cancer but are still not sufficient to enable the generation of expansive tumor growth. Human cells carry an intrinsic cell-autonomous program that limits their multiplication in general which appears to operate independently of cell-to-cell signaling pathways. Already in 1965 L. Hayflick observed that normal diploid cells lose their ability to proliferate normally after 50-70 cell divisions in vitro. This phenomenon, also known as Hayflick limit or cellular senescence, underlies the natural shortening of telomeres during each cell cycle caused by the inability of DNA polymerases to completely replicate the 3' ends of chromosomal DNA [13]. If the chromosomes become too short, the cell generation enters a state termed crisis which is characterized by karyotypic disarray associated with end-to-end fusion of chromosomes and finally massive death. Only in germ, stem and certain white blood cells the progressive erosion of telomeres is prevented by an enzyme that adds hexanucleotide repeats onto the telomeric DNA, the telomerase. It is supposed that normal somatic cells lack of this enzyme to prevent an aging cell from becoming malignant. In fact, virtually all types of cancer (85%-90%) [14] maintain their telomeres by upregulated expression of telomerase and thus, achieving an immortal status. It is assumed that this telomerase overexpressing cells in an advanced neoplasm only reflect finally immortalized clones from very few survivors of crisis. Interestingly, some malignant cells maintain or even increase the length of their telomeres in absence of telomerase activity by a mechanism referred to alternative lengthening of telomeres (ALT) [15]. It is suggested that ALT relies on a recombination-based mechanism whereat single stranded DNA at one telomere terminus invades another telomere and uses it as a copy template resulting in exchange and increase of sequence information. This inter-telomeric copying may becomes possible through abundant displacement loops (D-loops) which normally disrupt base pairing during DNA-synthesis at this locus. The exact mechanism of this pathway still needs to be clarified. However, circumvention of senescence is a basic necessity for full malignant transformation.

### 2.3.5 Sustained angiogenesis

The oxygen and nutrient supply provided by the vasculature system is crucial for the function and survival of any cells. The physiological process involving the formation of blood vessels during development, growth and wound healing is referred to as angiogenesis. Certain biological signals such as vascular growth factors (VGFs) and fibroblast growth factors (FGFs) activate surface receptors present on endothelial cells of pre-existing blood vessels initializing them to invade the surrounding matrix and to form sprouts towards the angiogenic stimulus. However, already in the late 1960s Folkman et al recognized that angiogenesis is a fundamental step in the transition of accumulated aberrant cells into a malignant neoplasm [10]. The necessity of blood vessel formation within a growing tissue relies on the basic physical issue that oxygen and nutrients can only be supplied up to 100µm through passive diffusion. Hence, a growing tumor has to acquire the capability of neovascularization which is particularly achieved through the common strategy of altered gene transcription. Many tumors evidence increased expression of VEGFs and FGFs or the downregulation of angiogenic inhibitors such as thrombospondin-1 or β-interferon respectively [16] .

Further it could be demonstrated that aberrant modulating proteins of pro- and antiangiogenic signaling molecules [17] and the dysfunction of onco- or/and tumor suppressor genes [18] are involved in the shifted balance of angiogenesis inducers and countervailing inhibitors reflecting the complex homeostatic regulation within tumor formation that still remains incompletely understood.

### 2.3.6 Tissue invasion and metastasis

The ability to invade adjacent tissue and to give spread to new colonies on distant sites is the key characteristic that discerns a benign tumor from malignant cancer. Approximately 90% of all human cancer deaths are caused by the distant settlement of tumor cells referred to as metastases [19]. Most benign tumors pose little risk to their host because particularly cell-adhesion molecules keep them localized to the originating tissue, what makes them an easier target for surgical resection. Through continuous growth, nutrient supply and space of a tumor become limited factors. The capability for invasion and metastasis enables cancer cells to escape these adverse

conditions and to colonize new terrains in the body. The genetic and biochemical determinants which enable the acquisition of these capabilities during carcinogenesis still remain incompletely understood but generally rely on redundant pathways that mediate elusion of cells from the primary tumor, survival and arrest in the bloodstream and progressive outgrowth at distant locations [20].Invasion is the initiating metastatic process that consists of changes in the physical coupling of cells to their microenvironment, activation of extracellular proteases and an acquired motility of tumor cells to physically move through the tissue. The binding of cells to other cells and the extra cellular matrix (ECM) is mediated by cell adhesion molecules (CAMs) such as cadherins and integrins. A frequently observed alteration in malignant tumors is the

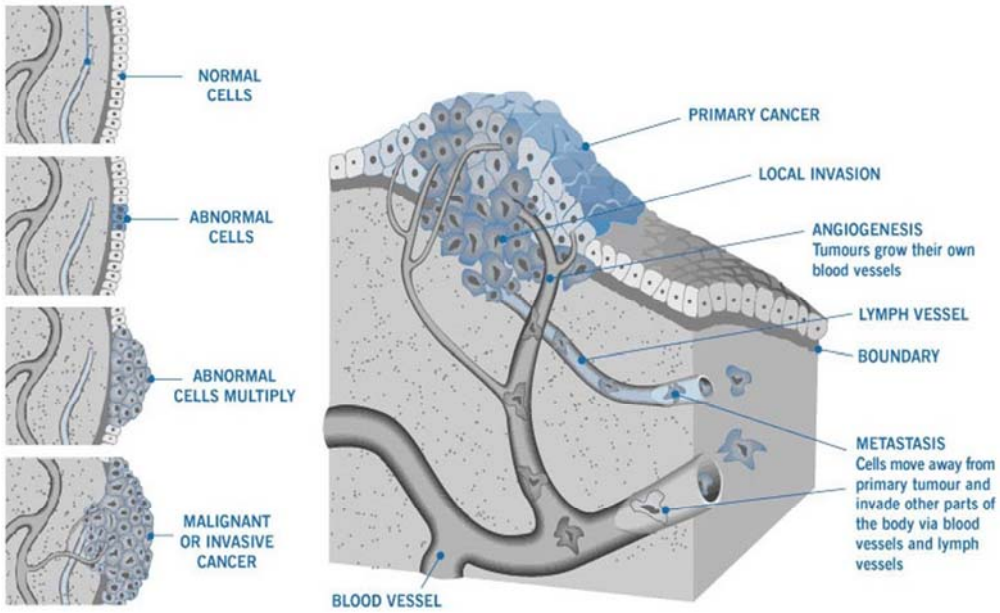



Figure 5. *Tumor development: From a normal cell to malignant cancer [20].*

inactivation of E-cadherin, a member of the cadherin family that is only expressed on epithelial cells. This molecule normally mediates the attachment of cells through homophilic protein-protein interactions of their extracellular domains and suppresses cellular motility. The intracellular domain of E-cadherin can be attached to β-catenin, forming a subunit which serves as intercytoplasmic bridge between cells and may be responsible for the transmission of antigrowth signals [11]. It was shown that forced expression of E-cadherin in cancer cells in vitro and in vivo impairs their invasive and metastatic potential, whereas suppression of E-cadherin function promotes both

capabilities [21]. Another type of signal- and attachment-mediating molecules are integrins which penetrate the plasma membrane and feature small cytoplasmic domains. These receptors ensure the binding to the ECM that usually provides structural support of involved tissue and regulates intercellular communication. The dysfunction of integrins is associated with the recruitment of ECM digesting extracellular proteases which provides the necessary pathway for invasion [22]. Another prominent factor that promotes the disintegration of the ECM is the forced expression of urokinase or urokinase receptors respectively which convert the proenzyme plasminogen into the active protease plasmin. Generally components of the plasminogen activation system are found to be correlated with tumor cell dispersal [23].

Once the anchorage of cells within a tumorigenic tissue is loosened and the path to nearby blood vessels or the lymphatic system respectively is vacated, changes in the cytoskeleton can facilitate physically movement of aberrant cells. Frequently affected targets of mutations are genes encoding Rho and other related GTPases which are able to drive extensions and contractions of actin/myosin filaments that

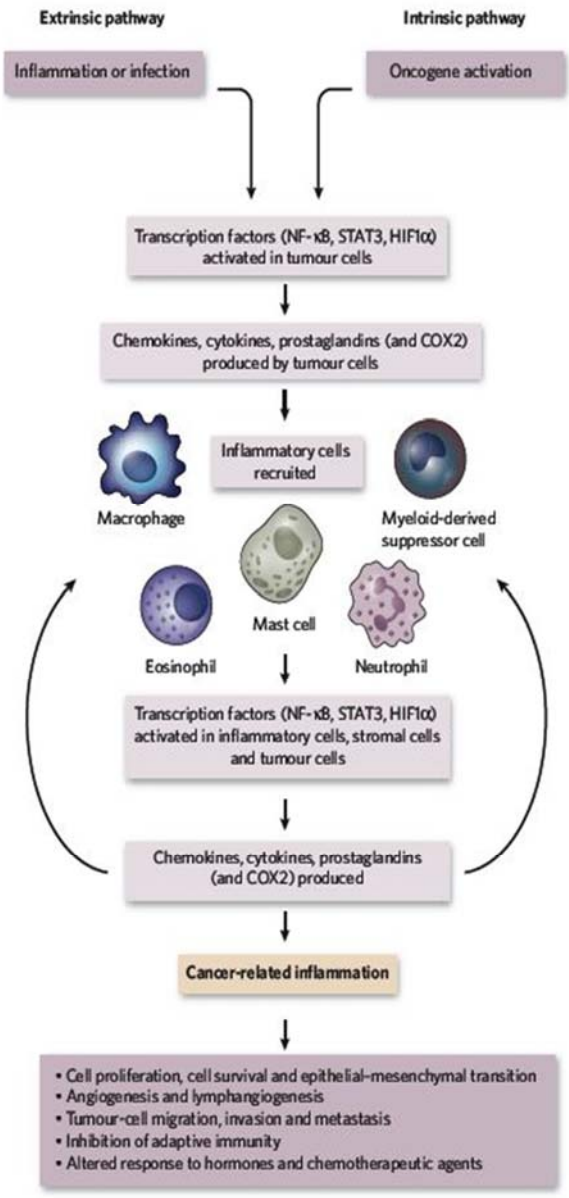



Figure 6. *Two main pathways connecting inflammation and cancer: The intrinsic pathway is activated by genetic events that lead to the production of inflammatory mediators, thereby generating an inflammatory microenvironment. In the extrinsic pathway, inflammatory or infectious conditions augment the risk of developing cancer. Both pathways converge and finally force the rise of smoldering cancer-related inflamemation that has many tumor-promoting effects [30].*

cause cellular locomotion. In addition, activated N-WASP, a protein that regulates the structure of actin filaments, helps to assembly protrusions referred to as invadopodias. These structures are very similar to podosomes formed for example by macrophages or monocytes, allowing cells to migrate on ECM fibers and to cross tissue barriers or penetrate blood vessel walls.

After entering the circulation system, cancer cells must survive and arrest in the harsh environment of the bloodstream for successful extravasation. The presence of immune cells, velocity-induced shear forces and the lack of nutrients consequently lead to the predominately death of escaped tumor cells. But few survivors of these destructive conditions may be able to bind coagulation factors (e.g. fibrinogen, fibrin, thrombin) and to arrest as enlarged clots in small capillary beds with help of specific adhesive interactions [24]. Generally it has long been recognized that some types of cancer show an organ-specific pattern of metastasis referred to as the 'seed and soil' hypothesis which was first published by Stephan Paget in 1889 [25]. It proposes that the location of settlement depends on seed (primary tumor) and soil (secondary organ) specific patterns [26]. A second theory suggests that circulatory patterns of the blood flow and the lymphatic system between primary tumor and final destination are responsible for the organ-specific metastasis of certain malignancies. In fact, both theories are not necessarily contrary and can apply according to the type of cancer as more recent studies confirm [27].

Experimental in vivo models show that metastasis is principally an inefficient process because approximately only 1% of injected cancer cells are able to form macroscopic tumors [28]. Successful colonization crucially depends on various factors: adequate interaction with the microenvironment, cellular structure of the infiltrated tissue as well as accumulation of genes necessary for survival and recruitment of growth and angiogenic signals within the new location. All these terms demonstrate the enormous complexity of metastatic formation, a process that is more and more elucidated but by far not completely understood.

### 2.3.7 Cancer related inflammation

Inflammation-associated cellular effectors and mediators are an important component of a tumorous environment. Some types of cancer are related to inflammatory events that already occurred before the appearance of malignant changes, others are attributed to induce an inflammatory microenvironment that enhances their development. Regardless of their diverse origin, inflammatory events are present in most, if not all tumors and for this reason are hypothesized to be a seventh hallmark of cancer [29].

The presence of an inflammatory microenvironment within a tumor is thought to be established by two different mechanisms: an extrinsic pathway, driven by an inflammation that augments the risk of cancer and an intrinsic pathway, driven by genetic alterations that lead to inflammatory conditions and neoplasia. The extrinsic pathway is associated with an inflammation that has been caused by harmful stimuli such as pathogens, irritants or damaged cells. Certain anatomical sites like the colon, prostrate, pancreas, liver and lung mesothelium (see asbestosis chapter 2.4.) are characterized by a high risk to develop malignancies after chronic inflammation [30]. In contrast, the intrinsic pathway generates an inflammatory environment through cancer typical aberrations of oncogenes, tumor suppressor genes and the chromosomal integrity in tumors for which there is no underlying inflammatory condition (e.g. breast tumors). Hence, these facts raise the question why carcinogenesis in general desires an inflammatory environment. Both pathways lead to the activation of transcription factors - mainly nuclear factor-kb (NF-kb), hypoxia-inducible factor 1α (HIF1α) and signal transducer and activator of transcription 3 (STAT3) - which coordinate the production of inflammatory mediators like cytokines, chemokines and cyclooxygenase 2 (COX2) (mediator of prostaglandin production). What follows is the attraction of a diverse leukocyte population including neutrophils, dendritic cells, macrophages, mast cells and lymphocytes. There is evidence that each of these components can be involved in carcinogenesis and/or tumor invasion and metastasis because their recruitment is always associated with the release of extracellular proteases, pro-angiogenic factors and growth signals [31-34]. Normally an inflammation is a self-limiting process because pro-inflammatory signaling is closely followed by the release

of anti-inflammatory factors. If this balance is turned in favor of an abundant production of pro-inflammatory cytokines the level of released growth and angiogenic stimuli can potentiate the development of neoplasms [30]. Hence, an inflammatory environment is frequently recognized in tumorigenic tissues because it can constitute as second type of stimulation that promotes the uncontrolled growth of initiated cells.

On the other hand there is also growing evidence that a chronic inflammation can be the origin of a neoplastic transformation. Due to persistent infections or a disrupted termination of the inflammatory response leucocytes and other phagocytic cells exhibit a prolonged activity and keep releasing toxins such as reactive oxygen (ROS) and nitrogen species (RNS) that are normally produced to fight pathogens. These agents can react as mutagens, leading to DNA damage and reduced DNA repair. Thus, consistent repeated tissue damage and regeneration through healing in the presence of these species may result in permanently acquired genomic aberrations, the basis of cancer development.

Naturally the immune system has the assignment to protect the organism against diseases by identifying and killing pathogens and also tumor cells. Cancer can only occur by successful evading from immune surveillance which is performed through multiple pathways. Although the molecular mechanisms are not fully understood, a typical alteration of tumor cells are a reduced number of major histocompatibility complex class I (MHC class I) molecules on their surface, thus avoiding detection by cytotoxic T cells (CTLs) which represent the main defense against aberrant cells [35]. In addition, a failed second activation of CTLs, referred to as costimulation, may lead to an ineffective immune response. Tumor-associated macrophages (TAMs) are a significant component of inflammatory infiltrates in neoplastic tissues and appear as a double edged sword. Although they may kill tumor cells, TAMs also produce interleukin-10 (IL-10), a cytokine that down-regulates costimulatory molecules. The consequence is the development of an immunological tolerance against tumor antigens and hence evasion from immune surveillance [36]. Besides suppression of adaptive immunity, TAMs express a number of potent vascular endothelial growth factor (VEGFs), thereby facilitating angiogenesis and the development of lymphatic vessels. Further these factors contribute to the recruitment of monocytes which are the origin of

TAMs, thus enhancing their accumulation in neoplastic tissue. Since there is knowledge about the wide influence of TAMs and other parts of the immune system on carcinogenesis, this issue is suggested to be a point of application in cancer therapy.

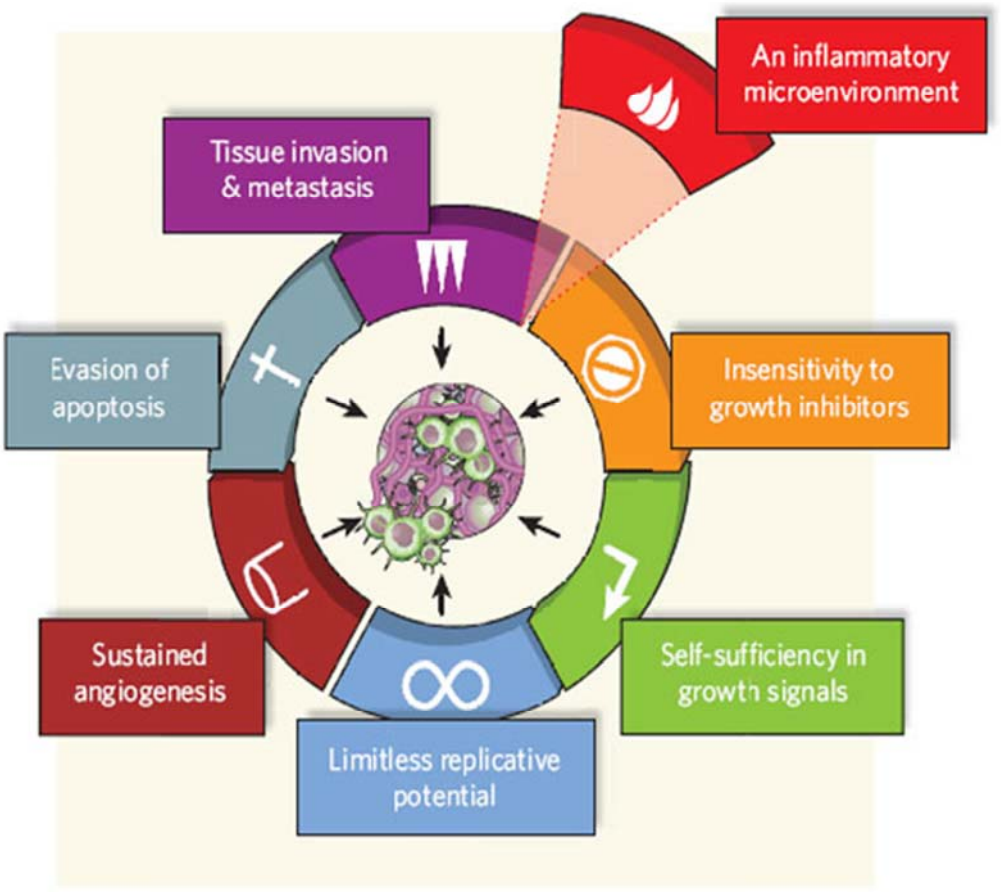


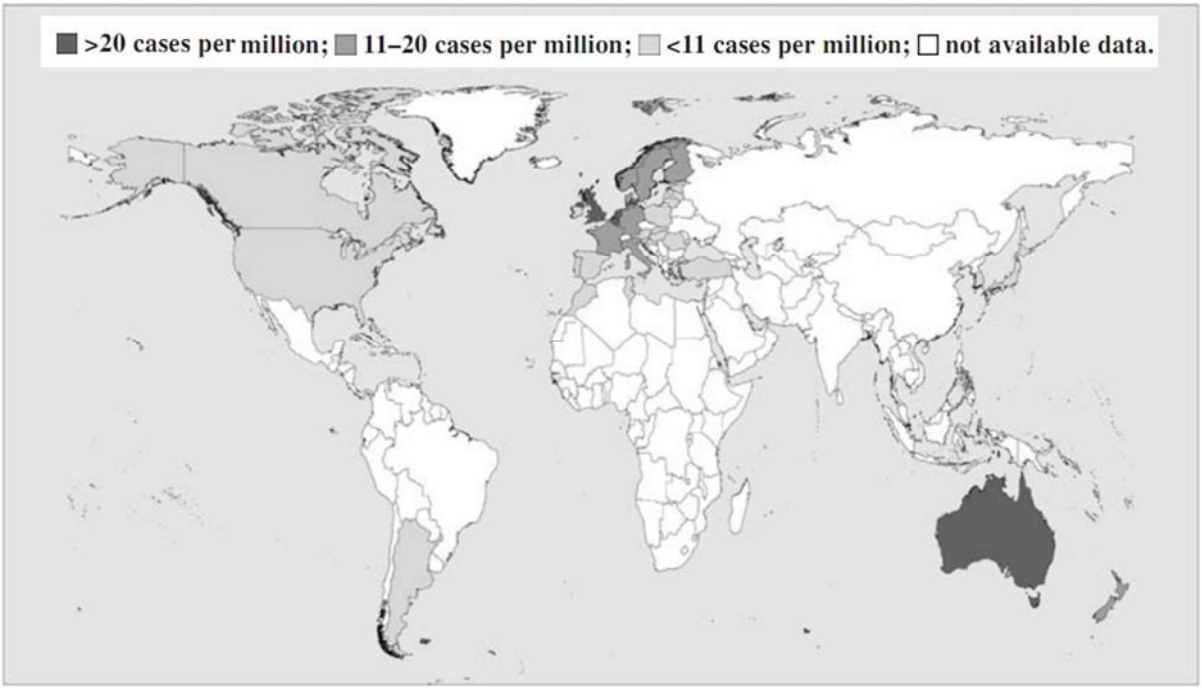

Figure 7. *Modified illustration of D. Hanahan's and R. Weinberg's six hallmarks of cancer by Mantovani et al. including cancer-related inflammation as an additional major attribute of cancer development [29]..*

## 2.4 Human Malignant Mesothelioma (HMM)

### 2.4.1 Epidemiology and risk factors

Human Malignant mesothelioma (HMM) is a highly lethal neoplasia that affects the lining of certain large serosal body cavities. In most cases mesothelioma is related to asbestos exposure although some studies support the role of radiation and the Simian Virus 40 (SV40) as causative factors [37-39]. HMM was an extremely rare disease until the second half of the 20$^{th}$ century. The incidence of this very aggressive type of cancer increased significantly and currently there are about 2.000 to 3.000 cases in the U.S. and approximately 10.000 worldwide per year [40].Two-thirds of HMM patients are between 50 to 70 years of age. Males are at a much higher risk for this cancer than females (70-80%), likely due to occupational asbestos exposure. Especially between the 40s and 80s in the U.S. and Europe, asbestos was widely used in the construction and shipbuilding industries due to its excellent material properties. The latency period between the time of initial exposure and diagnosis of HMM ranges from 20 to 50 years and reflects the significant rise of cases in the last decades. In Europe, the anticipated peak year of HMM incidence is in the period of 2015–2020, with a predicted incidence of 250,000 cases over the next 40 years [41].

The incidence of HMM shows noticeable variations from one country to another. The hot spots for mesothelioma often exactly correspond to the sites of industries with high asbestos use, although many countries which are anticipated for high incidences show an unexpected low HMM rate. Further on the one hand it has been proven that around 80% of patients with HMM in the U.S. have been exposed to asbestos. On the other hand epidemiologic studies propose that only 5% with heavy prolonged asbestos exposure develop mesothelioma. The reasons for this fact are not clear. It is assumed that the different technical approaches used to attribute asbestos exposure worldwide may be the principal reason for these discrepant results. However, geographic differences, genetic susceptibilities and different distribution of the SV40 between populations cannot be excluded as reason for this heterogeneity.




| Country | IR | Main source of the data | Country | IR | Main source of the data |
|---|---|---|---|---|---|
| Australia | 30 | Mesothelioma Registry | Romania | 6 | Researchers estimates |
| Great Britain | 30 | Mesothelioma Mortality Registry | Austria | 5.6* | Cancer Registry |
| Belgium | 29 | Researchers estimates | Poland | 4* | Mortality data |
| The Netherlands | 23* | Mortality data | Slovakia | 4 | Researchers estimates |
| Italy | 17* | Mortality data | Slovenia | 4 | Cancer Registry |
| Norway | 16* | Cancer Registry | Spain | 4* | Mortality data |
| New Zealand | 15 | Cancer Registry | Estonia | 3 | Researchers estimates |
| Denmark | 13 | Cancer Registry | Israel | 3 | Cancer Registry |
| Germany | 13 | Various | Latvia | 3 | Researchers estimates |
| Sweden | 12* | Cancer Registry | Lithuania | 3 | Researchers estimates |
| France | 10–13* | Mesothelioma Surveillance Program | Macedonia | 3 | Researchers estimates |
| Finland | >10* | Cancer Registry | Portugal | 2–3 | Researchers estimates |
| Canada | 9 | Cancer Registry | Argentina | 2.2* | Health Ministry Statistics |
| Cyprus | 9 | Researchers estimates | Singapore | 2 | Cancer Registry |
| United States | 9* | SEER Program | South Korea | 1–2 | Cancer Registry |
| Hungary | 8 | Mesothelioma Registry | Morocco | 0.7 | Researchers estimates |
| Turkey | 7.8 | Researchers estimates | Tunisia | 0.6 | Researchers estimates |
| Croatia | 7.4* | Cancer Registry | | | |
| Japan | 7 | Mortality data | | | |

IR = estimated annual crude incidence rates per million; * = pleural mesotheliomas only; †) = Christodoulides G, personal communication; ‡) = Barış Y, personal communication.

Figure 8. *Illustration of estimated annual crude incidence rates of human malignant mesothelioma in the world and some highlighted countries in the table below [40].*

### 2.4.2 Anatomical sites of HMM

Anatomically HMM is situated within the tunica serosa, a smooth membrane that lines and encloses several body cavities and organs [42]. Generally this lining consists of a secretory epithelial layer and connective tissue underneath. The latter can be divided into the lamina propria serosae which provides blood vessels and nerves and the tela subserosae that serves as fat depot. The squamous epithelial layer which is faced towards the body cavity produces the lubricating serous fluid that allows the smooth movement of enclosed organs. It originates from the mesodermal coelomic epithelium and is for this reason referred to as mesothelium. If cells within this layer become malignant, the developing tumor is termed as mesothelioma. Overall there are four different types of HMM referring to its localisation within the body:

I. The malignant pleural mesothelioma (MPM) affects the serous lining of the thoracic cavity, the two-layered pleura. The outer parietal pleura is attached to the chest wall and the inner visceral pleura covers the lung. The thin space between both layers is termed as pleural cavity containing pleural fluid that averts the lung from collapse. In the majority of cases neoplastic development arises in one of both layers and can spread into adjacent tissues. MPM is the most frequent type and affects approximately 70% of all patients [43].

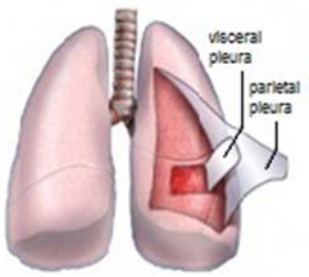



Figure 9. *Peritoneum [42].*

II. Malignant peritoneal mesothelioma affects the serous lining of the abdominal cavity, the peritoneum which is divided into a parietal and visceral layer like the pleura. The visceral peritoneum encloses most inner organs of the abdomen such as the liver, gallbladder, spleen, stomach and nearly all parts of the small and large intestine. Malignant peritoneal mesothelioma is by

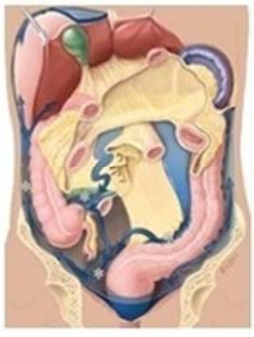


Figure 10. *Peritoneum[42].*

far less frequent than MPM and affects 10-20% of all HMM patients [44].

III. Pericardial mesothelioma affects the visceral (epicardial) and the parietal layer of the serous pericardium which contains the heart and the roots of the great vessels. Approximately the half of all heart tumors are pericardial mesotheliomas but only 1-6% of all HMM patients suffer from this type.

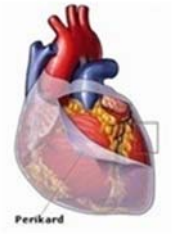



Figure 11. *Pericardium[42].*

IV. Testicular mesothelioma affects the two-layered serous tunica vaginalis which covers the testis. It represents with about 100 described cases the most uncommon type of HMM.

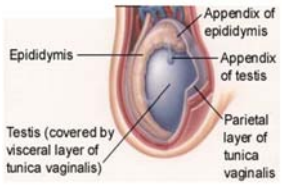



Figure 12. *Tunica vaginalis [42].*

### 2.4.3 Histological classification

Based on the diverse constitution of the pleural or peritoneal tissue, human malignant mesotheliomas can be classified into three histologic subtypes: epithelioid, sarcomatoid and biphasic (mixed epithelioid and sarcomatoid). The epithelioid subtype with an incidence of 50-60% is the most frequent, followed by biphasic with 20-40% and sarcomatoid with approximately 10% [45]. Several studies have correlated the specific subtypes with different survival rates proposing a better prognosis for patients with epithelioid mesothelioma [46, 47]. Within the malignant tissue the epithelioid subtype can show diverse histological characteristics such as tubulopapillary, microcystic and well-differentiated papillary structures with generally well-defined and uniform shaped cells. Due to its similarity to another form of cancer known as adenocarcinoma, the diagnostic process can be difficult for histopathologists. The sarcomatoid subtype is characterized by elongated spindeloid cells, which are typically irregular and not uniform in shape. A variety of this subtype represents the desmoplastic mesothelioma with bland fibroblastic like cells than sometimes can be misdiagnosed as benign fibrous

tissue. The biphasic mesothelioma is the mixture of more or less fractions from one subtype. To actually verify the biphasic form, it is important to collect larger tissue samples from different locations during biopsy.

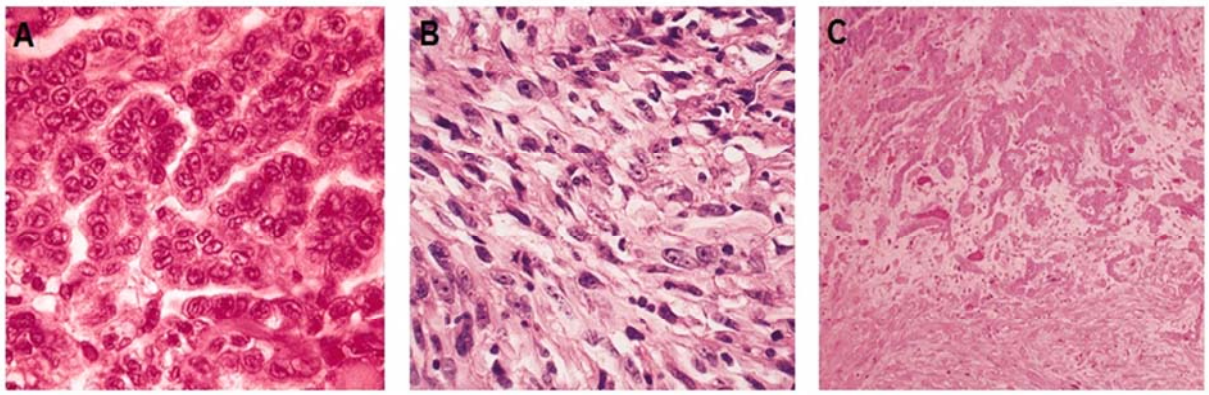



Figure 13. *Three histological HMM subtypes:* (A) *epithelioid*; (B) *sarcomatoid*; (C) *biphasic [46].*

### 2.4.4 The nature of asbestos and its impact on HMM

Asbestos (from Greek ἄσβεστος meaning "inextinguishable") is a collective name for a group of fibrous silicate minerals that share certain physical characteristics. Those include tensile strength, flexibility, sound absorption and its resistance to seawater, heat, electrical and chemical damage. Hence, asbestos, the "magic mineral", was especially used for various applications in the construction and shipbuilding industry but also e.g. for automobile brake pads, clutch discs, electric ovens and shoes.

Concerns about the hazardous effects on health of asbestos have been discerned early on. Already in 1943 lung cancer as a result of asbestos exposure was recognized as occupational disease but it took about 30 years when the first related products were banned. In 1987 the International Agency for Research on Cancer (IARC) has classified various types of asbestos fibres as human carcinogens (Group I) [38]. Since 1990 asbestos usage has been entirely forbidden in Austria and since 2005 in the whole European Union. Although safer materials have replaced many products that once were made of asbestos, a large number of countries still use, import and export asbestos and asbestos-containing products.

Generally there are six types of asbestos: amosite, crocidolite, anthophyllite, actinolite and tremolite, which belong to the group of amphibole minerals, also known as brown asbestos and chrysotile that belongs to the serpentine group. The latter is also known as white asbestos and has been the most commonly used type. Although the link between the development of HMM or lung cancer respectively after asbestos exposure is generally accepted, the relative carcinogenicity of the various fibre types and a dose response relationship remain controversial. Some studies showed that larger fibres, such as amphibole, rather accumulate in the lung tissue, while shorter fibres, mainly chrysotile, were found in the pleura, suggesting that the latter may be predominantly responsible for the development of HMM [48]. Although these results appear

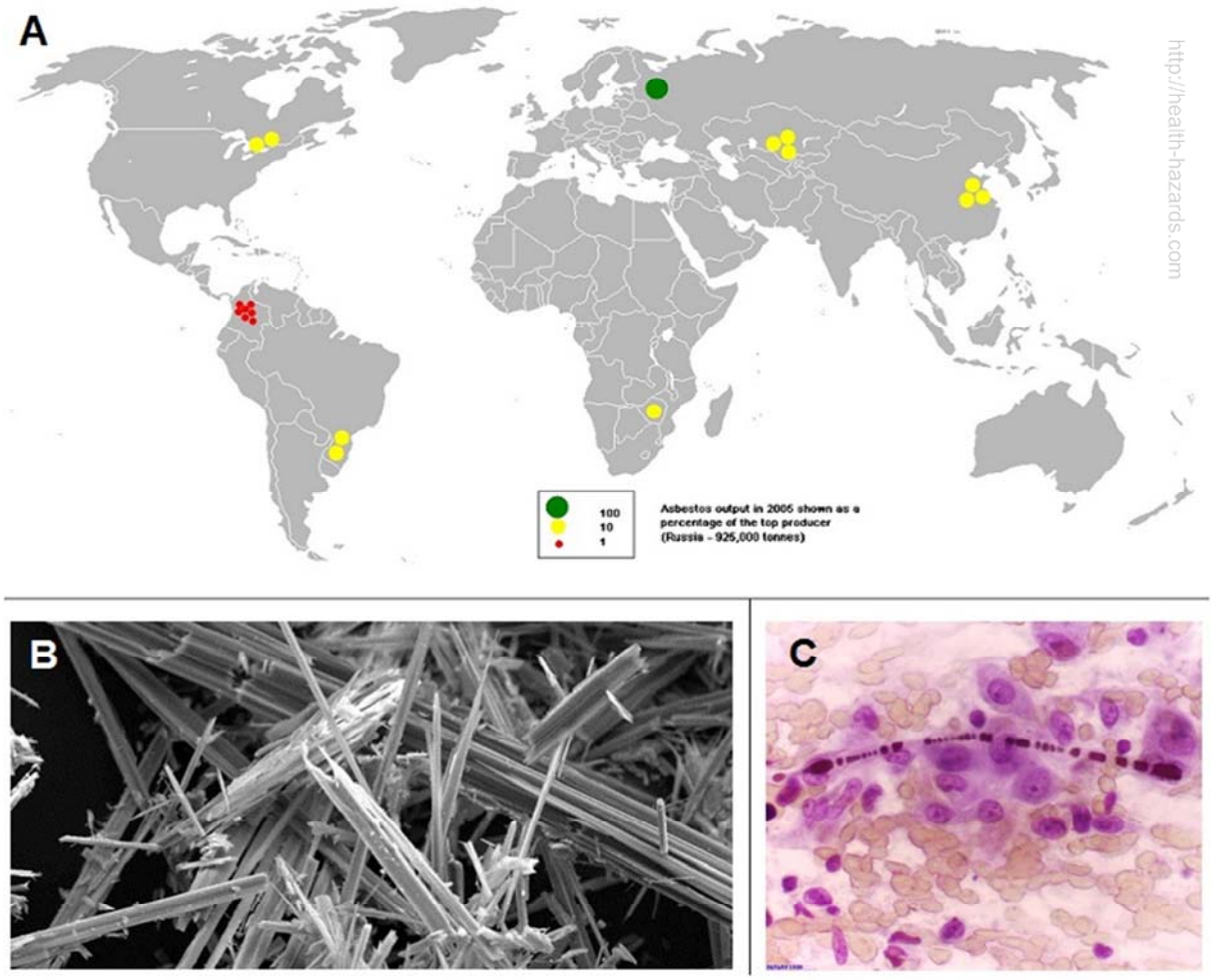



Figure 14. *(A) In 2006 the production of asbestos was estimated at 2.3 million tons, with approximately 40% of that being mined in Russia. Illustration shows further hot spots of asbestos production around the globe. (B) Asbestos Fibers using a Scanning Electron Microscope [38]. (C) Lodged asbestos fiber in a human lung [38].*

conclusive, to date there is not sufficient evidence to exactly associate certain fibre types with the development of cancer on specific sites [38]. Also the dose of asbestos required for a malignant outcome remains uncertain, not least because historical studies are based on interviews with patients which represent an unsatisfying source for scientific dose evaluations. Since the determination of asbestos exposure is based on different methods and inter-laboratory comparisons are unreliable because of heterogeneous procedures, controversial conclusions may remain.

The link between asbestos and HMM is highly supported by numerous animal studies. The majority of all in vivo experiments are based on the intrapleural and intraperitoneal injection of asbestos resulting in a dose dependent outcome of malignant pleural and peritoneal mesothelioma [49-51]. Although these injection experiments propose plausible results, they have been criticized because that way of exposure differs vehemently from the actual process. Hereby the most discussed issue is the bypassing of clearance and lung defence mechanisms leading to unrealistic heavy accumulations of asbestos in the pleura and peritoneum. For this reason also chronic inhalational experiments have been applied which today represent the best comprehensible in vivo studies on asbestos carcinogenicity. Depending on exposure duration and used species these experiments resulted in mesothelioma incidences from 1.2-20% [48, 52]. However, mesothelioma development was not significantly affected by the fibre type as it has been observed in injection studies. Regarding to dose response curves, all animal studies suggest that mesothelioma development is directly related to the amount of asbestos exposure and that tumors will not develop below a certain threshold level [53]. In contrast epidemiological studies in human do not support this observation [54].

#### 2.4.4.1 Molecular mechanisms of asbestos induced HMM development

The molecular mechanisms of asbestos pathogenicity are not fully understood. However, with the extensive work of in vitro and in vivo model systems direct and indirect mechanisms of asbestos induced mesothelioma have been proposed.

Direct mechanisms of asbestos related carcinogenesis can be generally separated into genotoxic and nongenotoxic pathways. The basic toxic characteristic of asbestos fibres relies on the appearance of reactive oxygen species (ROS) which are known as

high class mutagens. It is hypothesized that clearance mechanisms that try to solubilize inhaled fibres cause the release of asbestos containing metals such as iron. Those may serve as oxidation-reduction catalyst in a Fenton-type reaction to produce ROS [55]. Since amphiboles are not soluble, this theory may only apply to chrysotile. In fact, amphibole fibres are thought to serve as sites of precipitation within the tissue through their unfeasible removal. After a period of time the fibres become coated with iron-rich material and minerals trough mineral-fluid interactions. What follows is the formation of particles known as asbestos bodies or ferruginous bodies with radically changed surface characteristics of the fibres. Consequently this surface can serve as catalyst between fluid constituents changing their physical properties which may lead to an aberrant and dysfunctional fluid chemistry [38].

It has been shown that asbestos fibres can also mechanically interfere with the mitotic spindle, chromosomal segregation, and cytokinesis in cell culture [56-59]. Consequences are altered chromosome morphology and ploidy as well as produced DNA strand breaks. These in vitro studies have been confirmed by several animal experiments which showed increased mutation frequencies [60] and elevated levels of hydroxyl [61] and lipid radicals [62] in affected tissues, after asbestos injection or inhalation.

According to direct nongenotoxic effects after both, chronic and acute asbestos exposure, an increased proliferation of epithelial and mesothelial cells accompanied by activated growth factor receptors and intracellular signaling pathways have been generally observed [38]. This has been confirmed in human and rodent mesotheliomas which show a frequently constitutive expression and activation of growth-factor pathways including those of insulin-like growth factors (IGFs), platelet-derived growth factor (PDGF), vascular endothelial growth factor VEGF, and transforming growth factor beta (TGF-$\beta$) [38]. Responsible mechanisms remain uncertain. However, it is known that ROS-mediated injury and/or direct physical damage of cells through contact with asbestos fibers trigger apoptosis or necrosis. This cell death may be hyper-compensated by enhanced repair and cell proliferation within affected tissue. A continuous repeat of cell injury and repair may give rise to accumulation of mutations especially in the presence of a toxic environment. This could expand a preneoplastic

proliferating cell population during the early stages in the development of HMM [38].
Indirect mechanisms of asbestos carcinogenesis are believed to rely on a triggered chronic inflammation, a hallmark of cancer. The permanent inflammatory response is caused by persistent fibers within the tissue which are countered by activated macrophages through the release of ROS and NOS. This secondary induced toxicity may triggers mutations within proliferating cells and enhances the development of HMM [63]. Furthermore, epigenetic silencing of tumor suppressor genes after asbestos exposure has been described in HMM [64-66]. This is supported by the fact that ROS have also been proposed to contribute to altered DNA methylation [67, 68].
Several cofactors with asbestos fibers in the induction of HMM such as tobacco smoke, ozone inhalation and the SV40 are still subject of controversial discussions. Acquired genomic aberrations especially of key genes involved in DNA repair after asbestos exposure may facilitate accumulation of further mutations by tobacco-smoke carcinogens [38]. Ozone inhalation was at least shown to impair clearance and increase retention of asbestos fibers in the lungs of rats [38].

### 2.4.5 Possible relations of SV40 and HMM

The Simian virus 40 (SV40) is a DNA virus that has been assigned to processes in cancer development especially of HMM. It belongs to the family of polyomavirus and is thought to be endogenous in rhesus monkeys while epidemiological studies have shown that it is now widespread among the human population. A possible transfer from monkey to human may has occurred between 1955 and 1963 when contaminated polio vaccines were applied to millions of people [69]. Also the contagiously transmission by horizontal infection through hematic, sexual, and orofecal routes is suggested by epidemiological evidence [69].
The mechanisms of SV40 tumorigenesis in human are only partially understood. Generally it has been shown that SV40 expresses two viral oncoproteins, the large T antigen (Tag; 90kDa) and the small t antigen (tag; 17kDa) which are able to block certain proteins involved in cell cycle regulation. Tag is capable of binding to the major tumor suppressor proteins p53 and Rb and consequently inhibits their regulatory function of inducing apoptosis and/or cell cycle arrest when a cell is damaged. More

than 50% of all human tumors contain a mutation or deletion of the p53 encoding gene *TP53* [70] verifying its enormous importance in preventing from cancer. Furthermore, Tag was shown to be responsible for chromosomal aberrations in host cells and also induces insulin like growth factor (IGF), a hormone that stimulates cell proliferation [71]. The main function of the small tag is the inhibition of the protein phosphatase PP2A whose major targets belong to signaling cascades such as Raf, MEK, AKT and Wnt which are frequently deregulated in proliferating malignant cells.

The link between SV40 and development of HMM has been a particularly controversial area of research because in this point opinions range from strong to no evidence for a causal relationship. Several studies detected SV40 viral DNA sequences in up to 80% of HMM [71] but this findings have been partly refuted based on technical concerns by some investigators. Upon others López-Ríos et al. showed evidence against a role for SV40 infection in HMM and claimed high risk of false-positive PCR results because of the presence of SV40 sequences in common laboratory plasmids [72]. Also a cross-reactivity of serologic tests with related viruses such as BKV (Human Polyomavirus 1) and JCV (Human Polyomavirus 2) is discussed to produce false positive results. These viruses also belong to the family of polyomavirus and are very common in the general population, infecting 70–90% of humans but do not cause disease in immune-competent individuals. Recently development of more sensitive tests which are based on RT-PCR assays that are specific for SV40-encoded microRNAs (miRNAs) were unable to identify an expression of these markers in HMM samples [73]. However, some in vitro and in vivo experiments propose an association between SV40 exposure and HMM development. Human mesothelial cells were shown to have a different susceptibility to infections with JCV, BKV and SV40. While JCV did not infect the targeted cells, BKV caused mesothelial cell lysis. Only the infection of SV40 resulted in high rates of malignant transformed mesothelial cells indicating its actual presence in former analyzed HMM specimen rather than the ubiquitous human JCV and BKV [74]. In animals, SV40 was shown to be a strong carcinogen causing the development of mesotheliomas in nearly 100% of hamsters following intrapleural injection or 60% following intracardial exposure respectively [75]. In contrast, transgenic mice that express SV40 Tag in the mesothelial compartment showed a relatively low level of

spontaneous tumor development. Interestingly, when these mice were exposed to asbestos they rapidly developed mesotheliomas which were faster growing and more invasive than asbestos induced tumors in wild-type mice. Additionally in vitro analyses demonstrated that SV40-transfected human mesothelial cells exposed to asbestos maintain a malignant transformed phenotype while wild-type cells died rapidly due to asbestos cytotoxicity [76]. These data indicate a co-carcinogenicity between SV40 and asbestos in the development of mesotheliomas at least in in vitro and in vivo experiments. However, these results are not sufficient to prove causality. Further epidemiological studies and analyses of tissue specimen have to be performed to give deeper insight into the role of SV40 in HMM.

### 2.4.6 Diagnosis of HMM

In case of a clinical suspicion of thoracic/pleural HMM, the endoscopic examination of thorax (thoracoscopy) or peritoneum (laparoscopy), respectively, with an attendant biopsy represents the most important procedure to prove diagnosis. Modern imaging via computed tomography (CT), magnetic resonance tomography (MRT) and/or positron emission tomography (PET) is also a common part of the diagnostic process [77-79]. The mode of subsequent therapy depends on the specific parameters given by examination. The progress level of MPM is staged referring to the commonly used TNM classification of malignant tumors [80]. This system describes the size of tumor (T) and whether it has invaded nearby tissue/organs, the involvement of nearby lymph nodes (N) and the appearance of distant metastases (Tab.2). Today there is no standard staging system for peritoneal mesothelioma or other types of HMM.

Table 2. *International TNM staging system for MPM [80].*

**(T) Tumor**

| | |
|---|---|
| T1a | Tumor limited to the ipsilateral parietal pleura, including mediastinal and diaphragmatic pleura<br>No involvement of the visceral pleura |
| T1b | Tumor involving the ipsilateral parietal pleura, including mediastinal and diaphragmatic pleura<br>Scattered foci of tumor also involving the visceral pleura |
| T2 | Tumor involving each of the ipsilateral pleural surfaces (parietal, mediastinal, and diaphragmatic, and visceral)<br>Involvement of diaphragmatic muscle<br>Confluent visceral pleural tumor (including the fissures) or extension of tumor from visceral pleura into the underlying pulmonary parenchyma |
| T3 | Locally advanced but potentially resectable tumor; tumor involving all of the ipsilateral pleural surfaces (parietal, mediastinal, diaphragmatic, and visceral)<br>Involvement of the endothoracic fascia<br>Extension of tumor into mediastinal fat<br>Solitary, completely resectable focus of tumor extending into the soft tissues of the chest wall<br>Nontransmural involvement of the pericardium |
| T4 | Locally advanced technically unresectable tumor; tumor involving all of the ipsilateral pleural surfaces (parietal, mediastinal, diaphragmatic, and visceral)<br>Diffuse extension or multifocal masses of tumor in the chest wall, with or without associated rib destruction<br>Direct transdiaphragmatic extension of tumor to the peritoneum<br>Direct extension of tumor to one or more mediastinal organs<br>Direct extension of tumor into the spine<br>Tumor extending through to the internal surface of the pericardium with or without a pericardial effusion; or tumor involving the myocardium |

**(N) Lymph nodes**

| | |
|---|---|
| Nx | Regional lymph nodes cannot be assessed |
| N0 | No regional lymph node metastases |
| N1 | Metastases in the ipsilateral bronchopulmonary or hilar lymph nodes |
| N2 | Metastases in subcarinal or ipsilateral mediastinal lymph nodes, including ipsilateral internal mammary nodes |
| N3 | Metastases in contralateral mediastinal, contralateral internal mammary, and ipsilateral or contralateral supraclavicular lymph nodes |

**(M) Metastasis**

| | |
|---|---|
| Mx | Distant metastasis not assessable |
| M0 | No distant metastasis |
| M1 | Distant metastasis present |

**Stage**

| | | |
|---|---|---|
| Stage I | | |
| | 1a | T1aN0M0 |
| | 1b | T1bN0M0 |
| Stage II | | T2N0M0 |
| Stage III | | Any T3M0 |
| | | Any N1M0 |
| | | Any N2M0 |
| Stage IV | | Any T4 |
| | | Any N3 |
| | | Any M1 |

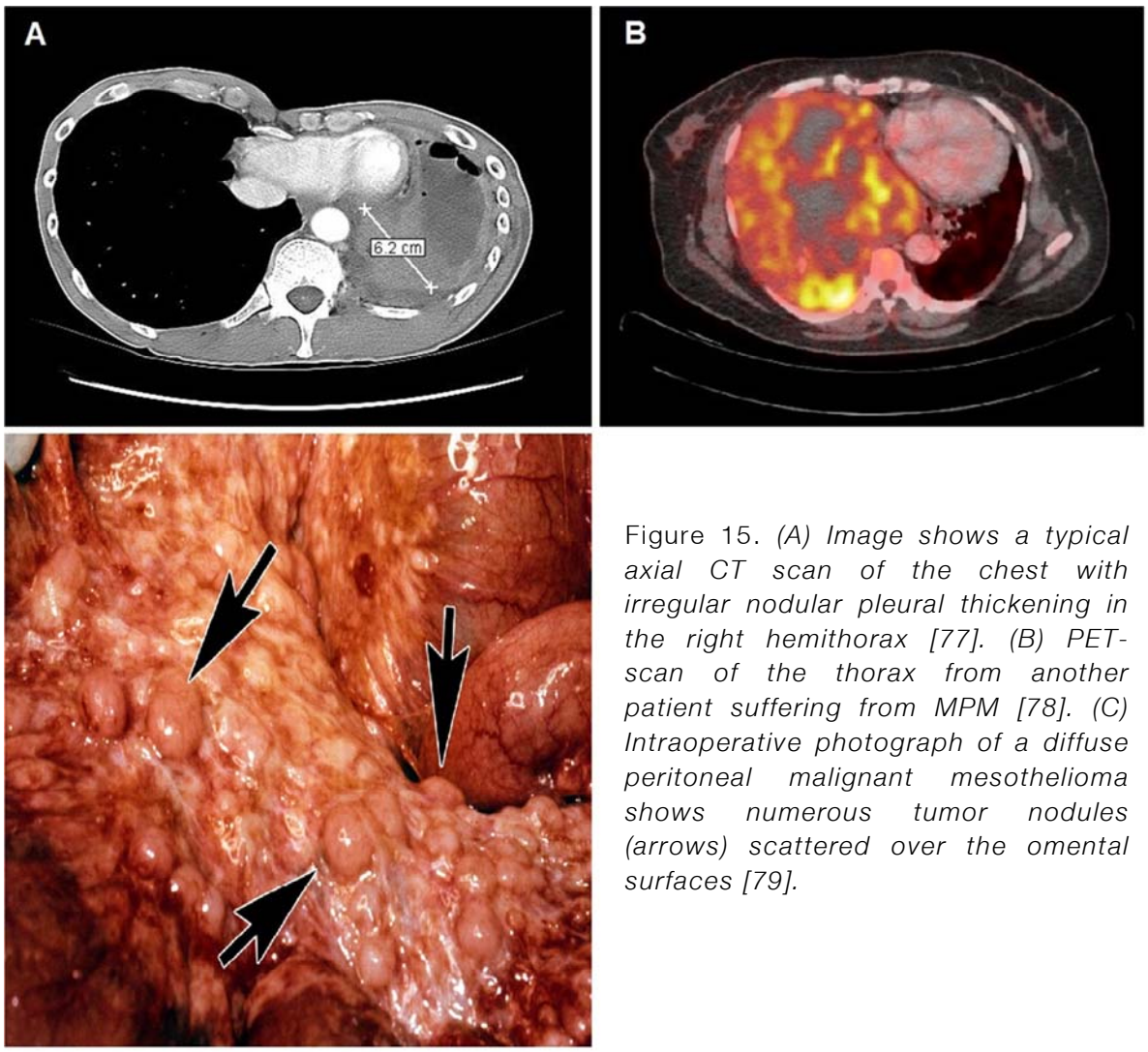



Figure 15. *(A) Image shows a typical axial CT scan of the chest with irregular nodular pleural thickening in the right hemithorax [77]. (B) PET-scan of the thorax from another patient suffering from MPM [78]. (C) Intraoperative photograph of a diffuse peritoneal malignant mesothelioma shows numerous tumor nodules (arrows) scattered over the omental surfaces [79].*

### 2.4.7 Etiopathology and prognosis of HMM

In the majority of cases HMM is diagnosed at progressed stage because of very unspecific symptoms like shortness of breath (dyspnea), cough and abdominal or thoracic pain. Unfortunately these symptoms do not occur until a relatively advanced tumor progression and thus most cases of HMM are primary diagnosed at stage II. For this reason mostly only palliative therapy with the objectives of prolonging lifetime and improving quality of life can be offered. Only few patients are considered of surgical tumor resection with a curative intention. Although this collective features a better prognosis, the five-year survival rate of this patient group lies below 15% in case of MPM [81]. The median survival time of patients with adverse prognosis lies between five and eight months, those with favorable prognostic parameters survive between ten and eighteen months in case of MPM [82]. Normally respiratory insufficiency and secondary pneumonia are the actual causes of death. Patients diagnosed with malignant peritoneal mesothelioma obtain comparable median survival rates with approximately one year [83]. These poor prospects show that the development of new and more efficient treatment options is urgently needed.

### 2.4.8 Current therapy of HMM

Due to the prevalently multifocal and diffuse expansion of HMM only very extensive surgical resections result in a detectable survival improvement. However, in the last years the perioperative mortality dropped under 10% because of more advanced surgical techniques and post-surgical treatment in specialized clinics [84]. To improve therapeutic success multimodal therapies are always preferable to sole surgery. At the moment only the combination of resection, chemo- and radiotherapy provides the best possible way to enhance survival of MPM patients. In case of malignant peritoneal mesothelioma cytoreductive surgery (CRS) combined with perioperative intraperitoneal chemotherapy (PIC) is proposed to be the best standard of care to this date [85].

### 2.4.8.1 Surgery

The goal of surgery is to provide a macroscopic resection of all grossly visible tumors as completely as possible. In case of MPM two different operative techniques have evolved: extrapleural pneumonectomy (EPP) and pleurectomy/decortication (P/D). EPP involves the removal of the affected parietal and visceral pleura, the complete underlying lung, parts of the diaphragm and pericardium. P/D represents a less extensive surgery in which the lung is left in place and only parietal and visceral pleura are resected [86]. Within both methods the removed linings are replaced by prosthetic material (usually Gore-Tex™) granting a breathable and protective compensation.

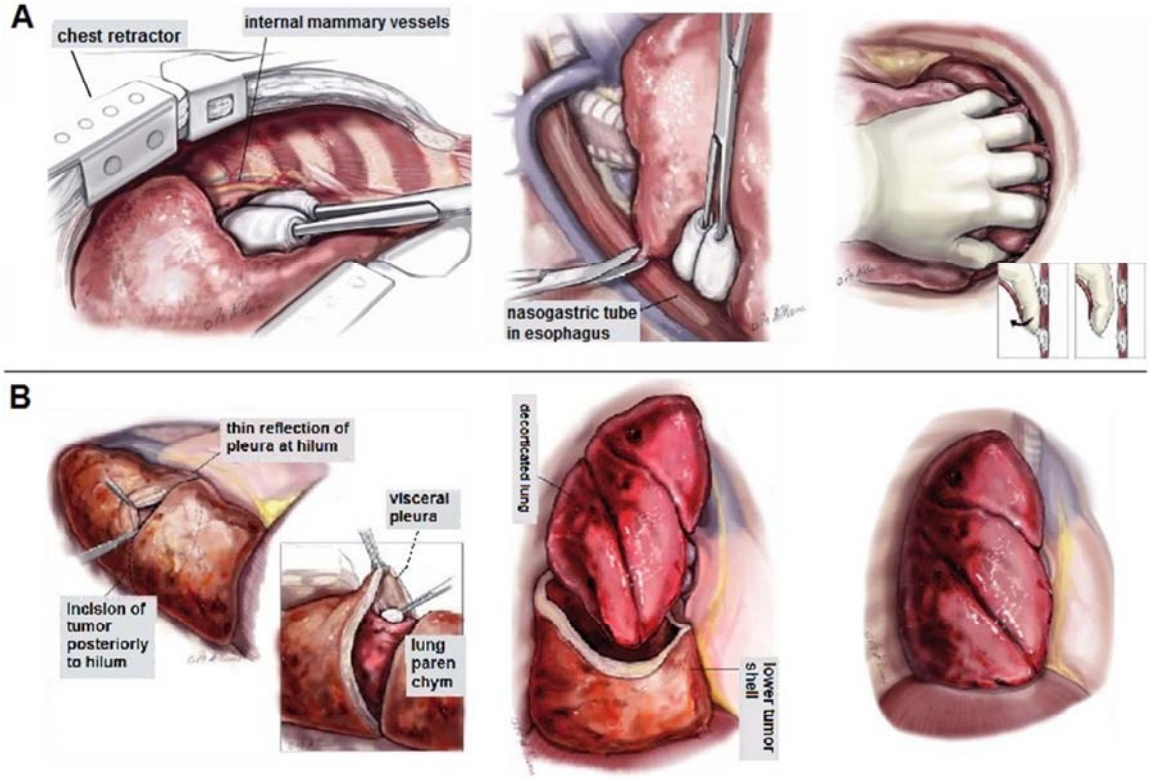



Figure 16. *(A) Extrapleural pneumonectomy: First chest retractors are placed to increase access to the initial exposure of the tumor. A nasogastric tube is inserted in the esophagus to prevent injury while the tumor is being resected off the esophagus. Then the diaphragm is avulsed or bluntly separated from the chest wall muscular attachments. (B) Pleurectomy/decortication: The second type of surgical intervention is accomplished in two stages. First, the tumor is incised posteriorly to the hilum and a plane is developed between the visceral pleura and the underlying lung parenchyma. The fully decorticated lung is retracted out of the lower tumor shell and removed from the operative field. In this case the diaphragm stays intact [85].*

The choice of applied technique relies on the tumor characteristics, whether the patient can tolerate the surgery and which procedure achieves a macroscopically complete resection. In case of malignant peritoneal mesothelioma which represents a relatively rare clinical entity, there is no standardized surgical procedure at the moment. Operations for this type of HMM are generally based on an extensive tumor debulking reducing all foci to a microscopic level. An accompanying complication of such operations in all cases may be a widespread hematogenous or lymphangogenic dissemination of microscopic tumors [85]. Additionally in some parts of the chest it is not possible to remove the neoplastic tissue including sufficient surgical margins. The frequent consequence is a recurrence of the tumor at local sites or even at former uninvolved areas within the body. Thus, the treatment of microscopic residuals with adjuvant therapy is essential.

#### 2.4.8.2 Radiation therapy

The principal mechanism of radiation therapy relies on the induction of DNA lesions in cancer cells which generally are characterized a deficiency in error correcting mechanisms. Ionizing high-energy rays generate toxic ROS in the focused area and lead to inhibition of cell-cycle progression mainly induced by DNA double strand brakes. Radical radiation therapy shows excellent benefits in certain cancer types [87, 88], but unfortunately not in the treatment of MPM and malignant peritoneal mesothelioma. Due to critical organs such as the lung, heart, esophagus and liver in the hemithorax, the administered total dose of radiation is limited. Several studies have shown that hemithoracic irradiation alone results in significant toxicity leading to hazardous inflammations of affected tissues without any survival benefit [89]. For this reason, currently there is no evidence to support the use of radical radiation therapy alone, administered with curative intent, in the management of patients with MPM [89, 90]. Hence, this kind of antineoplastic treatment is only applied within a multimodal therapy regimen as perioperative option to prevent fast relapse of microscopic tumor residues or in fewer cases as palliative care. Due to hardly any benefits of radiation therapy on malignant peritoneal mesothelioma patients, this treatment option is generally not applied in this case [91].

### 2.4.8.3 Chemotherapy

With respect to disseminated cancer, chemotherapy in general is the cytotoxic or cytostatic treatment of tumors using chemicals which mostly work by impairing mitosis or inducing apoptosis in fast dividing cells. As cancer cells reveal increased proliferative rates and deficiency in error correcting mechanisms, they show a higher susceptibility to such agents than healthy cells. Some types of neoplastic disease show an excellent response to chemotherapeutic treatment regimens, especially in early stages, and are able to enhance the chances of cure [92-94]. Unfortunately, HMM exhibits only a moderate chemo-sensitivity. Older chemotherapeutic agents such as cisplatin and adriblastin only achieved response rates below 20% [95]. In the last years, newer drugs such as pemetrexed, gemcitabine, vinorelbine and oxaliplatin extended the lineup of agents and improved tolerability but did not show significantly increased response rates in monotherapy regimen. Within a randomized phase III trial including a cohort of 456 patients the combined treatment with cisplatin and pemetrexed showed a significant advancement compared to cisplatin monotherapy referring to response rate (41.3% versus 16.7%) and median survival (12.1 months versus 9.3 months) [96]. Currently this combined treatment represents the standard chemotherapy for patients in good general condition.

The cytotoxic effects of cisplatin (cis-diamminedichloroplatinum(II)) was already discovered in 1965 by Barnett Rosenberg when he noticed that electrolysis of a platinum electrode produces cisplatin which inhibits binary fission in E.coli by disrupting cell division [97]. Generally it is accepted that the effects of cisplatin arise from binding the DNA causing crosslink adducts that on the one hand interfere with DNA synthesis during mitosis and on the other hand trigger apoptosis due to blocked DNA repair [98].

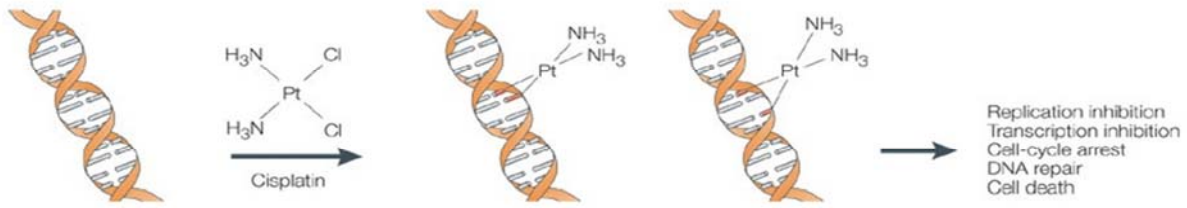



Figure 17. *Formation and effects of cisplatin adducts. The platinum atom of cisplatin binds covalently to purines to form intra- and interstrand crosslinks [97].*

Recently it has been shown that the mechanisms involved in the cytotoxicity of cisplatin are much more complex than DNA damage alone. It is proposed that cisplatin can directly interact with regulatory proteins [99] and the plasma membrane [100] leading to the dysfunction of certain signaling pathways. Moreover there is evidence that cisplatin induces the formation of ROS representing an additional trigger of cell death [101].

In the majority of cases chemotherapeutic drugs are systemically applied and pass several body tissues. For this reason also cells that divide rapidly under normal

Table 3. *The primary molecular mechanisms involved in cisplatin-resistance [100].*

| Molecular mechanisms | Consequence |
|---|---|
| Decreased drug uptake (e.g. downregulation of copper transporter CTR1) | Reduced intracellular platinum accumulation |
| Increased drug efflux (e.g. overexpression of cMOAT and copper transporters: ATP7A, ATP7B) | |
| Increased level of GSH and increased activity of GSH-related enzymes | Reduced bioavailability of platinum |
| Increased level of metallothioneins | |
| Alterations in mismatch repair | Increased DNA repair capacity and/or increased capacity to replicate through adducts |
| Alterations in nucleotide excision repair | |
| Alterations in anti-apoptotic factors (e.g., BCL-2 and IAPs) | Failed induction of cell death |
| Alterations in pro-apoptotic factors (e.g., p53, caspases, Fas, BAX, and BAK) | |
| Alterations in the MAPK cascade pathway | |
| Alterations in the PI3-K/Akt signaling pathway | |
| Alterations in transcription factors | |
| Alterations in cell cycle–related factors | Varies depending on the altered factor(s) |

circumstances as for example in the bone marrow, digestive tract and hair follicles, are harmfully affected. Hence, cisplatin and other related chemotherapeutic agents cause severe side effects. Another application limiting factor represents intrinsic and acquired resistance leading to a low responsiveness or tumor relapse, a fact that is frequently observed in HMM treatment [102]. Several molecular mechanisms that can be activated by tumor cells to survive cisplatin treatment have been described (Tab.3). Upon other, upregulated expression of efflux transporters and/or decreased level of uptake activity are proposed to be mainly responsible for a reduced intracellular platinum accumulation causing the ineffectivity of chemotherapy. Furthermore cisplatin-resistant tumors are suggested to obtain increased DNA repair and tolerance of DNA-damage which may be responsible to finally overcome cell death.

For the treatment of HMM the chemotherapeutic antifolate pemetrexed is generally recommended to be applied with cisplatin, as the combination showed more efficiency than either drug alone [96]. Since folic acids are essential for the biosynthesis of certain nucleotides and methyl groups, their functional inhibition consequently impairs DNA and RNA synthesis as well as DNA repair. Like most other antifolates, pemetrexed is a folic acid analogue that is able to bind folate-metabolizing enzymes which normally progress the formation of precursor purine and pyrimidine nucleotides. Because replicating cells depend on the supply of these basic DNA units, pemetrexed is cytotoxic during the S-phase of cell cycle especially in fast dividing cancer cells [103]. However, like in the case of cisplatin also normal cells are toxically affected leading to their dysfunction or apoptosis and causing adverse reactions. To some extent these

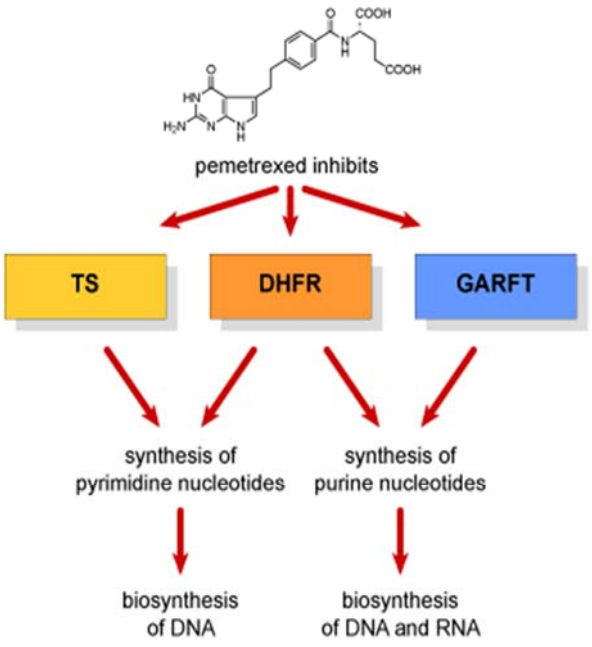



Figure 18. *Pemetrexed inhibits multiple targets involved in both pyrimidine and purine synthesis [102].*

side effects can be attenuated by high folate diets and supplemental folic acid. Although pemetrexed and other antifolates can lead to neoplastic cell death their long term effectiveness is diminished by cellular response. Over a period of time cells can compensate the blocked enzymes by e.g. increasing their expression and thus acquiring resistance to pemetrexed [104]. Some examples of molecular alterations concerning acquired pemetrexed-resistant are listed in Table 4.

Table 4. *Molecular alterations involved in pemetrexed-resistance [103].*

| Molecular alteration | Example |
|---|---|
| Qualitative and/or quantitative alterations in influx and/or efflux transporters of (anti)folates | inactivating mutations and/or down-regulation of reduced folate carrier (**RFC**) which is predominantly responsible for the cellular uptake of pemetrexed |
| Qualitative and/or quantitative alterations of folate-dependent enzymes | the target enzymes dihydrofolate reductase (**DHFR**), thymidylate synthase (**TS**) and folylpoly- $\gamma$ -glutamate synthetase (**FPGS**) |
| Overexpression of multidrug resistance proteins which may cause resistance to other anticancer drugs | Active efflux of drugs and cellular toxins by members of the ATP-binding cassette (ABC) transporters including P-glycoprotein (**Pgp/ABCB1**), multidrug resistance proteins (**MRPs/ABCC**) as well as breast cancer resistance protein (**BCRP/ABCG2**) |

## 2.5 Targeted therapy

Although in the last decades the research on cancer beard advanced treatment strategies it is generally accepted that current therapeutic options, which are still predominantly based on chemotherapeutic agents, are limited. For this reason a large field of contemporary research focuses on investigative attributes unique for cancer cells that my present key alterations of the malignant phenotype. This novel strategy of cancer treatment is generally termed “targeted therapy”, which is based on the interference with critical molecular pathways of tumors to inhibit their growth and force their demise [105]. In contrast to conventional chemotherapy this strategy is believed to be more effective against the actual source of malignancy attaining a better outcome for patients while reducing severe side effects. Experiences showed that most types of cancer are characterized by sets of different molecular changes responsible for their development and progression. For this reason one of the major challenges is to reveal essential, cancer type-specific altered signal pathways, to gain more inside into their role in malignant progression and to find compounds that directly target involved molecules.

By now two main categories of targeted therapeutics have been developed, monoclonal antibodies and small molecule inhibitors. The therapeutic effect of monoclonal antibodies is based on their binding to specific cell surface molecules or extracellular receptor ligands. In addition to target inhibition, monoclonal antibodies are able to induce

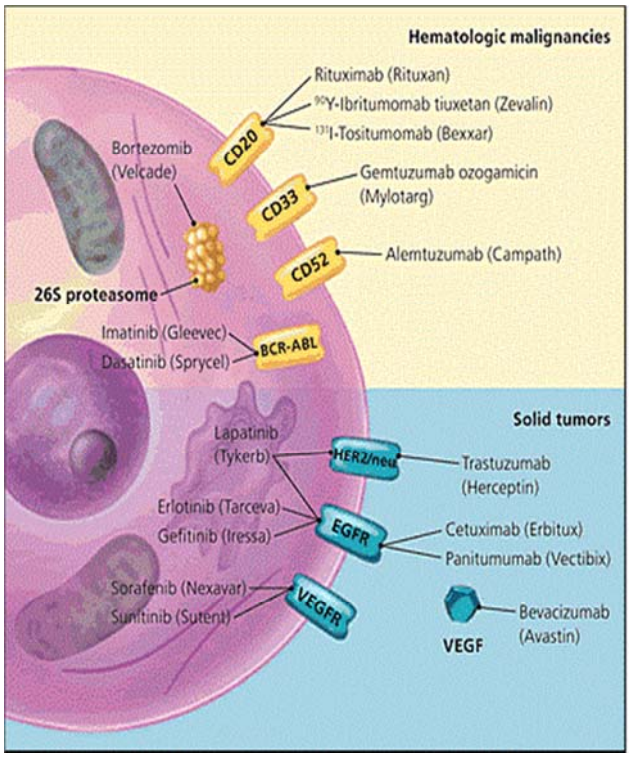



Figure 19. *The illustration shows a variety of monoclonal antibodies and their specific targets on the cell surface. Their field of application can be generally separated into two distinct classes of cancer: hematologic malignancies and solid tumors [104].* .

a mechanism referred to as antibody-dependent cell-mediated cytotoxicity (ADCC). This part of the adaptive immune response is mediated by effector cells of the immune system such as natural killer (NK) cells, neutrophils and eosinophils which actively lyse target cells that in this case have been labeled by monoclonal antibodies. Although this strategy is still under development, few drugs have already been licensed for clinical use (Fig.19) due to their enormous benefits to the treatment of some neoplastic diseases [106-110]. The second main group of agents is referred to as small molecules, a very general term in the fields of pharmacology and biochemistry for drugs that are able to rapidly diffuse across cell membranes and take effect on intracellular

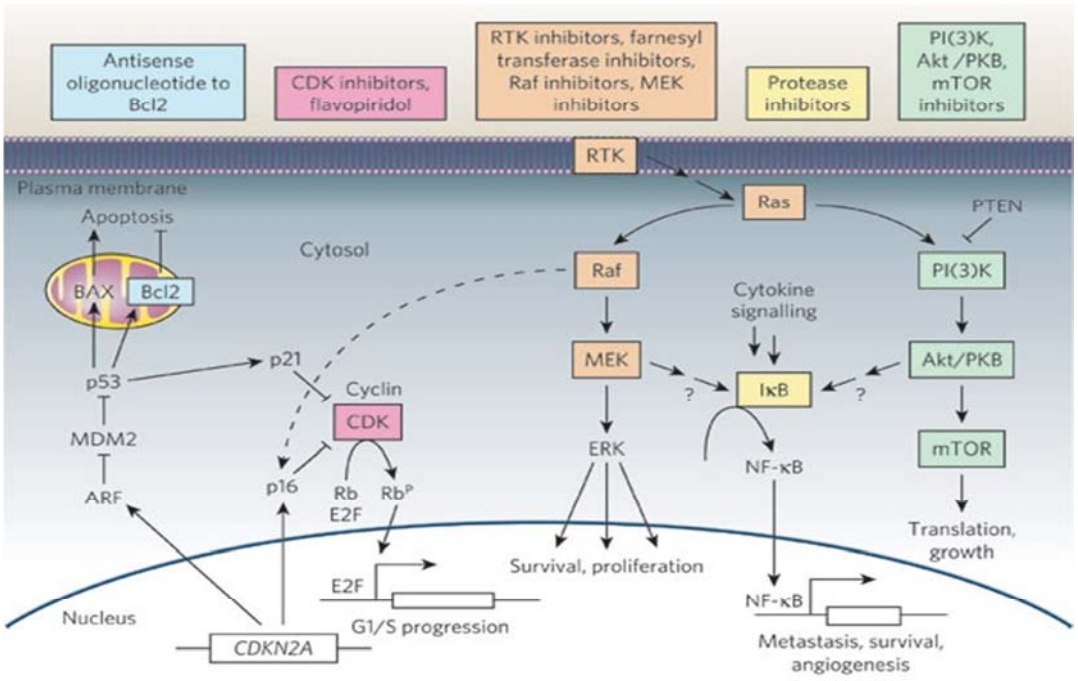



Figure 20. *Overview of different molecular targets of small molecules and the involved signaling pathways hit by targeted therapy [104].*

key players. Generally their aim is to interfere with aberrant signal transduction that leads to uncontrolled cell proliferation followed by metastasis, apoptosis resistance and/or tumor associated angiogenesis [111]. As attained chemotherapy resistance of cancer cells is also maintained by aberrant signaling pathways, targeted therapy may also be used to restore sensitivity to certain chemotherapeutical agents. Actually some targeted compounds are already in clinical use showing enhanced respond rates, less

side effects and prolonged survival of patients [112-114]. However, their field of application is limited only to certain malignancies and the effectiveness shows a broad variation. One of the main reasons for this issue is the still only superficial knowledge on key carcinogenic players and most notably their interactions with networking pathways. Thus the inhibition of one target may lead to the reinforcement of other signaling cascades allowing survival of cancer cells. Thus, a better understanding of reactions triggered by the interference with one targeted molecule may lead to improved therapeutical success. Further, patients with the same malignant disease might harbor tumors with different genetic mutations. Consequently inhibition of a specific molecule may lead to an enormous benefit for one patient but show only marginal effects for another due to the insignificance of the targeted pathway in this tumor. For example patients with chronic myelogenous leukemia (CML) show a dramatically improved survival after treatment with the small molecule imatinib when they were positive for Philadelphia chromosome [115]. Here the therapy relies on the inhibition of a critical molecular driver, the bcr-abl protein which is constitutively activated in 95% of all patients. Unfortunately cases with such a define target are rare especially in solid tumors. Nevertheless, this phenomenon, called “oncogene addiction” may be also present in other malignancies. Hence, their identification will be one future focus of targeted therapies, although may be combined treatment strategies will be required to prevent the escape of tumors from this “subjection”.

### 2.5.1 mTOR and its role in normal cells

During the last years an increasing number of molecular pathways which are strongly involved in oncologic signaling have been revealed. One of the most important represents the PI3K (phosphatidylinositol 3-kinase) pathway which is intracellularily mediated by AKT-kinase, one of the main activators of mTOR. In normal cells the

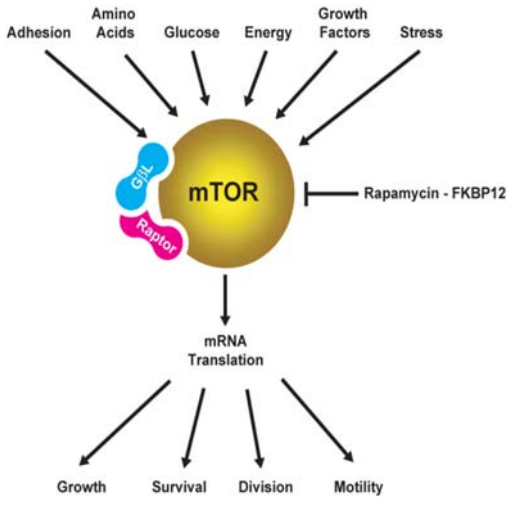



Figure 21. *Overview of multiple factors affecting mTOR and the resulting cellular responses [115].*

mammalian target of rapamycin (mTOR) is a kinase that acts as biological switch and helps to mediate an adequate response to changes of the cellular environment. It is linked to the upstream PI3K pathway that senses nutrient and energy levels, growth-regulating factors and cellular stress. mTOR mediates these signals as a master operator that regulates cell proliferation, cell survival, protein synthesis, and angiogenesis. In this manner, mTOR on the one hand forces anabolic processes and on the other hand prevents cells from proceeding when certain factors are not sufficient to support the effort [116].

### 2.5.2 Control mechanisms of mTOR

Reflecting the multiple roles of mTOR, its regulation is necessarily complex and involves several well-defined factors whose interaction with other molecules and signal pathways are still not fully understood. According to the current model, the main mTOR regulatory molecules include the tuberous sclerosis complex TSC1 (hamartin) and TSC2 (tuberin), which are able to form a heterodimer that inhibits mTOR activity [117].

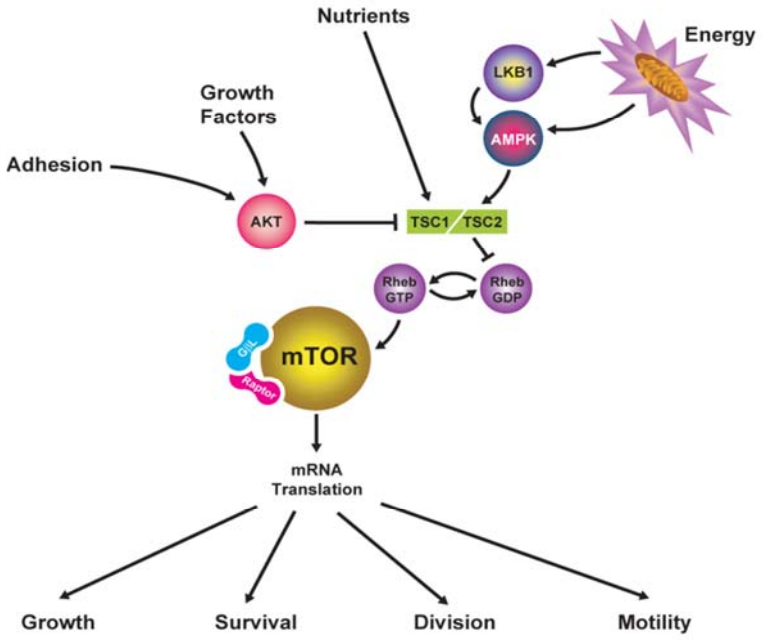



Figure 22. *Activation of mTOR. The GTPase Rheb activates mTOR and is in turn controlled by TSC1 and TSC2. The interaction of TSC proteins with Rheb is affected by signals from growth factors, amino acids, adhesion proteins and energy levels. AMPK is activated by an increase in AMP and activates TSC2, which inhibits Rheb. LKB1 additionally promotes the activation of AMPK in turn of low energy levels [115].*

When AKT-kinase, which is indirectly stimulated by growth factors, phosphorylates TSC2, the TSC heterodimer dissociates and thereby allows TSC2 to activate the small GTPase Rheb in turn to upregulating mTOR activity. Interestingly phosphorylation of TSC2 by AMPK (5' AMP-activated protein kinase), a regulator of cellular energy homeostasis which is activated through energy deficiency, forces the inactivation of Rheb and consequently the inhibition of mTOR activity [118]. Hence, on one hand indirect stimulation by growth factors results in mTOR activation and on the other hand low energy levels lead to its inhibition. Both situations are mediated by the same molecules, TSC2 and Rheb, depending on specific upstream factors.

Activation of mTOR subsequently allows the formation of the nutrient- and energy-sensitive mTOR Complex1 (mTORC1) which is at least composed of mTOR, regulatory associated protein of mTOR (Raptor) and G-protein β-subunit like protein (GβL). This complex controls mTOR activity by regulating the accessibility of its kinase domain to mTOR-substrates depending on the nutrient and energy supply in the cell [116]. The exact mechanisms involved in the sensing of nutrient and energy levels are still widely unknown and remain to be explored.

Besides the afore mentioned regulatory function of mTOR involved in cell growth and protein synthesis, it has been shown that this molecule is also associated with the formation of the cytoskeleton through its stimulation of F-actin stress fibers, RhoA, Rac1, Cdc42, paxillin and protein kinase C α (PKCα) [119-121] . Interestingly this functions are only mediated by another mTOR involving complex, mTORC2, which is composed of mTOR, rapamycin-insensitive companion of mTOR (Rictor), GβL, and mammalian stress-activated protein kinase interacting protein 1 (mSIN1). The regulatory mechanisms of the complex associated proteins related to mTOR activity are not fully understood but appear to be stimulated by nutrients, growth factors and insulin.

### 2.5.3 Aberrant upstream mTOR-signaling and its relation to cancer

A multitude of critical molecules involved in the regulation of mTOR upstream pathways are known to be frequently deregulated to a certain extant in cancer. This leads to a number of potential defects that can cause an inappropriate activation of mTOR-

signaling finally causing growth and proliferation of malignant cells. Due to the key position of mTOR in the transduction of aberrant signals, blocking their effects at the point of convergence is a rational approach and a promising cancer treatment strategy.
Aberrant mTOR signaling can obtain its origin from upstream deregulated growth factor receptors in the cell surface which maintain inappropriate signaling due to overproduced growth factors or mutations/translocations that lead to their constitutive activation or overexpression [122]. Also the dysfunction of cytokine and integrin receptors is a common characteristic of cancer cells [11]. Aberrant messages from receptors on the cell surface are then transduced to intracellular signaling pathways whose importance can vary depending on the cell type and therefore are often associated to specific malignancies. The PI3-K/AKT pathway is commonly deregulated in a series of cancer types and associated with tumor progression and increased metastatic ability [123, 124]. Independent of any receptor stimulation, PI-3 kinase can be overactivated by intracellular signaling such as from oncogenic mutated RAS which is found in 20-25% of all human tumors [125]. Also mutations in *PIK3CA*, the gene for the catalytic subunit of PI-3 kinase have been reported in many cancers [126] and are responsible for its constitutive activation.
A negative regulation of the PI3-K/AKT pathway is essential for a healthy cell to maintain control of its own proliferation and growth and is particularly provided by the phosphatase enzyme PTEN (Phosphatase and Tensin homolog on chromosome 10). It removes the phosphate groups added by PI-3 kinase to its substrates and consequently turns off subsequent signaling. However, PTEN is one of the most frequently lost tumor suppressors in several cancer types [127] which reflects the fundamental significance of this pathway in malignant progression.
Another critical kinase that mediates its regulatory function through mTOR stimulation represents AKT, a key player associated with cell growth and survival. Activating mutations of AKT are frequently found in several cancer types and help to maintain antiapoptotic signaling [123]. It is believed that constitutively activated AKT counteracts apoptotic programs in response to the genetic insults caused by chemotherapy and therefore maybe contributes to drug resistance. This demonstrates again that blocking the signaling downstream of over activated AKT seems to be a reasonable therapy

strategy and has been confirmed by several studies showing a correlation between the presence of activated AKT and sensitivity to mTOR inhibitors [128, 129].

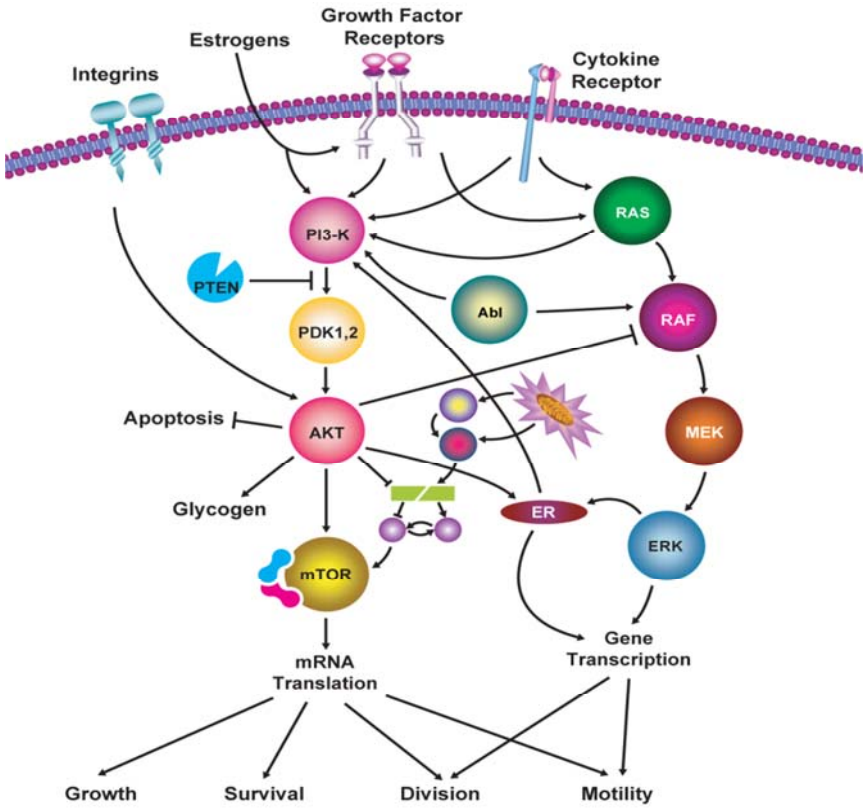



Figure 23. *Schematic overview of connected oncogenic pathways involved in mTOR signaling. Only some of the molecules of known functions and importance are shown. AKT is particularly important because of its role in modulating the mTOR pathway and maintaining of antiapoptotic signaling [115].*

### 2.5.4 Influences of mTOR on protein synthesis and cell proliferation

mTOR is known to be responsible for the regulation of more than 100 genes, many of which are involved in cell growth and cell proliferation-associated processes [130]. It acts at the level of gene transcription and protein translation and provides for this reason a rapid response to changes in the cellular environment and situations of stress. The direct targets of mTOR are the serine/threonine kinase P70-S6K1 (Ribosomal protein S6 kinase beta-1) and 4E-BP1 (4E-Binding Protein 1), a member of translation repressor proteins. mTOR phosphorylates P70-S6K1 which in turn phosphorylates the ribosomal protein S6 that activates the translation of 5'terminal oligo-pyrimidine (TOP)

mRNAs. Critical components of the protein translation machinery such as ribosome forming proteins, elongation factors (eEF1A, eEF2) and the poly(A)-binding protein (PABP) are coded by these TOP mRNAs. mTOR represents the regulatory molecule of all these factors due to its sensing of amino acid concentration within the cell which are essential for translation [131]. The inhibition of mTOR allows the phosphatase PP2A to inactivate P70-S6K1 and consequently stops the synthesis of ribosomes and proteins involved in metabolic processes.

The phosphorylation of 4E-BP1 by mTOR causes the release and thus the activation of eIF-4E (eukaryotic Initiation Factor 4E) which forms with other initiation factors the CAP-mRNA recognizing complex eIF-4F. These CAP-mRNAs obtain a 7-methyl guanosine nucleotide triphosphate 'cap' at their 5'end and polyadenosine tail at their 3'end. The eIF-4E complex brings together ribosomes and the 5'ends of CAP-mRNAs and therefore initiates the translation of key proteins that regulate cell cycle, cell growth and survival. The key role of mTOR in regard to the transduction of signals leading to the translation of these proteins may even more significantly related to the progression of cancer than the activation of P70-S6K1, as data suggest [132].

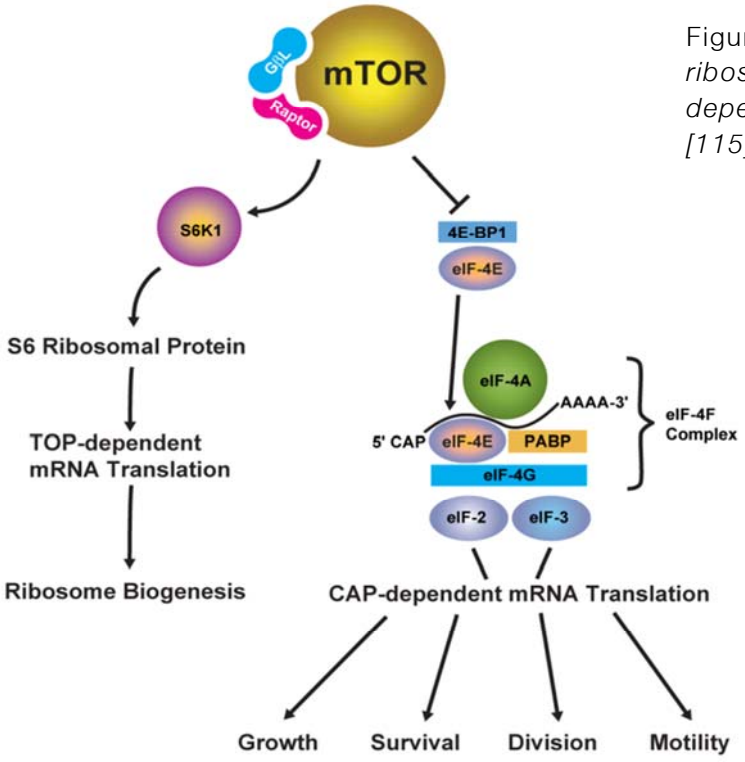



Figure 24. *mTOR controls ribosome biogenesis and CAP-dependent mRNA translation [115].*

### 2.5.5 Influences of mTOR on cell cycle progression

The translational activity forced by mTOR involves a large amount of different mRNAs including those coding for cyclins (cyclin D1) which regulate the activity of cyclin-dependent kinases (CDKs) thus driving the cell cycle [133]. Normally cyclin D1 is synthesized during G1-phase and drives the transition into S-phase if the cell is sufficiently supplied with nucleotides and necessary enzymes. Overexpression of cyclin D1, which is the case in many cancer types, can lead to a shortened G1-phase and drives the cell through the G0/S checkpoint, even when the necessary growth factors are not present. In addition, there is evidence that cyclin D1 is involved in epigenetic regulatory mechanisms leading to an enhanced accessibility of the genome to several transcription factors implicating its synthesis [134]. The subsequent inhibition of cyclin D1 mRNA translation by mTOR inhibitors may suppress the growth of malignant cells, a therapy strategy which has been already investigated in mantle cell lymphoma and other cancers associated with cyclin D1-overexpression [135].

### 2.5.6 Influences of mTOR on angiogenesis

One of the most important proteins involved in the stimulation of blood vessel formation represents HIF-1α (Hypoxia-Inducible Factor 1α) which is encoded by capped mRNAs whose translation is influenced by mTOR-signaling. It positively regulates the transcription of the gene for VEGF-A (Vascular Endothelial Growth Factor A), a potent angiogenesis stimulating factor. Its overexpression has been associated with aggressive disease and poor prognosis in several types of cancer [136-138]. Increased expression of HIF1α also showed in many cases a significant correlation with de-regulated mTOR-related pathways found in different cancers [139]. In normal cells with sufficient blood supply and the presence of adequate oxygen level, HIF1α is rapidly ubiquitinated and subsequently degraded by proteosomal activity. In contrast, within a hypoxic environment, which commonly occurs in the center of tumor nodules, HIF-1α translocates to the nucleus and binds to HIF-1β, initiating the transcription of VEGF-A and numerous gens involved in anaerobic glycolysis, glucose transport and metastasis. Consequently a hypoxic tumor increases its oxygen supply by the formation of new blood vessels and ensures its survival through the generation of sufficient energy [140].

Inhibition of mTOR activity reduces the expression of genes stimulated by HIF1α, affecting tumor angiogenesis especially by suppressing the proliferation of vasculature-associated smooth muscle cells which are required for vessel maturation [141].

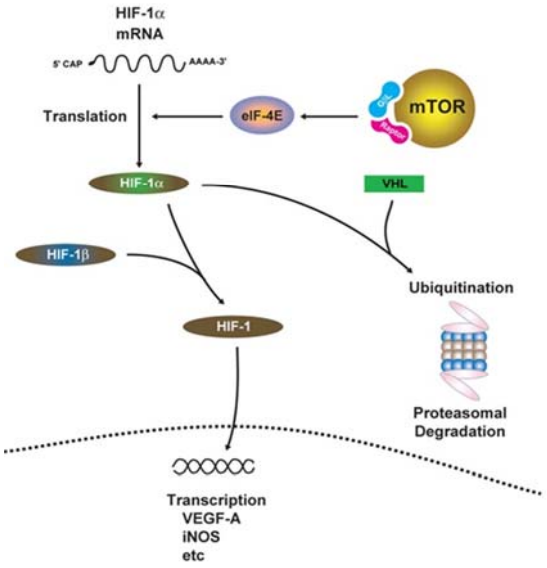



Figure 25. *mTOR, angiogenesis and response to hypoxic stress [115].*

### 2.5.7 mTOR-inhibitors

Rapamycin (sirolimus) is a macrocyclic lactone (Fig.26) produced by the bacterium *Streptomyces hygroscopicus* which was discovered for the first time on the Easter Island Rapa Nui [142]. In 1975 rapamycin was synthesized as a white water-insoluble substance and already first evaluations of the National Institute of Health (NIH) could demonstrate its cytostatic characteristics. However, further investigations on rapamycin focused primarily on immune suppressive properties [143] rather than development for cancer therapeutical implementations. Currently, rapamycin is clinically used after certain transplantations to prevent heavy immune reactions [144, 145]. Only the identification of mTOR as specific target of rapamycin and concerns about novel anticancer strategies forced further investigations towards this field. An important step for its further development was taken when temsirolimus (CCI-779), a water-soluble derivate, was synthesized and offered the systemic application of this new targeted compound [146]. In 2007 temsirolimus was approved by the U.S. Food and Drug Administration (FDA) [147] . The mode of action of rapamycin and its analogs relay on its binding to the cytosolic immunophilin FK-binding protein 12 (FKBP12), forming a complex that only binds mTOR and therefore grants its specificity. As described before (s. ch. 2.5.2) activated mTOR forms two complexes which maintain different regulatory functions, mTROC1 and mTORC2. The latter contains the protein RICTOR (Rapamycin-insensitive companion of mTOR), which was originally named based on the ineffectiveness of rapamycin to inhibit mTORC2 activity. However, recent studies

have demonstrated that chronic exposure to rapamycin also inhibits the formation of new mTORC2. It is believed that pre-existing mTORC2 is not affected but rather binding of rapamycin to free mTOR is promoted which results in reduced adherence of RICTOR [148].

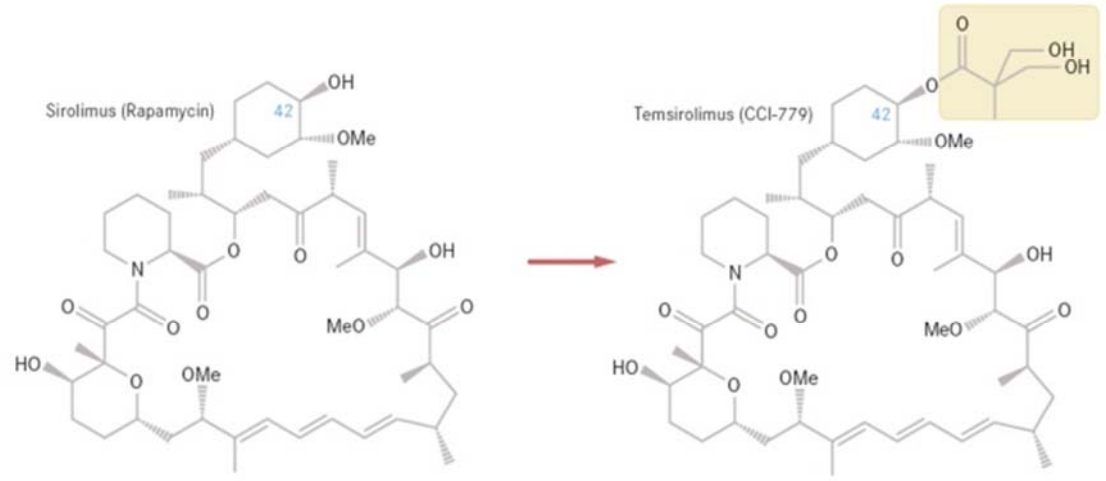



Figure 26. *Comparison between the chemical structures of sirolimus (rapamycin) and temsirolimus (CCI-779) [145].*

# 3. AIM OF THE STUDY

Human malignant mesothelioma (HMM) is an aggressive tumor of serosal surface such as the pleura and peritoneum with generally poor prognosis. It is strongly associated with asbestos exposure and shows a 20 to 50 year latency period. Clinical treatment of mesothelioma patients with conventional chemotherapeutics as cisplatin and pemetrexed shows poor response. For this reason the development of new therapeutic concepts with higher efficiency is urgently needed. Within carcinogenic development, pathologic activation of the mTOR (mammalian target of rapamycin) pathway takes a central position. This pathway is strongly associated with increased cell proliferation, angiogenesis, cell motility and raised survival potential leading to apoptosis resistance. Various types of cancer cells show an abnormal level of this molecule including malignant mesothelioma cells as existing data confirm [149, 150]. Consequently, the targeted inhibition of these oncogenic signals represents a main focus of modern cancer therapy. Temsirolimus, an inhibitor of mTOR, is a “targeted compound” with widespread antineoplastic effects and may be a promising candidate in the treatment of HMM.

The aim of the here presented study was

1) To gain more insight into the role of mTOR in HMM and determine its constitutive activation status in surgical specimens and mesothelioma cell models. Further, we investigate for genetic aberrations in HMM cell lines using aCGH with the main focus on genes involved in the mTOR pathway.
2) To investigate the anti-cancer activity and the mode of action of temsirolimus as single agent against HMM cell lines. To this end, several analyses regarding cytotoxicity, cell proliferation, impact on DNA synthesis, cell cycle distribution as well as cell death induction in monolayer and three-dimensional (3-D) cell culture systems are performed.
3) To compare the anti-tumor potential of temsirolimus with cisplatin and pemetrexed and to test whether the combination of the targeted compound with conventional chemotherapy results in synergistically anti-cancer effects. These aspects are investigated in vitro as well as in in vivo xenograft models.

# 4. MATERIALS AND METHODS

## 4.1 Cell culture

All MPM cell lines were maintained in RPMI 1640 or M10 with 10% FCS in a humidified atmosphere of 5% $CO_2$ at 37°C. The list of used cell lines, their histological subtype and source are summarized in Table X. The cisplatin-resistant P31res1.2 subline was established from the parental P31 by in vitro cisplatin selection to a final concentration of 1.2mg/L [151]. Cell lines were authenticated by array comparative genomic hybridization (aCGH) in all cases in 2009. The identical origin of the P31parent and P31res1.2 were additionally proven by DNA finger printing.

Table 5. *List of used cell lines and their growth medium*

| Cell line | Histologic subtype | Growth Medium | Source |
|---|---|---|---|
| P31 | epithelioid | M10 | K. Grankvist Umeå University |
| P31res1.2 | epithelioid | M10 | K. Grankvist Umeå University |
| SPC111 | biphasic | RPMI 1640 | R. Stahel University of Zurich |
| SPC212 | biphasic | RPMI 1640 | R. Stahel University of Zurich |
| NCI-H28 | epithelioid | RPMI 1640 | ATCC |
| NCI-H2015 | epithelioid | RPMI 1640 | ATCC |

## 4.2 Clinical samples

Formalin-fixed, paraffin-embedded tumor tissue was available from 70 patients suffering from histological proofed malignant pleural mesothelioma. All patients underwent resection between 01/1993 and 01/2010 at the Medical University of Vienna, Department of Thoracic Surgery. The tumor tissue blocks were embedded during pathological routine diagnostic work up and coded by identification numbers given from the pathologic department during routine investigation. From each tumor block, sections were cut at 4 µm. H&E-stained sections were used to confirm the presence of invasive carcinoma. Informed consent was obtained from all patients.

## 4.3 Drugs

Temsirolimus was obtained from Wyeth Phamaceuticals Inc., Cambridge, USA, cisplatin from Sigma, Saint Louis, USA and pemetrexed from Eli Lilly Pharmaceutical, Indiana, USA. For in vitro experiments temsirolimus was dissolved in DMSO at 25ng/µl, cisplatin in PMF at 20mM and pemetrexed in 0.9% NaCl at 25mg/ml stock. Before application all drugs were further diluted in culture medium. For in vivo experiments temsirolimus was diluted in 5% PEG/5% Tween and cisplatin in serum-free culture medium. DMSO concentrations were always below 1% in vitro and proven to be ineffective at those concentrations in all cases.

## 4.4 Immunohistochemistry

*Background:*

Immunohistochemistry (IHC) is a method to visualize proteins of interest (antigens) within tissue samples and cell compartments via specific antibodies. For oncological approaches this technique helps to identify and classify tumors obtained from biopsies which express certain known antigens. Thus, it is possible to distinguish tumors by important parameters, so called “biomarkers” such as growth rate, metastatic activity or responsiveness to therapy even when they show a similar morphology. Within basic biology research, IHC represents also an important tool to verify proteins of interest and to examine the expression status in the investigated tissue.

The main principle of IHC relays on the affinity of antibodies to their specific epitope (antigen) within the tissue (antigen-antibody interaction). To visualize the presence of targeted antigens, antibodies are coupled with a detection system that is most frequently an enzyme, such as peroxidase, which catalyzes a color-producing reaction after adding a dedicated substrate. Alternatively, also fluorophores can be used to visualize an antigen-antibody interaction using a immunofluorescence microscope. If already the antibody that binds directly its antigen is linked with a detection system, the technique is referred to as “direct method” which is quite simple and rapid. However, in this case it is not possible to amplify the signal which becomes particularly important for a lowly expressed antigen. For this reason a more common used technique represents the “indirect method” that involves at least two different antibodies. In the first step an unlabeled primary antibody reacts with its antigen and can only be visualized after adding a secondary antibody that is conjugated to an enzyme or tagged to a fluorophore. The secondary antibody only reacts with the primary antibody which features multiple binding sites making this indirect method more sensitive through signal amplification. The second step of this procedure involves finally the indirect visualization of the targeted protein by different methods depending on the used type of detection system. The most common applied immunohistochemical indirect method for the analyses of surgical tumor specimen is the Labeled Streptavidin-Biotin method (LSAB). It utilizes a biotinylated secondary antibody that links primary antibodies to

multiple streptavidin-horseradish peroxidase conjugates due to their high affinity to biotin. By adding 3, 3'-Diaminobenzidine (DAB), a brownish staining is catalyzed wherever secondary antibodies are attached. This method has become widely used since it is able to overcome some of the limitations of earlier fluorescence antibody methods especially in commonly formalin-fixed paraffin-embedded tissues samples.

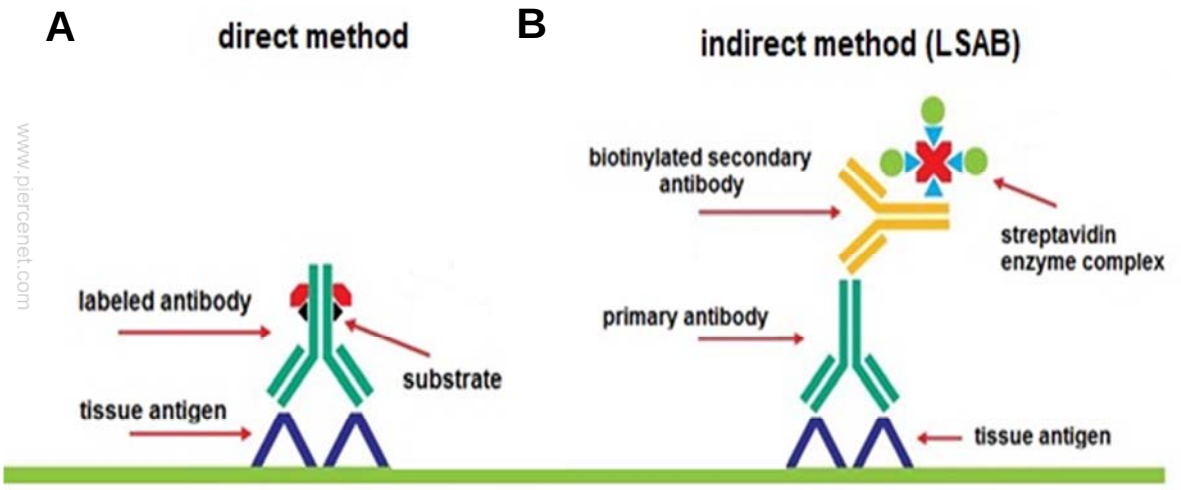



Figure 27. *Comparison of two immunohistochemical methods. (A) The direct IHC method involves antigen specific antibodies (AB) which already feature a detection system. Then a dedicated substrate is added to visualize the targeted antigen. (B) The Labeled Streptavidin-Biotin method (LSAB) as example of the indirect IHC system. In this case an unlabeled primary AB binds its specific antigen. Then a secondary biotinylated AB specifically binds the primary AB. Added streptavidin binds with high affinity to biotin the secondary AB. Finally adding 3, 3'-Diaminobenzidine (DAB) catalyzes a brownish staining visualizing the targeted antigen.*

*Preparation:*

Briefly, tissue sections were deparaffinized and rehydrated. After heating for 10 min in 10 mmol/L citrate buffer (pH 6.0) in a pressure cooker for epitope retrieval, the tissue sections were incubated over night at 4°C with primary antibodies (p-mTOR Ser2448; Cell Signaling; Ki67, Dako; dilutions 1:100). Antibody binding was detected by means of the UltraVision LP detection system according to the manufacturer's recommendations (Lab Vision Corporation). Color development was done by 3,3′-diaminobenzidine and counterstaining by hematoxylin. p-mTOR immunostaining was independently evaluated by two authors (Amir Mohamed, Ulrike Setinek) in at least five

representative areas per section. Interpretation of the results was limited to proven tumor tissue and only cytoplasmic staining was considered positive. Staining intensity was scored as strong=3, moderate=2, weak=1. Intensity scores were multiplied by the respective percentages of cells and divided by 3, resulting in an H-score from 0-100. The results were correlated with clinical/histological data using the SPSS 17 software package.

## 4.5 Genome analyses

### 4.5.1 Array comparative genomic hybridization

*Background:*

Malignant cell transformation is guided by an increasing number of mutations in genes controlling cell growth and cell death as well as genomic stability. Array comparative genomic hybridization (aCGH) is a powerful technique to detect genome-wide gains and losses of genes caused by chromosomal aberrations in tumor cells. The method is based on co-hybridization (1:1 ratio) of fluorescent-labeled tumor (green) and reference DNA (red) onto an array of immobilized DNA oligonucleotide fragments (represent main sequences of the human genome) printed on a glass slide. After scanning the slide, software calculation allows to determine the ratio between both fractions, giving under-represented hybridization levels of tumor DNA as gene losses and dominant signal intensities from individual spots as gene dose gains compared to the reference fluorescence levels. Array CGH represents a further development of conventional CGH which relies on the same principals but in this case hybridization takes place on whole metaphase chromosomes limiting detection resolution for aberrant DNA changes to that of light microscopy (~5MB). In contrast, aCGH allows zooming with an intermediate (44K) or high resolution (244k) array into chromosomal regions at the level of single genes.

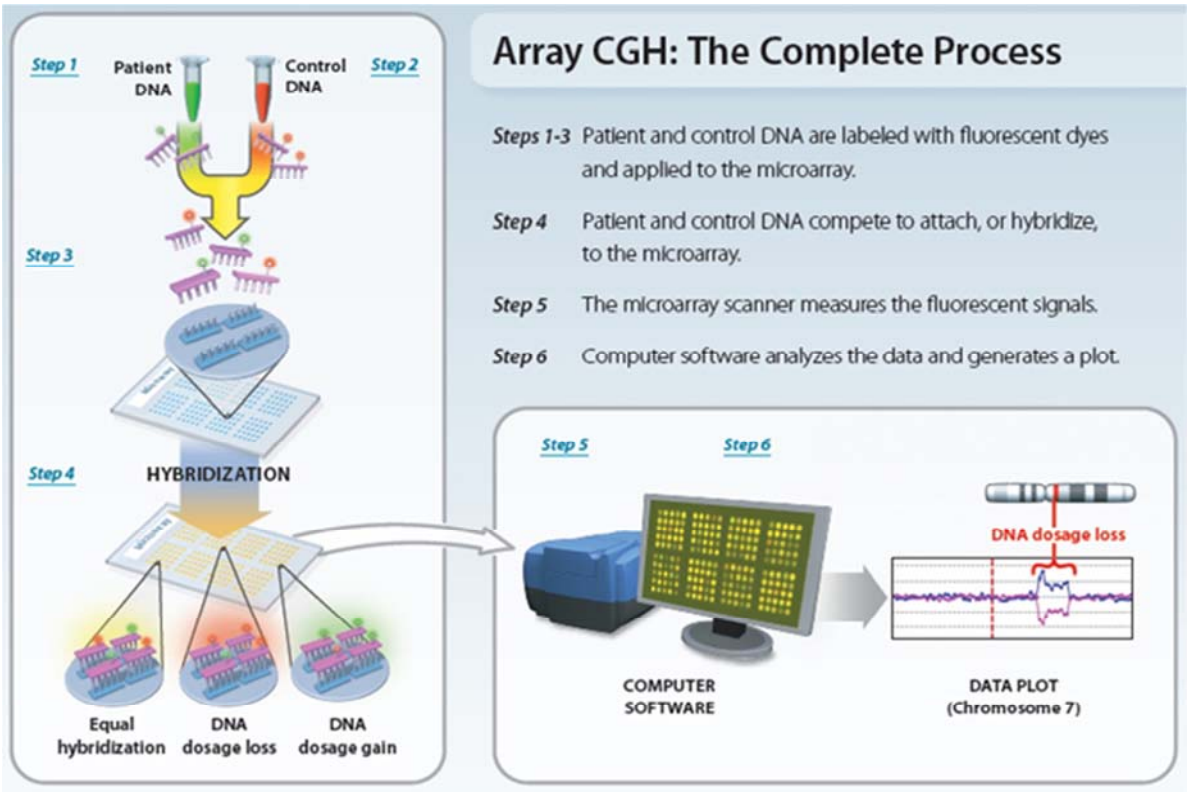



Figure 28. *Illustration of the main steps involved in array comparative genomic hybridization (aCGH)*

*Preparation:*

aCGH was performed using 4x44K whole genome oligonucleotide-based arrays for humans (Agilent). Labeling and hybridization procedures were performed according to the instructions provided by Agilent. Shortly, reference DNA and test DNA were digested with AluI and RsaI (both Promega), then differentially labeled by random priming with Cyanine 3- and Cyanine 5-dUTP (Perkin-Elmer). Human genomic male DNA (Promega) was used as reference DNA. After purification with Microcon YM-30 filter units (Millipore) the two labeled products together with Blocking Agens, Hybridization Buffer (both included in the Oligo aCGH/Chip-on-Chip Hybridization Kit, Agilent), and human cot-DNA (Roche) were combined and hybridized onto the 4x44K oligonucleotide arrays for humans (Agilent). Hybridization was carried out for 48h at 65°C in a hybridization oven. Afterwards, slides were washed according to the protocol and scanned with a G2505B Micro Array Scanner (Agilent). Feature extraction and data analysis were carried out using the Feature Extraction and DNA Analytics software, respectively.

## 4.6 Cell proliferation and cell vitality assays

### 4.6.1 Cytotoxicity assays

*Background:*

A common method to determine indirectly the cytotoxicity of different drugs as single agent or in combination against cell lines in vitro represents the MTT-assay. This colorimetric assay is based on the enzymatic reduction of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) (MTT) to formazan giving a deep orange color. Because this reduction requires active and functional mitochondrial reductase enzymes, only viable cells will give an orange coloration while dead cells remain uncolored. The staining intensity is then determined by absorbance measurement at a wavelength of 450 and 620 nm in a spectrophotometer. Finally the comparison between coloration of the untreated control and treated cells reveals a ratio that indicates the cytotoxic or cytostatic effects of drugs.

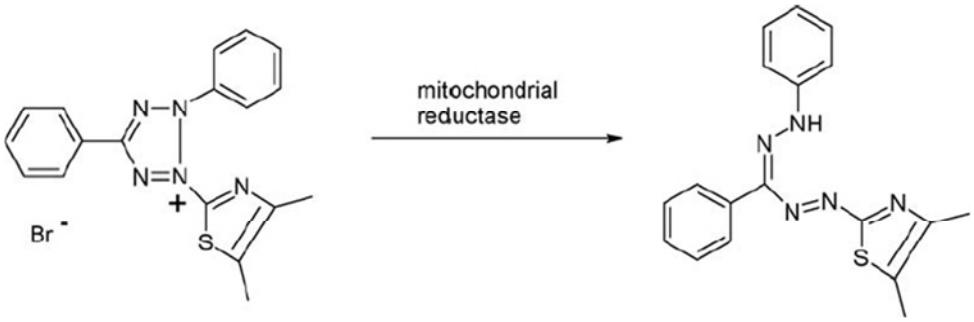



Figure 29. *MTT is reduced in formazan by a mitochondrial reductase*

*Preparation:*

MM cells were seeded in 96-well microtiter plates at a density of $2x10^3$ cells in 100µl growth medium per well and allowed to recover for 24 hours. Dissolved drugs were diluted in growth medium and added to the cells in different concentration ranges for 24h to 72h at 37°C in humidified atmosphere and 5% $CO_2$. Subsequently the proportion of viable cells was determined by MTT assay following the manufacturer's procedure (EZ4U, Biomedica, Vienna, Austria). After incubation for 1-4 hours, depending on the metabolic capacity of the different cell lines, plates were gently

shaken before absorbance measurement at 450 nm or 620 nm as reference respectively. Cytotoxicity was expressed as $IC_{50}$ values (half maximal inhibitory concentration) and calculated by the software GraphPad Prism 5.0 from dose-response curves.

### 4.6.2 Clonogenic assays

*Background:*

To study long-term effects of specific agents on the survival and proliferation of single tumor cells, their capacity to form colonies after drug exposure can be analyzed by clonogenic assays. Therefor single cells are plated at a very low density in drug containing medium and the formation of colonies is evaluated subsequently. For better visualization, cells can be stained with crystal violet (hexamethyl pararosaniline chloride) which has the ability to intercalate DNA.

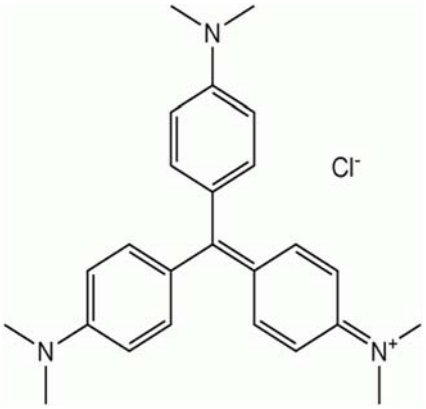



Figure 30. *Chemical structure of hexamethyl pararosaniline chloride*

*Preparation:*

Cells were seeded into 6-well plates at densities of $2x10^3$ to $5x10^3$ cells/well 24h before treatment. Dissolved drugs were diluted in growth medium and added to the cells in different concentration ranges. After 7 days drug containing medium was removed, cells were washed three times with PBS and fixed for 10 minutes with ice-cold methanol at 4°C. Subsequently the cells were stained with crystal violet (stock: 10% crystal violet in ethanol; diluted 1:1000 in PBS) for ~ 5 minutes and again washed with PBS. Finally the clones were dried at room temperature (RT) and evaluated by microscopical counting the clones.

*Recipes:*

10x PBS:

95 g 0.53 M Na2HPO4 x 2 H2O

32 g 0.23 M NaH2PO4 x H2O; Σ 1 l ddH2O

### 4.6.3 Hoechst 33258-PI staining

*Background:*

Hoechst 33258 is a fluorescent DNA intercalating nuclear dye that can be used to observe different states of the nucleus during apoptosis, necrosis or cell cycle by visualizing apoptotic bodies and condensed chromosomes during mitosis. Due to its ability to pass through intact membranes of living or fixed cells, it easily intercalates into double stranded DNA and produces a bright blue fluorescence when excited with ultraviolet light at 465nm wavelength. In cell vitality assays, the Hoechst dye is commonly combined with propidium iodide (PI), another DNA-intercalating stain that can be excited with U.V. at 488nm giving red fluorescence. In contrast to Hoechst 33258, PI is membrane impermeable and generally excluded from viable cells. Hence it represents a factual verification of dead cells (necrotic cells and late apoptosis).

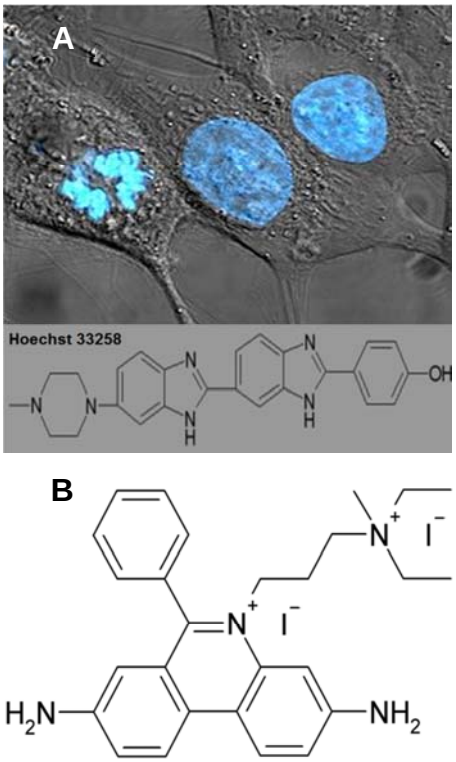



Figure 31. *(A) Hoechst 33258 intercalated into DNA of cells and its chemical structure. (B) propidium iodide (PI).*

*Preparation:*

Cells were plated in 6-well plates at a density of $2x10^5$ cells in 2ml growth medium per well and allowed to recover for 24 hours. Dissolved drugs were diluted in growth medium and added to the cells in different concentration ranges. After drug exposure, cells were trypsinized, collected, washed once with PBS, centrifuged for 5 minutes at 1100 rpm and resuspended in 300 µl PBS. Cytospins (Shandon Cytospin 3 Centrifuge Cell Preparation System) were performed with three slides for each sample which had been aliquoted (50-75µl) before. Then the cells on the slides were fixed using a 1:1 methanol-aceton solution for ~10 minutes and stained with DAPI containing antifade solution (Vector Laboratories, Inc., Burlingame, CA). Nuclear morphology was examined using a Leica DMRXA fluorescence microscope (Leica Microscopy and

System, Wetzlar, Germany) equipped with appropriate epifluorescence filters and a COHU charge-coupled device camera.

### 4.6.4 TUNEL assay

*Background:*

Terminal transferase dUTP nick end labeling (TUNEL) is a method to detect DNA strand breaks in apoptotic cells. During to the late stage of apoptosis nucleic DNA is fragmented by endonucleases ensuring the programmed cell death. Hence, a multitude of 3'-hydroxyl DNA termini accrues within the nuclei which can be labeled using terminal deoxynucleotidyl transferase (TdT) and commonly fluorescent-tagged deoxyuridine triphosphate nucleotides (dUTP). TdT catalyzes the template-independent addition of dUTPs to the 3'-OH ends of nicked DNA. In this study we used standard immunohistochemical techniques to visualize the labeled DNA breaks of MM xenograft tissue.

*Preparation:*

In this study TUNEL assay was used to visualize drug induced cell death in harvested xenograft tumors. Therefore tumor samples were primarily fixed in formalin and embedded in paraffin according to standard protocols. Then TUNEL assay was performed using the "In Situ Cell Death Detection Kit, Fluorescein" obtained from Roche Diagnostics GmbH, Germany. First paraffin-embedded tissue samples were dewaxed and rehydrated. After washing, tumor slides were incubated with TUNEL reaction mixture (enzyme solution + labeling solution) for 60 min at 37°C. Finally slides were again washed and analyzed by fluorescence microscopy.

### 4.6.5 $^{3}$H-thymidine incorporation assays

Deoxythymidin (dT), or short thymidine, is a 2'-deoxynucleosid composed of the pyrimidine nucleobase thymine and the pentose $_{D}$-deoxyribose. When phosphorylated, thymidine represents a component of the DNA (deoxyribonucleic acid) and pairs with deoxyadenosine to form a double helix. In contrast to other deoxynucleosides which also arise as nucleosides in the RNA (ribonucleic acid), thymidine is found only in DNA. Based on this fact, the quantification of the thymidine amount within cells represents an excellent indicator to draw conclusions from DNA synthesis and thus allows to determine whether a cell population is proliferating (enhanced thymidine level during S-phase) or dying (reduced thymidine level during cell lysis). During cell proliferation assays radioactive labeled thymidine (tritiated thymidine; $^{3}$H-thymidine) is supplied and can be detected using scintillator solution via a liquid scintillation counter measuring the fluorescence level of a transparent crystal which fluoresces through radioactive radiation.

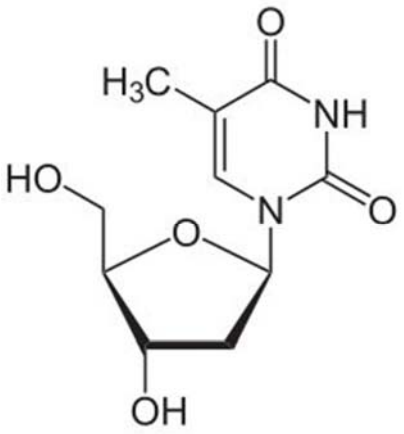



Figure 32. *Deoxythymidin*

*Preparation:*

Cells were seeded in 96-well microtiter plates at a density of 2x10$^{4}$ cells/well 24 h prior drug treatment. After 24 h exposure time the medium was removed and 100µl of a 2nM solution of $^{3}$H-thymidin (specific activity: 25ci/mM; GE Healthcare) was added to each well. Following incubation for 2h at 37°C cells were washed twice with ice-cold PBS, lysed in 100µl lysis-buffer and transferred into scintillator tubes. The 96-wells were washed again with 100µl PBS and also transferred to the cell lysates in the scintillator tubes. After addition of 5ml liquid scintillator (KFORO Laboratories), the samples were mixed by turning upside down and finally measured using a liquid scintillation counter (Tri-Carb 1900TR, Packard).

*Recipes:*

Lysis buffer $^{3}$H-thymidine incorporation:

10 mM Tris-HCl, pH= 7.8

1% SDS

Σ 1 l ddH2O

### 4.6.6 HMM cell spheroid growth assays

(performed in collaboration with B. Hegedus)

*Background:*

Multicellular tumor spheroids are globular aggregates formed by several thousand malignant cells and can be used as three-dimensional (3-D) cell culture systems for a large range of molecular analysis. Compared to conventional cell culture experiments, spheroid based assays more closely resemble the in vivo situation with regard to cell shape and cellular environment. These characteristics can determine gene expression and the biological behavior of cells as well as an advanced degree of differentiation. Several studies have emphasized the different behavior of cells in 3-D cell cultures compared to monolayers. For example it was shown that isolated and conventionally recultured tumor cells from drug resistant in vivo xenograft lost their therapy resistance in vitro, while grown as multicellular spheroids maintained a comparable insensitivity to the applied agent as in vivo [152, 153]. This phenomenon is referred to as contact effect which underlies a multitude of mechanisms such as an improved accessibility of repair enzymes to their substrates. Further the specific nuclear shape and DNA packing of spheroids may also contribute to a more efficient DNA-repair resulting in an enhanced drug resistance especially to DNA intercalating agents.

Besides the shape-associated characteristics of 3-D cell cultures, spherical colonies established from a multitude of different malignancies showed significant overlap with the genetic programs of progenitor/stem cells after transcriptional profiling [154]. Thus, it is believed that spheroids consist of a distinct subpopulation of cancer stem cells or tumor initiating cells that feature self-renewal capability and are responsible for the generation of more differentiated progeny cells. These characteristics were originally demonstrated in neural cells and cell lines derived from primary glioblastomas and

successively also verified for other tumors such as melanoma, ovarian and mamma carcinomas [155-157]. However, further studies revealed that spheroid-formation capacity is a property that strongly differs between various types of tumors and that some spheroids lack of significant cancer stem cell markers [9]. Although it is unclear whether the spheroid assay can be generally associated with cancer stem cells due to differing data and arguments, it has formed an important basis to study tumor-initiating cancer stem cells in a variety of solid tumors. Considering the possible differences between monolayer and 3-D cell culture based analysis, multicellular tumor spheroid in vitro models represent an alternative strategy for enhanced studies on tumor cell response to therapy and self-renewal capacity according to the cancer stem cell hypothesis (s. chapter. 2.2).

Several methods are applied to induce the formation of a three-dimensional structure by single cells. In this study we used microtiter plates with super-hydrophilic non-adherent surfaces which physically force the cells to generate aggregates.

*Preparation:*

To establish spheroid cultures from HMM cell lines, $5x10^3$ cells were seeded in triplicate in DMEM/Ham's F-12 medium (PAA Laboratories, Austria) supplemented with 20ng/ml basic fibroblast growth factor (bFGF, Eubio, Austria), 20ng/ml epidermal growth factor (EGF; Sigma) and 2% B27 supplement (PAA Laboratories, Austria) in ultra-low attachment 24-well plates (Corning, NY). Temsirolimus was added at 100ng/ml. 96 hours after plating all spheroids of each well were photographed. In order to exclude small cell clumps from the analysis, only the spheres with a diameter over 100µm were counted and their diameter measured on the digital photographs using Image-J software. In order to assess the self-renewal capacity of spheroid-derived cells, the spheres were treated for 10 min with trypsin (0.1%) and EDTA (0.01%). Cells were resuspended in DMEM/FBS medium and washed once in serum-free medium and passed through 70 µm filter (BD Biosciences, Bedford, MA). After centrifugation the single cell suspension was re-plated under the aforementioned conditions.

## 4.7 Cell cycle analyses

### 4.7.1 Flow cytometry

*Background:*

The cell cycle is referred to as the process of cell reproduction by performing an orderly sequence of events that involves the duplication of its contents and finally leads to the division into two daughter cells by mitosis. After division each cell starts the cell cycle in the interphase, a stage which can be subdivided into $G_1$- (duplication of cellular contents), S- (synthesis of DNA) and $G_2$-phase (chromosome check and repair). If the genome has been properly duplicated and putative failures have been corrected the cell continues with mitosis closing the circuit. Multicellular organisms also contain cells which no longer divide and remain in a quiescent state, called the $G_0$-phase. Such terminally differentiated cells are e.g. neurons or heart muscle cells. In contrast immortalized tumor cell cultures in vitro normally do not fall in quiescence and continue unlimited proliferation.

When testing the effects of drugs on tumor cells, the examination of cell cycle changes can give a deeper insight into the mode of action of these agents. Cell cycle analyses are commonly performed using flow cytometry, a technique that provides the possibility to count fluorescent antibody- or dye-labeled cells and examine their DNA content which changes during phases of cell cycle. A frequently used staining is propidium iodide (PI) which intercalates the major groove of double-stranded DNA

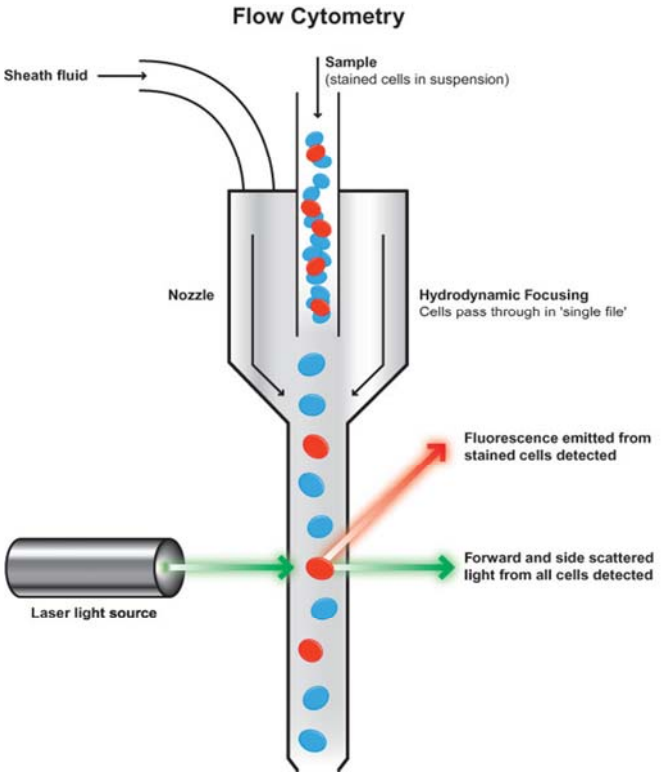



Figure 33. *A functional illustration of flow cytometry.*

and is able to fluorescence after excitation with 488nm. Hence, measuring the fluorescence level of drug-treated, PI-labeled cells depicts their proportional DNA amount, which gives an indication of whether the cells may have arrested in a certain cell cycle phase (Fig.34). Therefore stained cells are suspended in a stream fluid and solitary pass a laser beam within an electronic detection module that measures the excitation of every single cell (Fig.33).

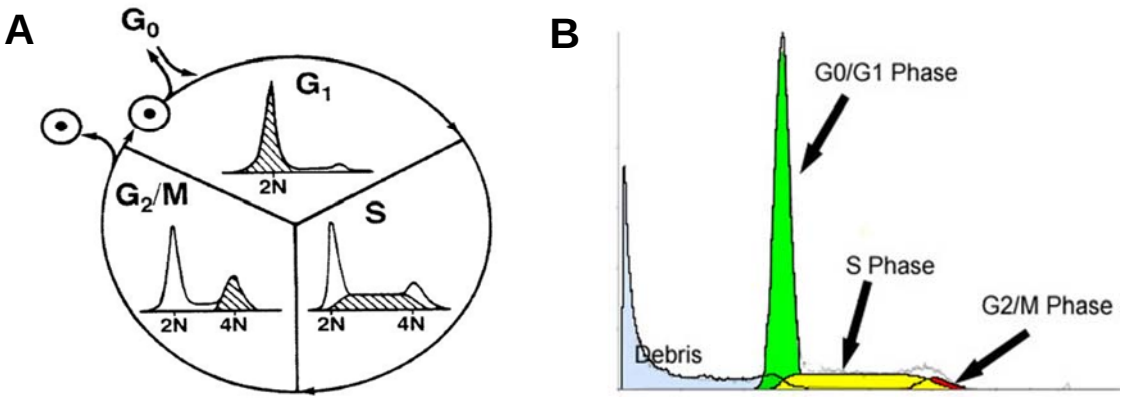



Figure 34. *(A) Schematic cell cycle overview highlighting genomic ploidy. (B) FACS diagram illustrating several different peaks indicating the amount of cells in the specific cell phase*

*Preparation:*

Cells were seeded in 6-well plates at a density of $5x10^5$ cells in 2ml growth medium per well over night prior 24h drug treatment. Further cells were trypsinized and washed once with FACS-PBS. The cell pellets were resuspended in 100 µl 0.9% NaCl and dropped slowly into 70% ice-cold ethanol. To prepare cells for PI staining, they were treated with NaCl and ethanol permeabilizing the cell membranes to provide the diffusion of PI into the cell. After at least 1h incubation at -20°C, cells were centrifuged again 1 minute at 8000 rpm and resuspended in 1 ml FACS-PBS. To prevent a possible intercalation of PI into RNA, 0.79 Kunitz units/ml RNAse A were added and samples were incubated at 37°C for 30 minutes. Then cells were filtrated into FACS tubes, stained with 1 mg/ml of PI for another 30 minutes at 4°C, and fluorescence was

measured by flow cytometry (FACS Calibur – Becton Dickinson, Palo Alto, CA) using Cell Quest Pro software.

*Recipes:*

FACS-PBS:

11.5 g Na2PO4 x 2 H2O

2 g KH2PO4

2 g KCl

80 g NaCl

Σ 1 l ddH2O

## 4.8 Protein analysis

### 4.8.1 Protein extraction

*Preparation:*

Cells were seeded as appropriate either in 6-well plates or 10cm plates at a density of $3x10^5$/well and $2x10^6$/plate, respectively, prior drug treatments. After 24h, 48h or 72h exposure time, the cells were collected by scraping into the medium and centrifuged at 1200 rpm for 5min. Next the cell pellet was washed once with PBS and then lysed with 30-50ml lysis buffer on ice for 10min. For enhanced destruction of cell membranes, the samples were treated in a sonicator for 3min and then centrifuged at 14.000 rpm for 15min at 4°C. After discarding the pellet containing nuclei and dense cell analyses, protein concentrations of collected supernatants were determined by using Micro BCATM Protein Assay Reagent Kit (Pierce Biotechnology, Rockford, USA). All protein lysates were stored in aliquots at -80°C.

*Recipes:*

Lysis buffer:

500 µl lysis buffer (s. ch. 4.6.5); 0.5% Triton X-100

10 µl/ml phenylmethanesulphonylfluoride (PMSF) –serine protease inhibitor, Roche-

25 µl/ml Complete (protease inhibitor cocktail tablets, Roche)

### 4.8.2 Western blot analyses

*Background:*

The investigation of protein levels generally plays an important role in life science and was used in this study to gain more insight into molecular changes of cells after drug treatment. Western blotting, the standard method for detecting specific proteins in a prepared cell lysate, is an immunochemical technique that allows to visualize targeted proteins via specific antibodies. As a first step the proteins in a given heterogenic protein lysate are separated by electric current using SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) consisting of two different polyacrylamide gels, a collecting and a separating one. The protein samples are loaded into the collecting gel where components become stacked into a very thin, sharp band prior entering the separating gel in which the proteins are fractionated by size. Both gels contain denaturing SDS buffer, an anionic detergent that binds proteins and unfolds them. Further SDS also masks the native charge of proteins by giving them a near uniform negative charge along their length. This results in an equal charge to mass ratio which is a necessary property for the unique migration of all different proteins in an installed electric filed towards the plus pole. An essential precondition for this movement is also the uniform shape of all proteins which is provided by the denaturing characteristics of SDS that destroys secondary and tertiary structures leaving only unfolded peptide bonds between amino acids.

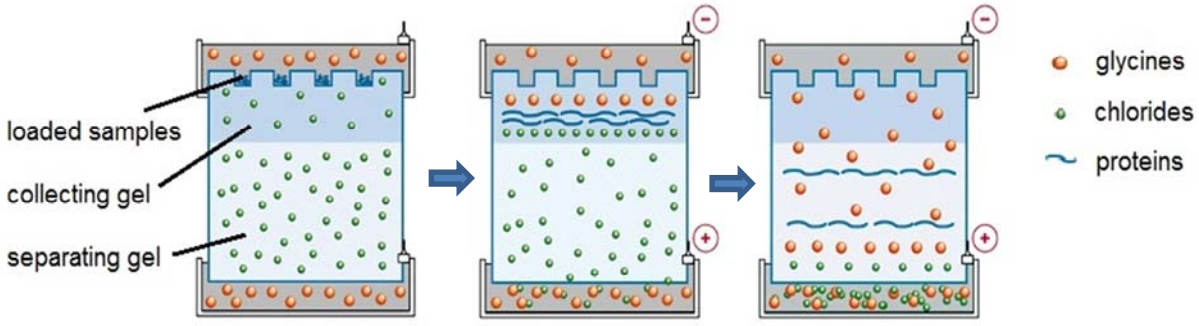



Figure 35. *SDS PAGE: At the beginning protein samples are loaded onto a chloride-containing gel which is situated in a tank with electrode-buffer that contains zwitterionic glycine. Due to electric current, the chloride ions start to migrate in front of the glycine ions which entered the gel. In the collecting gel the proteins form an intermediate layer between the two different ions. Finally, in the separating gel the proteins become fractionated by size.*

In this study we used a discontinuous buffer system that employs different buffers for the gels (collecting gel: Tris-HCl buffer- pH6.8; separating gel: Tris-HCl buffer – ph8.8) and the tank (electrode buffer: Tris-glycine buffer – pH8.8). During electrophoresis glycine ions, supplied by the electrode buffer, enter the collecting gel that slows down their mobility and leads to a zwitterionic form of the molecules at pH6.8. In contrast the chloride ions from the gel are smaller and negatively charged causing their much higher mobility and formation of a leading front. Due to the resulting voltage drop between glycine and chloride ions, the intermediate proteins become compressed into very thin layers creating the focused narrow zone at the top of the separating gel. Here the pH abruptly increases and leads to the ionization of glycine resulting into a faster movement of these now negatively charged molecules. Due to the higher acrylamide concentration in the separating gel, the smaller pores of the gel matrix decrease the mobility of proteins which now become separated by size.

Since the final gel is hard to conserve and is not accessible for antibody detection, the separated proteins have to be transferred on to a solid membrane. In this study we applied a polyvinylidene fluoride (PVDF) transfer membrane and the semi-dry blotting technique. This is an electrophoretic method that uses an electric field which forces the negatively charged proteins in the gel to migrate on to the membrane where they are bound manly due to hydrophobic interactions. Two stacks of filter paper, one at the top of the gel soaked with a cathode buffer, and another below the membrane soaked with an anode buffer, act as ion-reservoirs and maintain the electrical potential between the poles. By adding methanol to the anode buffer SDS is removed from the proteins providing their more efficient binding to the membrane.

To eliminate nonspecific background the membrane with bound proteins has to be further processed after blotting. First it is washed with TBST (tris-buffered saline with Tween) which removes all unbound components. Then the membrane is placed in a bovine serum albumin (BSA)- and non-fat dry milk -containing solution which provides an overage of proteins that attach all remaining polypeptide-free areas on the membrane. Consequently, antibodies obtain no possibility to unspecifically bind the membrane. After washing and blocking, finally the detection and visualization of targeted proteins is performed. Therefore the membrane is incubated with a solution

containing a primary antibody that is directed against monoclonal or several polyclonal epitopes at the protein of interest. Then a reporter enzyme (commonly horse radish peroxidase)-labeled secondary antibody that binds the species-specific region of the first antibody is added. In the presence of a chemiluminescent agent which is cleaved by the reporter enzyme, the antigen-antibody complex starts to produce luminescence. For the detection, a sensitive photographic film is placed on the membrane and the luminescence signal blackens the film at the specific protein spot.

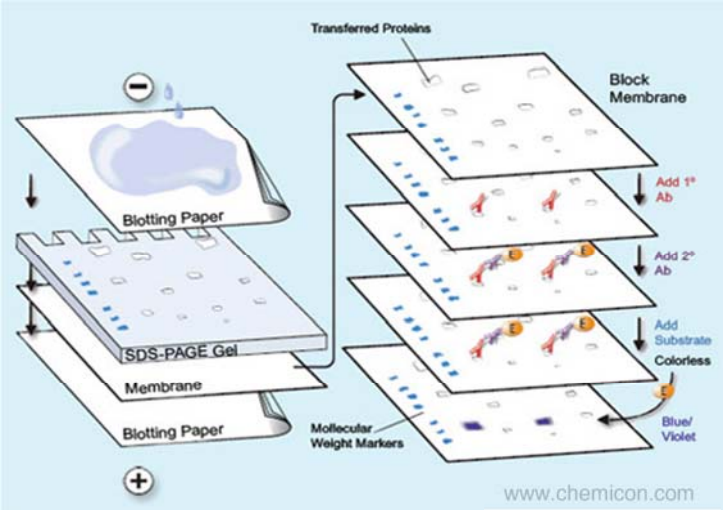



Figure 36. *Illustrated steps during the western blot procedure*

*Preparation:*

SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis) was performed using 10% separating gel and 4.5% collecting gel. Electrophoresis was conducted with 90V till protein bands reached the end of the western blot chamber. The gels have been blotted using Trans-Blot SD (Biorad) onto a polyvinylidene difluoride membrane with 0.08 mA for 45 minutes. After blocking with milk solution (0.5% BSA, 1% milk powder) for at least 1 hour, the membranes were incubated with the primary antibodies (1:1000 dilution with 3% BSA) against mTOR, pmTOR, S6, pS6, S6K, pS6K, 4EBP1, p4EBP1 and $\beta$-Actin (Santa Cruz; Cell Signaling) at 4°C overnight. Membranes were washed three times with TBST and incubated for 1 hour with HRP-coupled anti-rabbit antibody (1:10000 dilution in 1% BSA, Santa Cruz Biotechnologies). The membranes were washed again three times with TBST for 10min and the proteins were detected using Santa Cruz Biotechnology Detection Kit following the instruction of the manufacture.

*Receipts:*

**Tris-HCl 1.5 M, pH= 8.8:**

18.2 g Tris
Σ 100 ml ddH2O, pH= 8.8

**Tris-HCL 0.5 M, pH= 6.8**

3 g Tris
Σ 50 ml ddH2O, pH= 6.8

**Lysis buffer:**

50 mM Tris-HCl, pH= 7.6
300 mM NaCl
0.5% Triton X-100
Σ 500 ml ddH2O

**4x Sample loading buffer:**

4 ml 10% Glycine
2 ml 2-Mercaptoethanol
0.92 g SDS
2.5 ml 1 M Tris-HCl (pH= 6.8)
Σ 10 ml ddH2O

**10x TBS:**

120 g Tris
90 g NaCl
Σ 1 l ddH2O, pH= 7.6

**1x TBST:**

100 ml 10xTBS
900 ml ddH2O
1 ml Tween 20 ( Bio-Rad)

**10x Laemmli-Electrophoresis buffer:**

30 g Tris
144 g Glycine
10 g SDS
Σ 1 l ddH2O

**Bjerrumbuffer with SDS:**

5.82 g Tris
2.93 g Glycine
0.375 g SDS
Σ 1 l ddH2O

**Bjerrumbuffer with Methanol:**

5.82 g Tris
2.93 g Glycine
200 ml Methanol
Σ 1 l ddH2O

**SDS-PAGE – 4.5% Collecting gel:**

1.56 ml ddH2O
0.281 ml Acrylamid
0.625 ml 0.5 M Tris-HCl, pH= 6.8
25 µl 20% SDS
12.5 µl 10% APS
2.5 µl TEMED

**SDS-PAGE – 10% Separating gel:**

3.65 ml ddH2O
1.875 ml Acrylamid
1.875 ml 1.5 M Tris-HCl, pH= 8.8
75 µl 20% SDS
5 µl 10% APS
5 µl TEMED

## 4.9 In vivo xenograft models

*Background:*

In cancer research the term xenograft transplantation referres to the transplantation of living malignant cells from one species to another. Generally it has become an indispensable in vivo method in tumor biology research and the screening of investigative anticancer drugs. In this study we used severe combined immunodeficiency (SCID) mice as hosts for HMM cell transplants to investigate the tumor reducing effects of temsirolimus and cisplatin on a mammalian system.

Due to a recessive mutation on chromosome 16 the humoral and cellular immune system of SCID mice fails to mature. Responsible for this immunodeficiency is the inactive catalytic subunit of DNA-PK (DNA-dependent protein kinase), an enzyme required for the non-homologous end joining (NHEJ) of DNA. Besides the repair of double-strand breaks, NHEJ is utilized for V(D)J recombination (Variable, Diverse, and Joining), a mechanism that nearly randomly combines and joins different genes resulting in a large variability of encoded proteins that match antigens from diverse pathogens and dysfunctional cells. Consequently SCID mice obtain an impaired ability to produce main players of the adaptive immune system such as B or T lymphocytes as well as to activate some components of the complement system. For this reason SCID mice cannot efficiently fight infections or reject tumor cell transplants what makes them ideal model organisms.

Fig. 37: *SCID mouse*

*Preparation:*

All procedures involving animals and their care were approved by the Ethic Review Board and performed following FELASA guidelines. 1x106 SPC 111 or P31 cells were injected in 200 µl serum free medium i.p. into female 5- to 7-week-old severe combined immunodeficiency (SCID) mice (in total N=80). Two weeks after inoculation, mice were assigned into treatment groups, each consisting of 5 or 10 mice: 1) treated with solvent; 2) temsirolimus (10 mg/kg) every day x 5 for two cycles with two days off;

3) cisplatin (1 mg/kg) once a week for two weeks; combined temsirolimus and cisplatin as aforementioned. Performance status and body weight were checked every second day. The first experiment was terminated after 38 days from transplantation when the first animal had to be sacrificed due to weight loss. To establish impact on survival, in another experiment all mice were kept up to individual tumor progression with weight loss and/or ascites development. Tumors i.p. were harvested, weighted and processed for histological investigations by routine methods.

# 5. RESULTS

## 5.1 Determination of constitutive mTOR activity

### 5.1.1 mTOR - phosphorylation status of MPM surgical specimens

Out of the 70 mesothelioma specimens analyzed, 43 (61.4%) exhibited epithelioid, 19 (27.1%) biphasic type and 4 (5.7%) sarcomatoid histology. Additionally, four patients with pseudomesotheliomatous adenocarcinomas were investigated in this study (5.7%). Fig. X shows expression of phosphorylated mTOR (p-mTOR) in representative cases of the different MPM histological subtypes detected by immunohistochemistry. Strong immunoreactivity was found predominantly in the epithelioid type tumors (Fig. 38A), while sarcomatoid cases were widely p-mTOR negative (Fig. 38C). A comparable restriction of p-mTOR immunoreactivity to the epithelioid histology was observed in the biphasic tumors (Fig. 38B). Quantitative evaluation by a scoring system confirmed a strong phosphorylation of mTOR in mesotheliomas harboring epithelioid histology compared to the sarcomatoid type (Fig. 39A). Interestingly, p-mTOR levels were particularly high in early-stage disease and tended to moderately decline in late-stage HMM (Fig. 39B). Overall patient survival did not correlate significantly with p-mTOR staining intensity but tended to be higher in p-mTOR-positive cases (Cox Regression: p=0.177) (Fig. 39C). This is not surprising considering the generally strong overexpression of p-mTOR in the epithelial subtype and in early-stage disease, both characterized by better prognosis [158, 159].

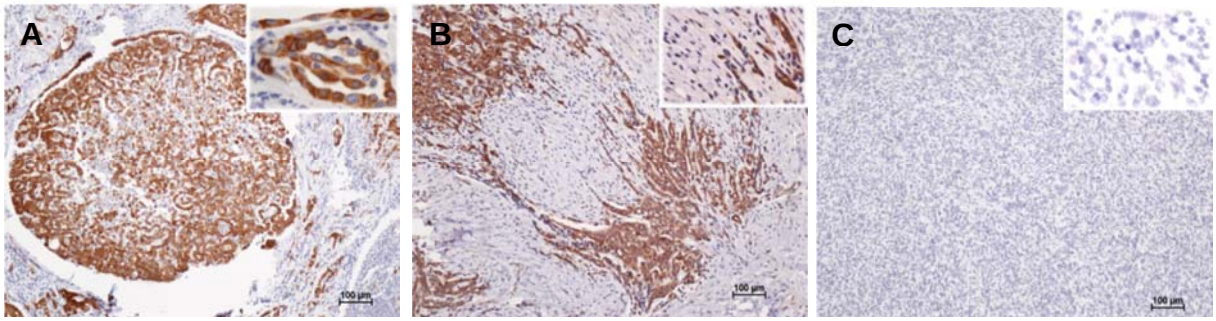



Figure 38. *mTOR phosphorylation in HMM.(A-C) Representative examples of human paraffin-embedded HMM surgical specimens of different histology immunostained for phosphorylated mTOR (brown) and counterstained with hematoxylin (nuclei in blue) (A, epitheloid; B, biphasic; C, sarcomatoid).*

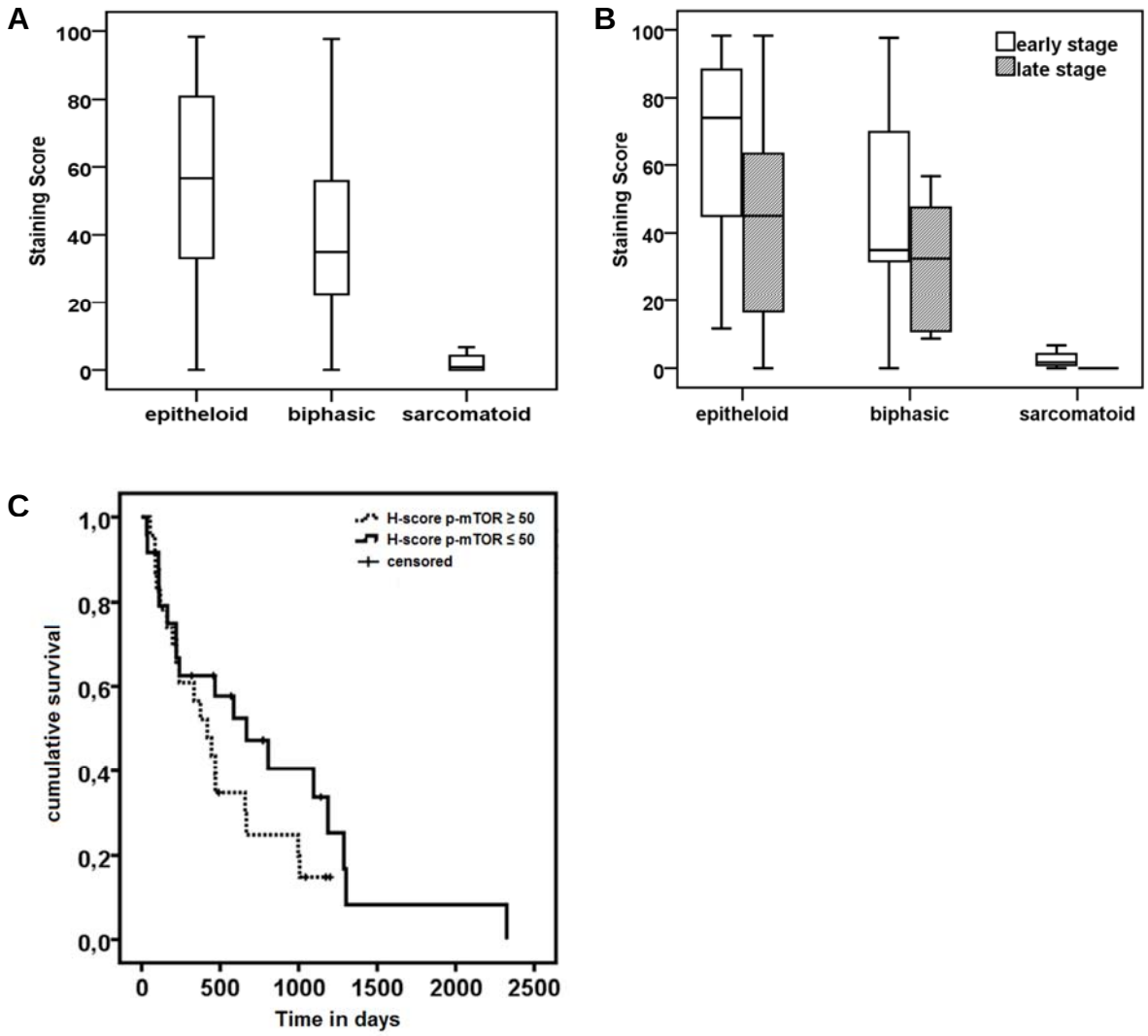



Figure 39. *(A-C) Evaluation of 70 patients was performed by H-score as described under Material and Methods. Hyper-phosphorylation of mTOR was predominantly detected (A) in tumors with epitheloid differentiation (mean staining score: 55±30 [epitheloid] vs. 2±3 [sarcomatoid]; p < 0.0001; unpaired t-test) and (B) in early stage epitheloid MPM (mean staining score: 66±27 [epitheloid early] vs. 46±31 [epitheloid late stage]; unpaired t test, p=0.031; not significant for all other early vs. late stage subgroup analyzes). (C) Kaplan-Meier curve shows cumulative survival of all patients grouped in relation to p-mTOR staining intensity [H-score p-mTOR ≤ 50 (dashed line); H-score p-mTOR ≥ 50 (continuous line);]. Overall patient survival did not correlate significantly with p-mTOR staining intensity (Cox Regression: p=0.177).*

### 5.1.2 The mTOR-pathway is activated in MPM cell lines

To determine if MPM cell cultures in vitro retain the observed activation of mTOR in MPM tumor specimens, five MPM cell models (SPC111, SPC212, NCI-H28, NCI-H2052, P31) and a cisplatin resistant subline of the latter cell line (P31res1.2) were investigated for activation of several mTOR pathway mediators by immunoblot (Fig.X). Generally, all investigated mesothelioma cell lines exhibited strong phosphorylation of mTOR corresponding to intense activation of the downstream effectors p70S6K, S6, and 4EBP1. mTOR activation was also present in two cell lines derived from biphasic mesothelioma (SPC111, SPC212) indicating that the in vitro cell cultures predominantly reflect the epitheloid histology of the original tumor. In the cisplatin-resistant subline of P31, phosphorylation of all investigated mTOR pathway mediators was equal or tended to be slightly reduced as compared to the parental cell line (Fig. 40).

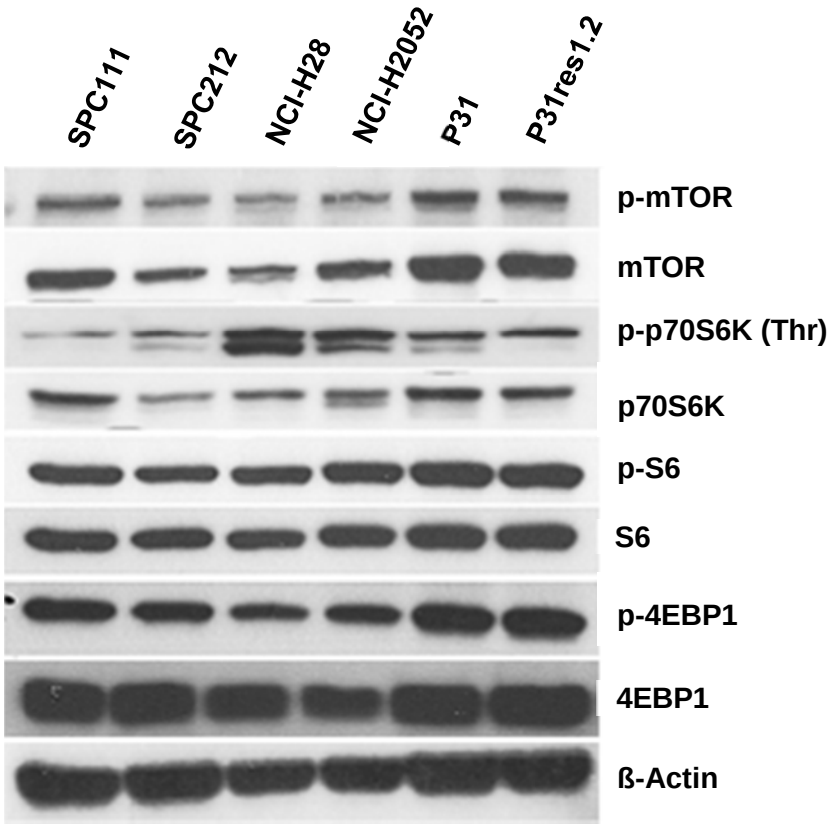



Figure 40. *Phosphorylation of mTOR and several downstream effectors as indicated in the investigated HMM cell lines by immunoblot.*

## 5.2 Comparison of standard chemotherapy and temsirolimus

### 5.2.1 Cisplatin as single agent against MPM cell lines

MTT proliferation assay of the investigated MPM cell models treated with cisplatin at concentrations from 0.5µM to 10µM for 72h resulted in hyper-sensitive to highly resistant effects. This was demonstrated by a broad variation of the half maximal inhibitory concentration ($IC_{50}$) as shown in Figure 41. The $IC_{50}$ value of the cisplatin-resistant sub-line p31res1.2 predictably could not be calculated in this setting and was higher than the maximum concentration of 10µM. A comparable minor response to cisplatin was also observed in NCI-H28 ($IC_{50}$ > 10µM), indicating an intrinsic cisplatin resistance. Including SPC212, with a relatively high $IC_{50}$ of 7.2µM, three of six cell lines were rather chemotherapy-resistant compared to the others in this analysis.

| Cell line | $IC_{50}$ values |
|---|---|
| SPC111 | 0.78µM |
| SPC212 | 7.2µM |
| NCI-H28 | > 10µM |
| NCI-H2052 | 3.3µM |
| P31parental | 3.9µM |
| P31res1.2 | > 10µM |

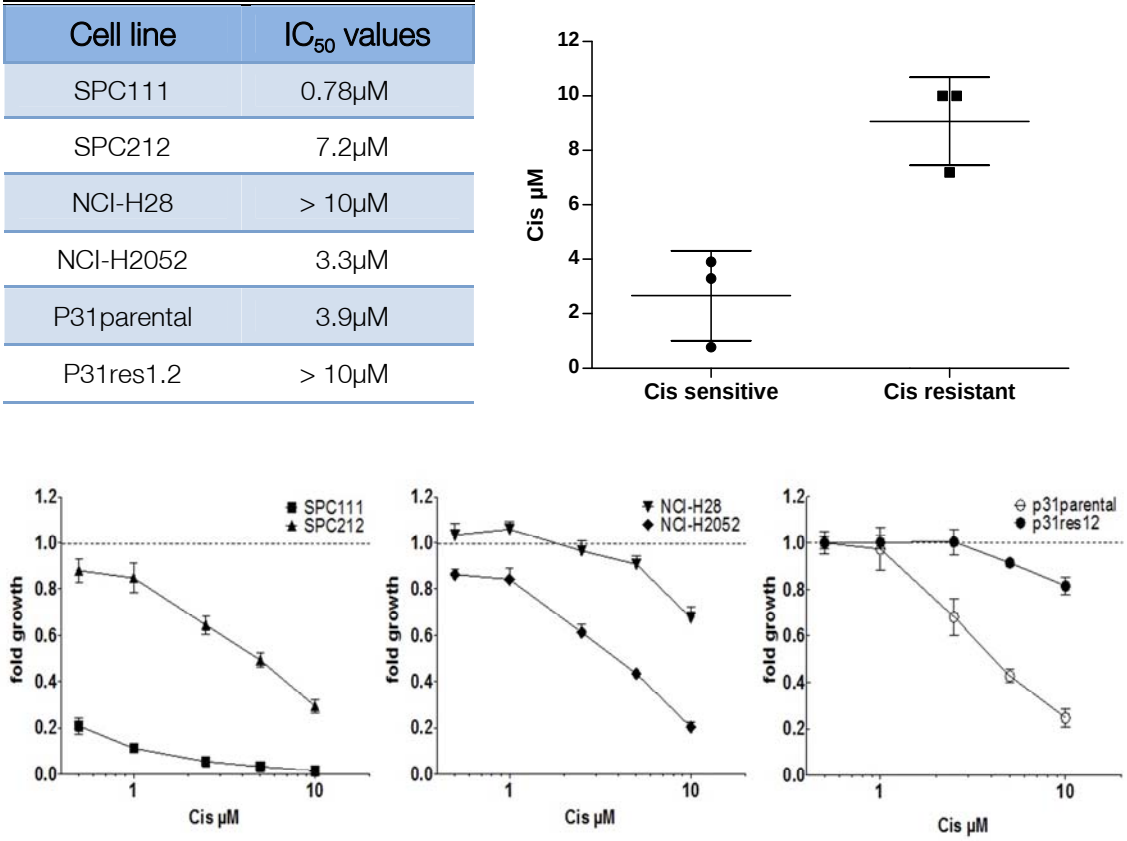



Figure 41. *Cisplatin-$IC_{50}$ values (upper panel) and changes in viability after 72h continuous drug exposure (lower panel) were measured by MTT assay. Data are given normalized to the untreated controls.*

### 5.2.2 Pemetrexed as single agent against MPM cell lines

All MPM cell models were treated with pemetrexed at concentrations from 50µM to 200µM for 72h within a MTT proliferation assay (Fig. 42) that demonstrated strong deviating results. The cell lines SPC212, NCI-H28 and P31parental showed no significant changes in cell viability and a stabilized cell number even at higher concentrations. With regard to SPC111 pemetrexed obtained moderate growth inhibitory effects but also in this case it was not possible to define an IC50 value. The number of viable cells was significantly reduced only in NCI-H2052 and P31res1.2 with $IC_{50}$ values already beyond the minimal dose. Interestingly those cell lines which obtained nearly no response to pemetrexed also showed high resistance to cisplatin (compare Fig. 41). This fact is highlighted by the cell models P31parental and P31res1.2, indicating a clear association between acquired cisplatin resistance and an arising insensitivity to pemetrexed.

| Cell line | $IC_{50}$ values |
|---|---|
| SPC111 | > 200µM |
| SPC212 | > 200µM |
| NCI-H28 | > 200µM |
| NCI-H2052 | 20µM |
| P31parental | 40µM |
| P31res1.2 | > 200µM |

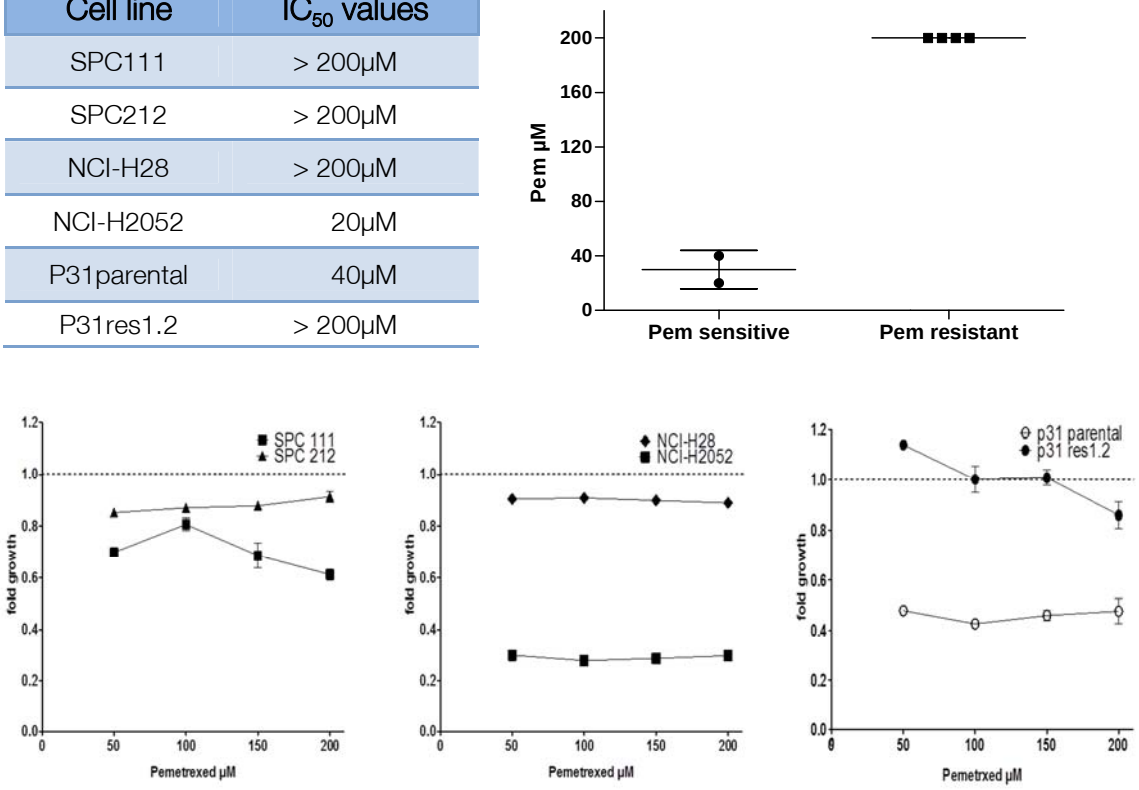



Figure 42. *Pemetrexed-$IC_{50}$ values (upper panel) and changes in viability after 72h continuous drug exposure (lower panel) were measured by MTT assay. Data are given normalized to the untreated controls.*

### 5.2.3 Temsirolimus as single agent against HMM cell lines

Based on the strong constitutive activation of mTOR in vitro, we set out to investigate the quality of this oncogenic molecule as a therapeutic target in HMM. The applied cell lines were treated with temsirolimus at concentrations from 1 to 250ng/ml for 72h within a MTT proliferation assay (Fig. 43) and resulted in two groups, with one more sensitive than the other. Two of six cell models showed high sensitivity to temsirolimus already at concentrations below 10ng/ml (SPC212, IC50 8.9; p31res1.2, IC50 9.8). In this application schedule the other cell lines (NCI-H28, SPC111, p31parental) led to a moderate to distinct but in all cases significant reduction of viable HMM cells. However, this assay obviously displayed that the cell lines with high resistance to cisplatin (NCI-H58, SPC212, p31res.1.2; compare chap. 5.2.1) as shown previously, were the most sensitive to temsirolimus.

| Cell line | $IC_{50}$ values |
|---|---|
| SPC111 | > max dose |
| SPC212 | 8.9ng/ml |
| NCI-H28 | > max dose |
| NCI-H2052 | > max dose |
| P31parental | > max dose |
| P31res1.2 | 9.8ng/ml |

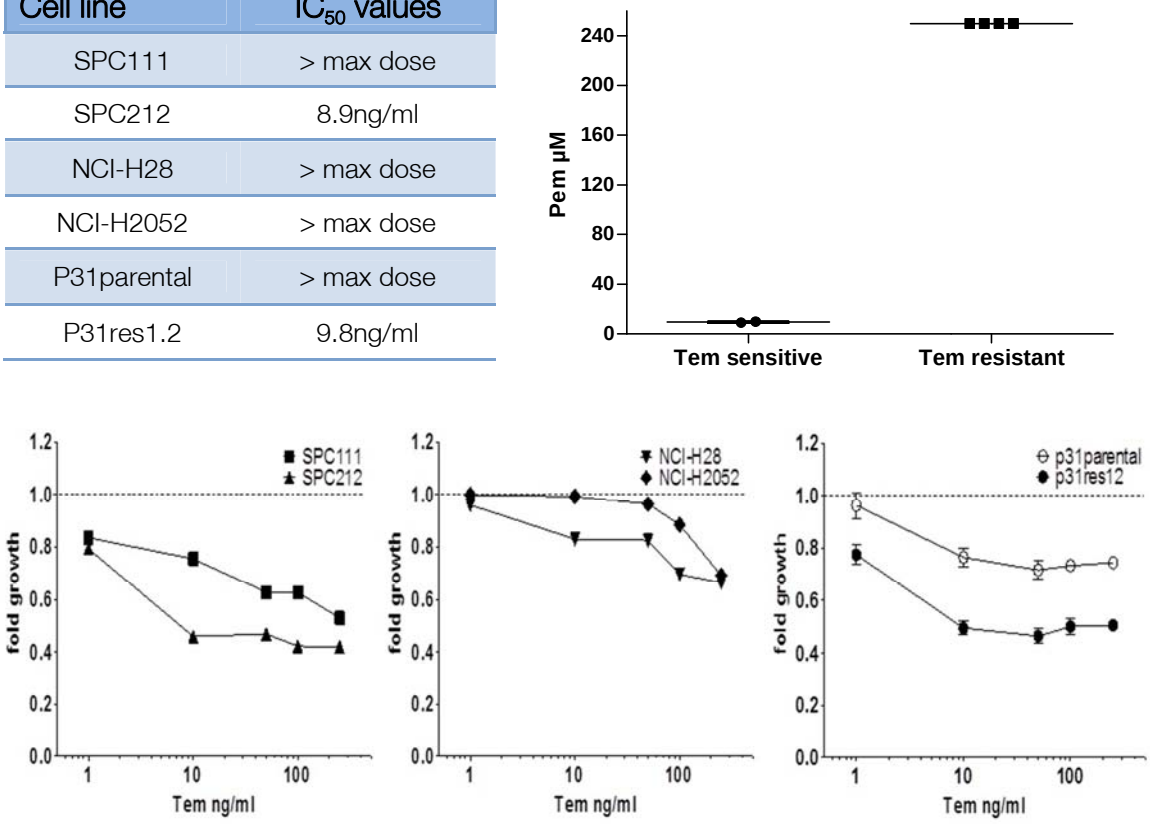



Figure 43. *Temsirolimus-$IC_{50}$ values (upper panel) and changes in viability after 72h continuous drug exposure (lower panel) were measured by MTT assay. Data are given normalized to the untreated controls.*

## 5.3 Investigation into the mode of action of temsirolimus

### 5.3.1 Analyses of genetic aberrations in MPM cell models

A main characteristic of cancer cells represents their genomic instability causing chromosomal aberrations including gains and losses of a multitude of different genes. Gene dose can play an important role in aberrant cellular signaling and may lead to pathway dysfunction that seriously contributes to the malignant progress and can become indispensable for continuous tumor growth. The distinct dependence of tumors on specific pathways reflects one of many determinants responsible for individual responsiveness to certain therapeutic agents of malignant cells even within the same type of cancer. Because this factor represents a possible source of the diverse response characteristics to temsirolimus, we performed aCGH analyses of the investigated cell lines to detect potential alterations of specific genes coding proteins involved in PI3K and mTOR signaling. Although analyses showed generally instable karyotypes and distinct variations of genomic aberrations between all cell models, there were no significant gains or losses detected in genes coding for mTOR, P70S6K, S6 and 4EBP1 (Fig. 44). The same result was obtained when analyzing further key molecules involved in mTOR signaling such as TSC1, TSC2, Rheb, and different PI3K isoforms (data not shown). Also the gene encoding for AKT1 was predominantly not affected by alterations in the investigated cell lines, except in NCI-H2052, which obtained a significant loss of this loci (Fig.44). This may represents one aspect contributing to the high sensitivity to cisplatin of this cell model, thus AKT signaling is thought to be essential for overcoming cell death induction after treatment with platinum based chemotherapy [160].

Besides genes encoding for key players of the mTOR pathway, we investigated for genetic aberrations frequently observed in HMM. Homozygous deletion of 9p21 has been reported as one of the most common deletions in malignant mesothelioma [71, 161]. This locus is located within a cluster of genes including *CDKN2A* (Cyclin-dependent kinase inhibitor 2A, p16) and *CDKN2B* (Cyclin-dependent kinase 4 inhibitor B), two tumor suppressor genes which play an important role in cell cycle regulation. Up to 72% of investigated HMM cell lines and tumor specimens have been identified to

obtain a *CDKN2A* deletion, which probably makes it to a reliable biomarker of this malignancy [162, 163]. Our analyses were absolutely consistent with these findings and showed that 5 of 6 cell lines (P31partental, P31res1.2, SPC212, NCI-H28 and NCI-H2052) had significant deletions of the 9p21 locus. Besides *CDKN2A*, also the adjacent gene encoding *CDKN2B* was absent in NCI-H28 and NCI-H2052, the latter showing further extensive deletions of neighboring loci indicating a complete loss of this chromosomal region. Only in SPC111 the 9p21 locus showed no significant aberrations compared to all other cell lines.

Another frequently identified target of genomic alteration associated with HMM represents *NF2* (Neurofibromin 2, Merlin), which is reported to be mutated in approximately half of the cases [162]. This tumor suppressor gene encodes for Merlin, a membrane-cytoskeleton scaffolding protein, which is thought to play a critical role in the inhibition of cell proliferation and motility through mediating contact-dependent growth arrest [164, 165]. In this study we could not detect any significant aberrations of the *NF2* locus (22q12.2) in none of the evaluated cell lines which indicates that the NF2 involving pathway rather plays a secondary role in the pathology of present cell models. Further aCGH analyses with regard to other important tumor suppressor genes such as *TP53* and *PTEN* let us draw the same conclusion. Although both, *TP53* and *PTEN* are among the most frequently observed deletions in a multitude of different cancer types, this study revealed no alterations of the involving loci in any investigated cell line (Fig. 45).

Multiple receptor tyrosine kinases including the epidermal growth factor receptor (EGFR), hepatocyte growth factor receptor (HGFR/MET) and insulin-like growth factor 1 receptor (IGFR), are known to be frequently overexpressed in various types of cancer [166]. The highly expression of these oncogenes has also been reported in the case of HMM [167-169]. However, aCGH analyses in this study of before mentioned growth factor receptors (Fig.45) and associated ligands (data not shown), revealed no significant gains of the involving genes.

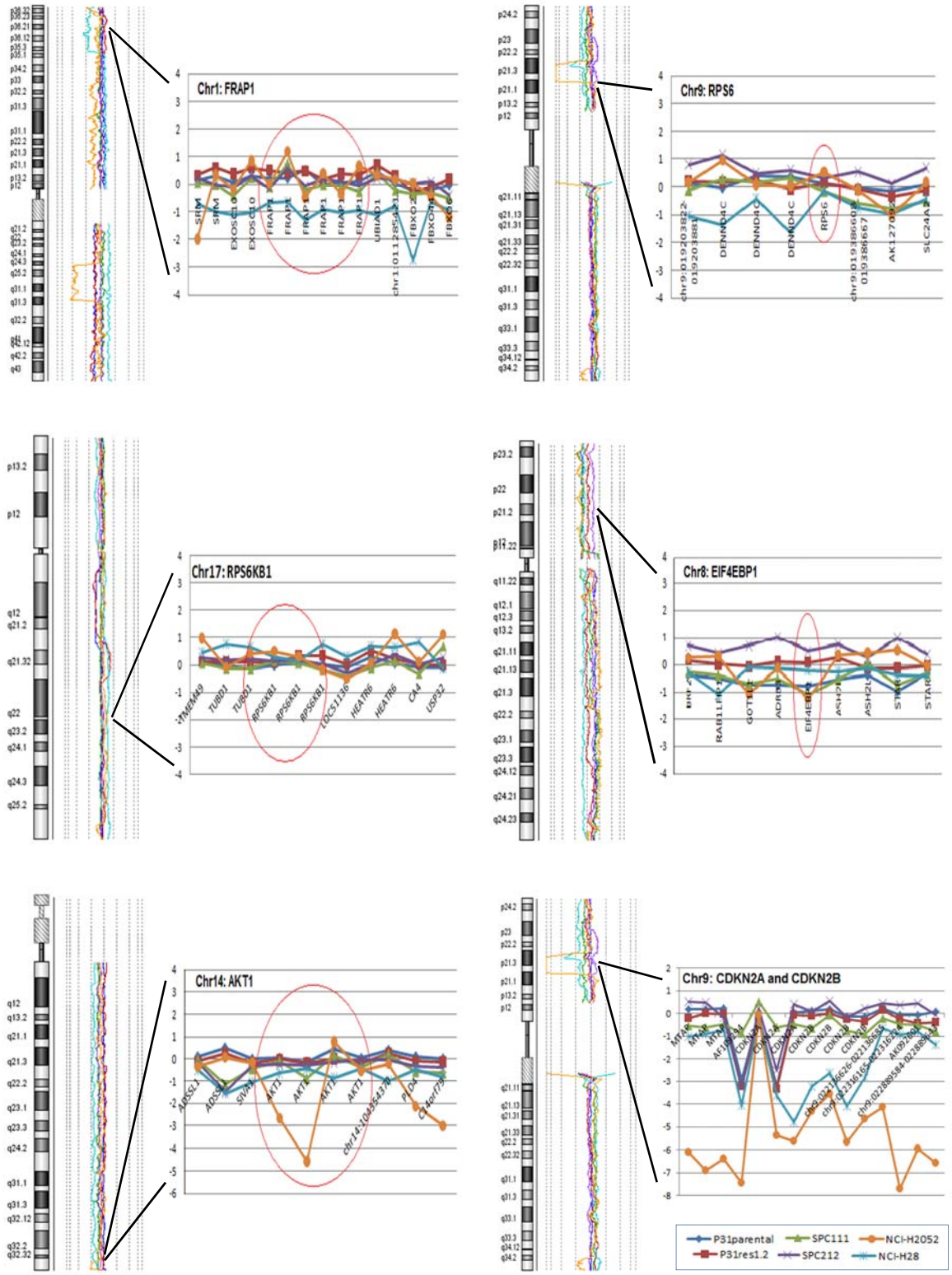



Figure 44. *aCGH analyses of investigated cell lines. Genes encoding the key molecules involved in mTOR signaling FRAP1 (mTOR), RPS6KB1 (S6K) and EIF4EBP1 (4EBP1) showed no significant alterations. Also AKT1, which belongs to the main mTOR networking pathways, was only significantly lost in NCI-H2052.*

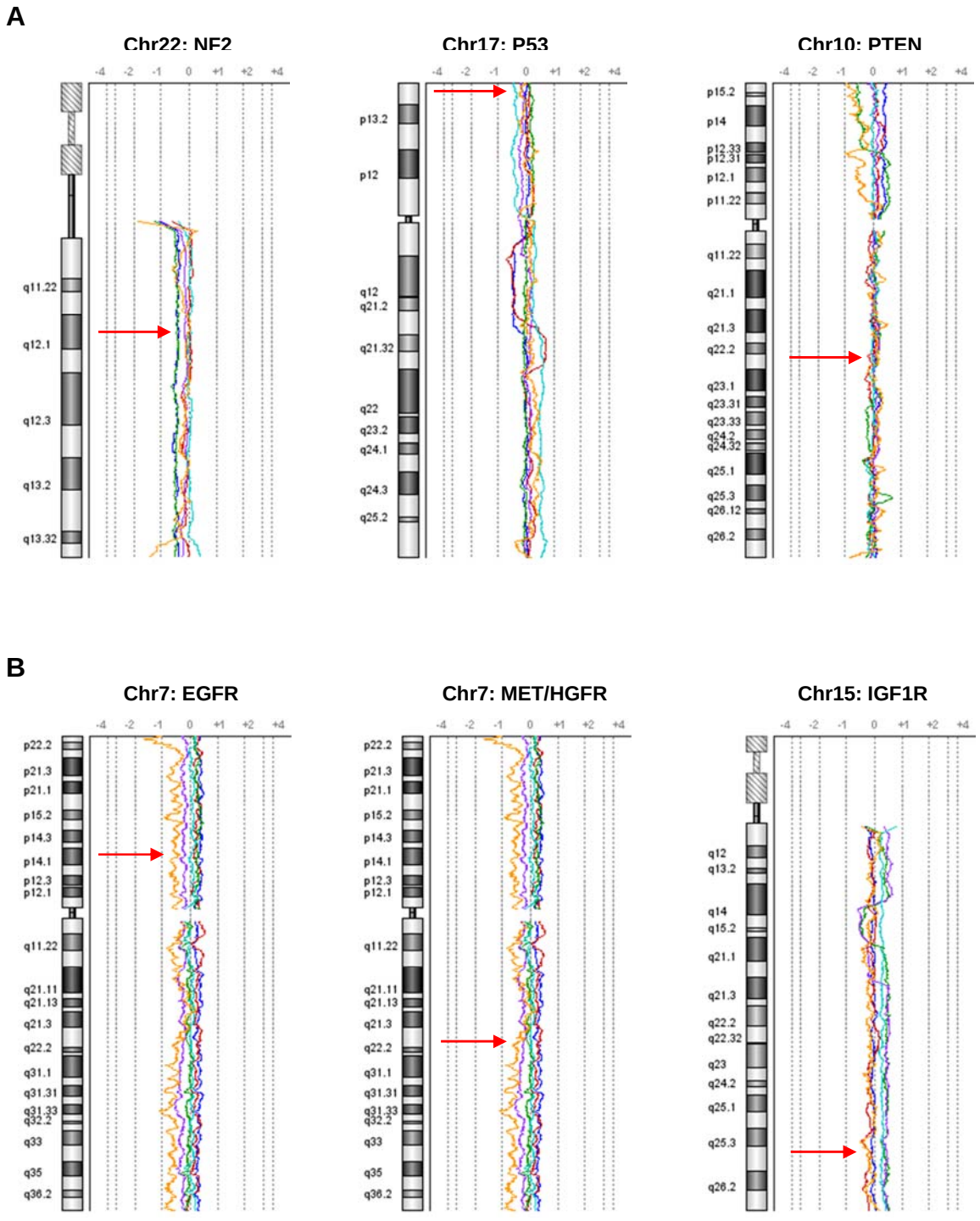



Figure 45. *Frequently observed genetic alterations in caner. (A) The tumor suppressor genes encoding NF2, p53 and PTEN showed no significant alterations. (B) An extract of investigated oncogenes. EGFR, HGFR(MET) and IGF1R showed no significant gene amplifications of these loci.*

### 5.3.2 Effects of temsirolimus on DNA synthesis

To gain more insight into the growth-inhibitory activity of temsirolimus especially in the cisplatin-resistant MPM cell models, impact on DNA synthesis was measured by $^{3}$H-thymidine incorporation. The cell models p31parental, p31res1.2, SPC111 and SPC212 were exposed for 24h to temsirolimus (range; 1-250ng/ml) and then incubated for 2h with radioactive labeled thymidine. DNA synthesis was only marginally affected in cisplatin-sensitive cell lines (SPC111, p31parental). In contrast cells more resistant to cisplatin (SPC212, p31res1.2) showed a clear tendency of decreased thymidine incorporation and a consequently reduced DNA synthesis.

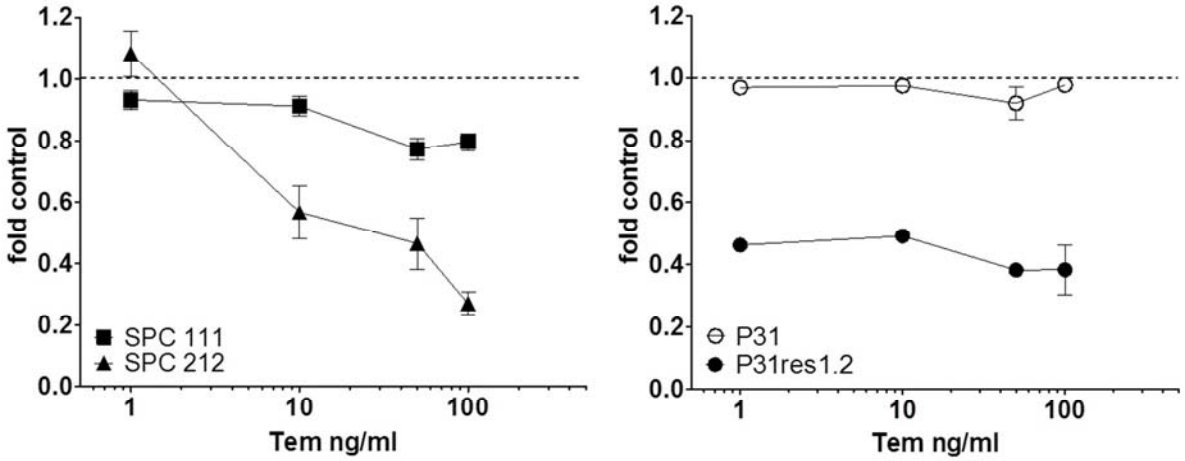



Figure 46. *Concentration-dependent effects of a 24 h drug temsirolimus exposure on DNA-synthesis determined by [$^{3}$H]-thymidine incorporation assay are given normalized to untreated controls. DNA synthesis inhibition was significantly stronger in the cisplatin-resistant subline P31res1.2 as compare to the parental cell line ($p < 0.0001$, two way ANOVA).*

### 5.3.3 Impact of temsirolimus on non-adherent, multicellular HMM spheroid formation

In a variety of malignancies serum-independently and non-adherently growing multicellular spheroids have been shown to be enriched in a subpopulation of cells displaying increased tumor-initiating capacity . In order to investigate whether inhibition of mTOR by temsirolimus affects this cancer stem cell-like cell population we have generated spheroid cultures from two mesothelioma cell lines SPC111 (Fig. 47 left) and SPC212 (not shown). Importantly, temsirolimus treatment already after four days caused progressive loss of HMM spheroid integrity (Fig.47 right upper panel) and significantly decreased number as well as mean diameter of spheroids (Fig. 47 right lower panel). Considering all these parameters it is apparent that temsirolimus treatment has a distinct impact on the tumor-initiating cell compartment of HMM cell lines.

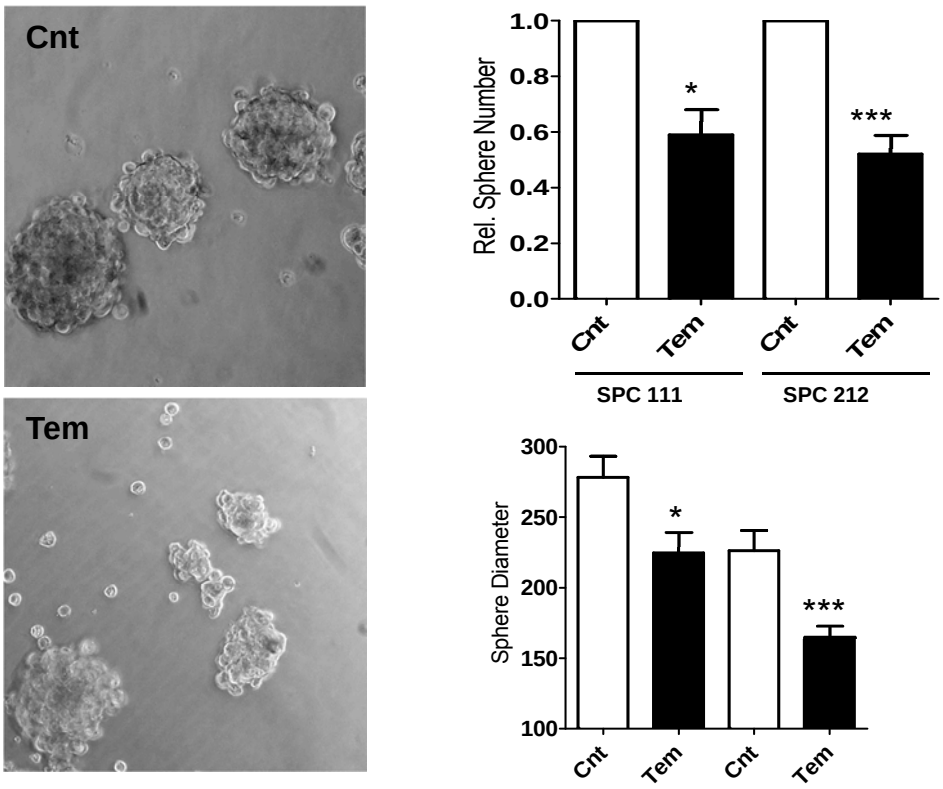



Figure 47. *Effects of temsirolimus exposure (100ng/ml) on non-adherent spheroid formation of HMM cells were determined after 96 h drug exposure. Representative photomicrographs of SPC111 cells are shown in the left panel elucidating the loss of spheroid integrity by mTOR inhibition. Impacts on spheroid size and sphere number are indicated in the right panels. In all cases, data are means of at least 3 independent experiments; bars, SD; *, $p < 0.05$, ***, $p \leq 0.0005$, significantly different from non-treated cells.*

### 5.3.4 Cell cycle alterations induced by temsirolimus

To elucidate whether temsirolimus induces alterations in cell cycle distribution, we performed cell cycle analysis using PI staining and flow cytometry after 48h drug treatment. In this analysis we focused again on the differences between rather cisplatin sensitive (SPC111, p31parental) and cisplatin resistant cell lines (SPC212, p31res1.2) to gain more insight into the striking inverse relationship with respect to temsirolimus sensitivity that became obvious in previous experiments. Compared to these significant differences as e.g. demonstrated by the impact of temsirolimus on $^{3}$H-tymidine incorporation primarily in cisplatin-resistant cell models (compare Fig. 46), the effects of mTOR inhibition on cell cycle distribution in investigated MPM cell models was rather moderate. However, analysis showed a slight increase in G0/G1 phase and decrease in S-phase of cell lines representing the rather cisplatin resistant and temsirolimus sensitive models (SPC212, P31res1.2; Fig.48). In contrast cell lines with less sensitivity to temsirolimus showed almost no changes of populations in G0/G1 phase but a distinct increase of the S-phase fraction. Nevertheless, with regard to cell models obtaining increased G0/G1 phase, these results demonstrate the expected effects on temsirolimus-sensitive cell models, as mTOR-inhibition is known to prevent transition into S-phase [170].

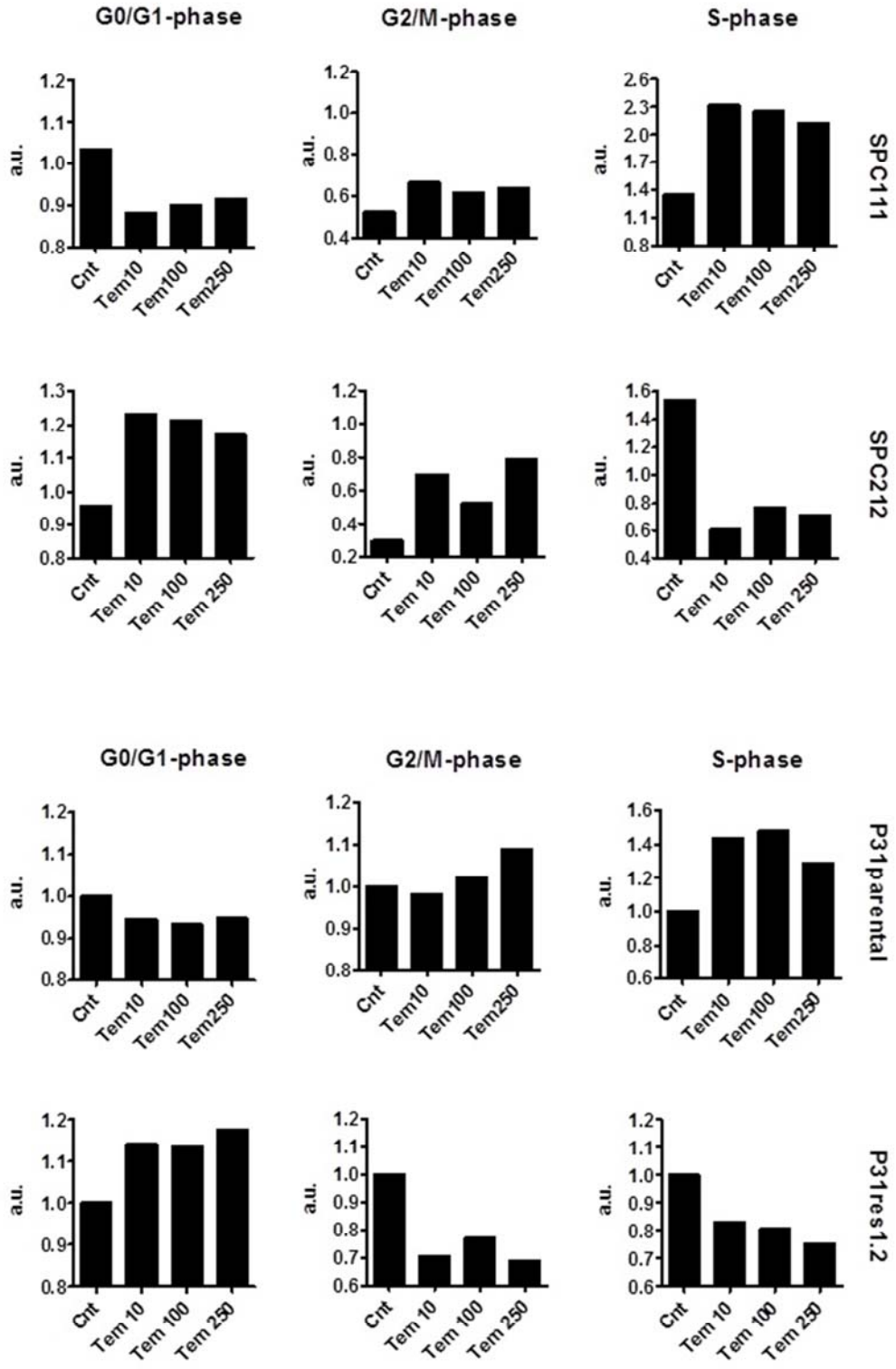



Figure 48. *Influence of temsirolimus treatment as single agent at the indicated concentrations, on cycle progression in HMM cells was determined by FACS analyses after 48h continuous drug exposure. Cell models are compared to appropriate temsirolimus sensitivities [SPC111(Tem-resistant) vs SPC212 (Tem-sensitive), upper panel; P31parental (Tem-resistant) vs P31res1.2 (Tem-sensitive)](a.u=arbitrary unit).*

## 5.4 Combination tests with temsirolimus and cisplatin

### 5.4.1 Impact on cell viability

The following assays were performed to investigate whether targeted inhibition of mTOR by temsirolimus in combination with cisplatin might exert synergistic antiproliferative effects. To implement a possible relation between growth inhibition and time of drug exposure we analyzed changes in cell viability by 72 h MTT survival assays (short-term) (Fig.49) and ~7d clonogenic assays (long-term) (Fig.51). The MTT assays showed that the combined treatment of temsirolimus and cisplatin overall significantly decreased the number of viable cells even at low concentrations demonstrating a stronger effect in all HMM cell lines than that of cisplatin or temsirolimus alone. In all investigated cell models, predominantly synergistic anticancer effects were observed independent of the responsiveness of the HMM cell lines to both single agents (compare Fig. 41 and 43). This was validated by the calculation of combination indices (CI) (compare Material and Methods) whereby values < 0.9 indicate synergism, >1.1 antagonism, and all other values additive activity (Fig. 50). However, the strongest synergistic activity of both agents was observed in cell lines with rather cisplatin resistance (SPC212, NCI-H28, and P31res1.2).

Clonogenic survival assays were performed with SPC111, SPC212, P31parental and P31res1.2 and confirmed both the synergistic anticancer activity of the combined treatment and the enhancement of temsirolimus sensitivity by induction of cisplatin resistance (Fig. 51). Besides the observed inverse responsiveness of investigated cell lines to both agents the combination of temsirolimus and cisplatin within this long-term analysis inhibited the proliferation of cell clones up to 99% compared to the untreated control.

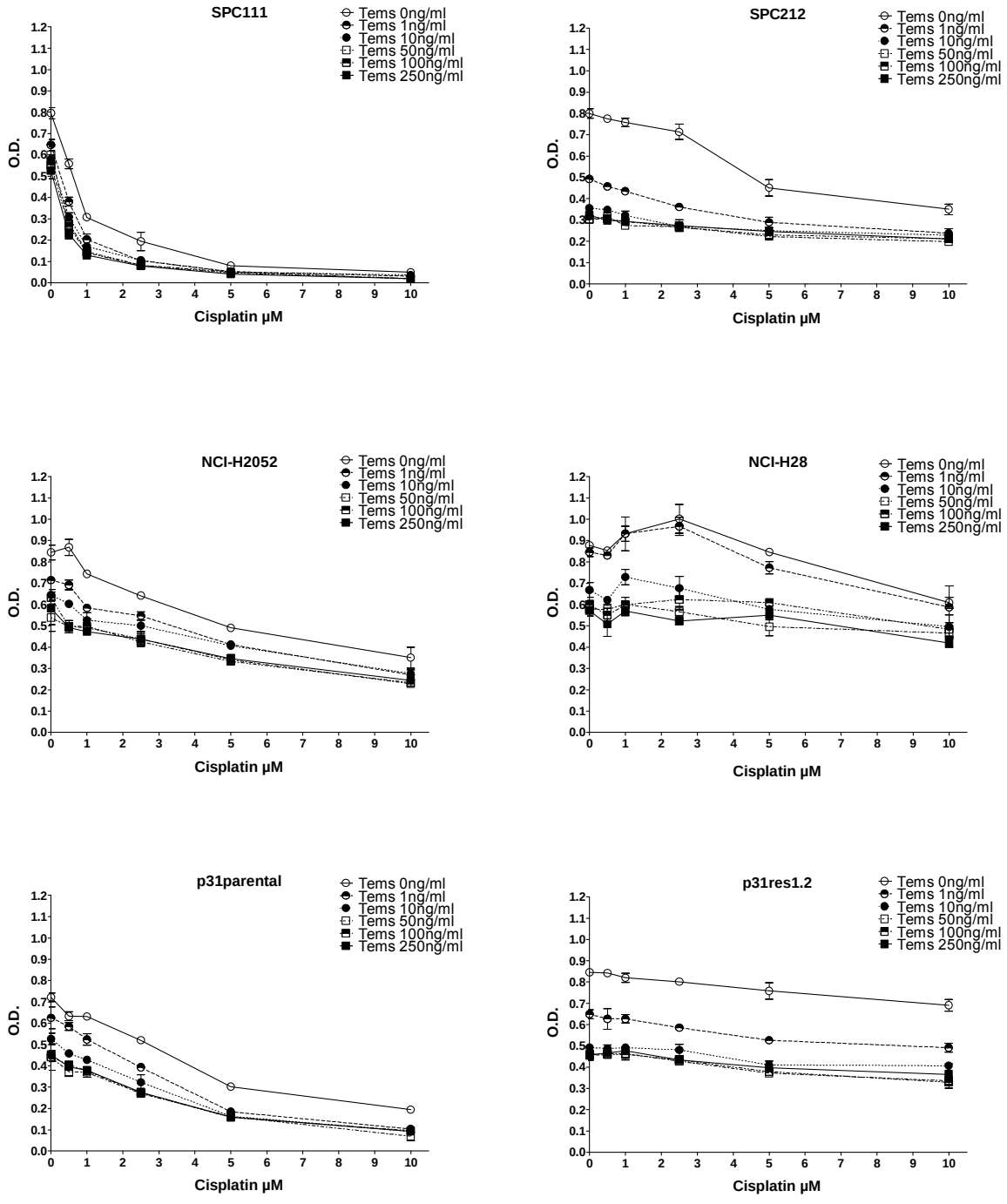



Figure 49. *Activity of temsirolimus (Tem) combined with cisplatin (Cis) against human HMM cell lines after 72h exposure. Changes in cell viability were measured by MTT assay.*

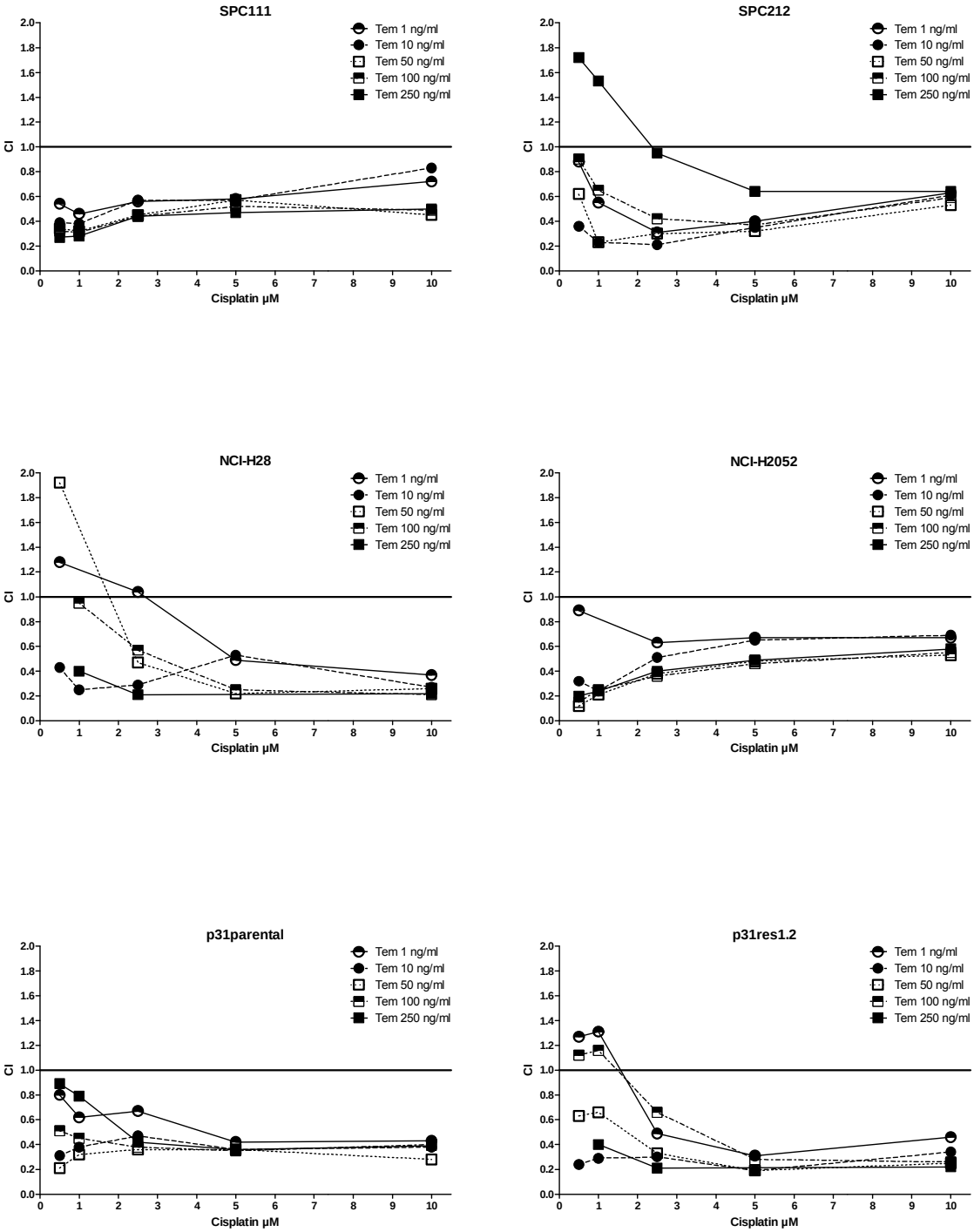
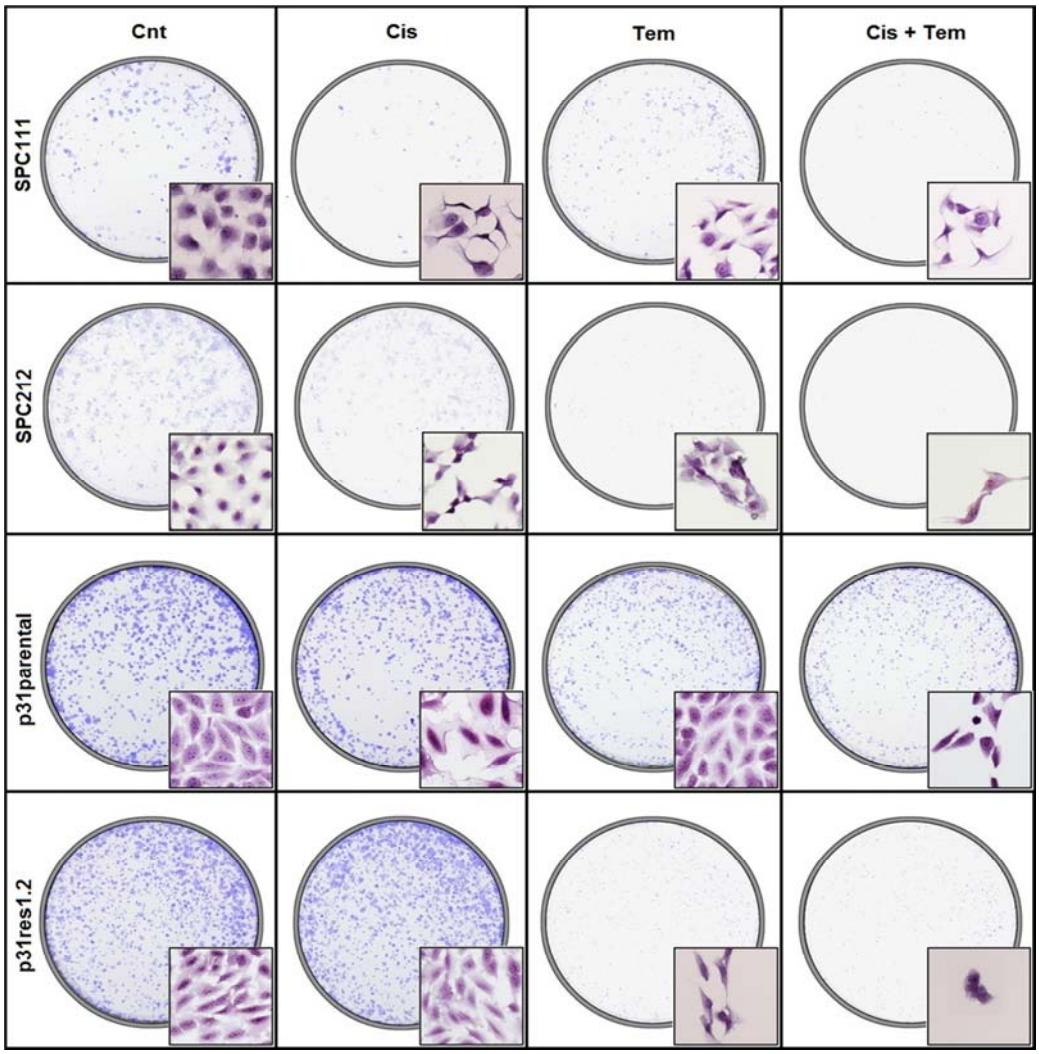



Figure 50. *Data from Fig.49 are given as combination indices (CI) calculated following the method of Chou and Talalay by using CalcuSyn Software. Values < 0.9 represent synergism, 0.9-1.1 additive activities, and > 1.1 antagonism.*



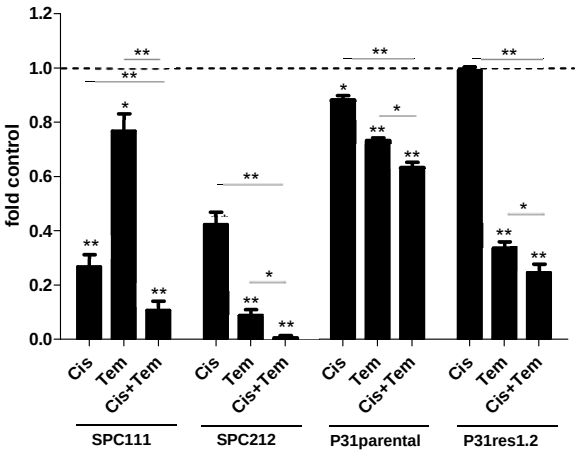




Figure 51. *Effects of long-term exposure to temsirolimus as single agent and in combination with cisplatin on colony formation of SPC111, SPC212, P31 and P31res1.2 were established. Cell lines sensitive to cisplatin (SPC111 ;P31res1.2) were treated with 1μM those comparably resistant (SPC212; P31res1.2), with 2.5 μM cisplatin. Temsirolimus was used at 100ng/ml. After seven days continuous drug exposure cells were stained with crystal violet, representative photomicrographs were taken (left) and colonies were counted. Data from three experiments are given normalized to controls (right). Statistic bars, SD; **, $p < 0.05$, ***, $p \leq 0.0005$, significantly different from non-treated cells. Significance levels immediately upon the SD bars represent comparisons with the untreated control.*

### 5.4.2 Impact on apoptosis induction

In order to investigate the role of apoptosis induction on the synergism of temsirolimus and cisplatin, Hoechst-propidium iodide staining of exposed MPM cells was performed and the proportion of cells with condensed chromatin counted (the P31 resistance model is representatively shown in Fig. 52). As expected, cisplatin at 5µM induced strong G2/M cell cycle arrest (large nuclei in the Hoechst staining) in both cell models but apoptosis predominantly in the parental cell line (quantification in Fig.53). Temsirolimus (250ng/ml) induced only in the cisplatin-resistant subline a significant increase of apoptotic cells, thus again confirming the inverse relationship of chemotherapy and mTOR inhibition response. Apoptosis induction was dramatically enhanced especially in P31res1.2 by combination of both agents inducing up to 75% apoptotic nuclei. This was also reflected by analysis of cleavage of the caspase substrate Poly (ADP-ribose) polymerase (PARP) which represents a notable marker for apoptosis. This enzyme is involved in a number of cellular processes such as DNA-repair and becomes cleaved by proteolytic degradation in cells sustaining extensive DNA damage and initiating programmed cell death.

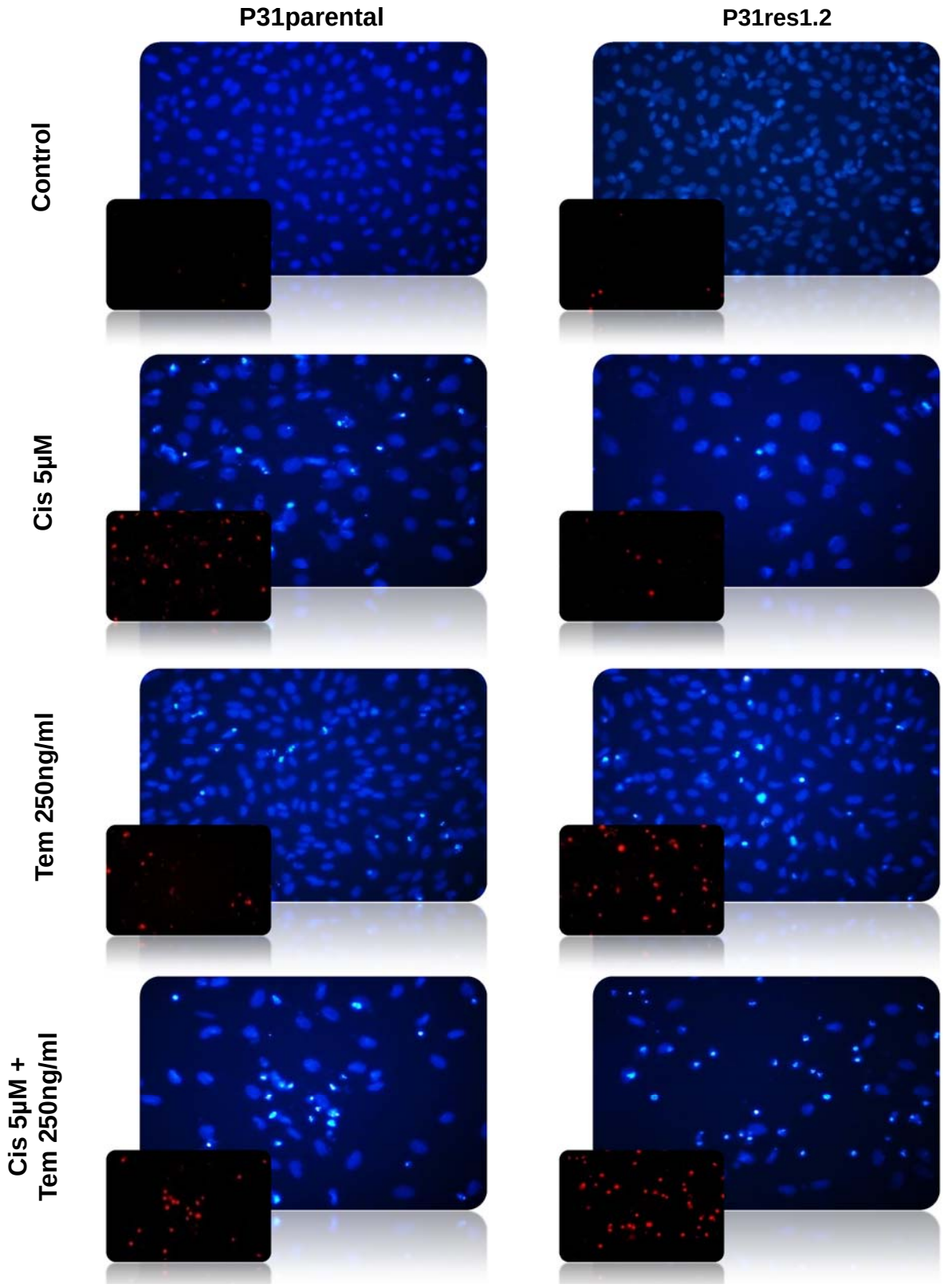



Figure 52. *Representative photomicrographs show impact of temsirolimus and cisplatin as single agents or in combination on apoptosis induction after 48h drug treatment. (large pictures show Hoechst-stained nuclei of all cells (viable/apoptotic/dead); small pictures show PI-stained nuclei of dead cells. For quantitative analyzes see Fig.53.*

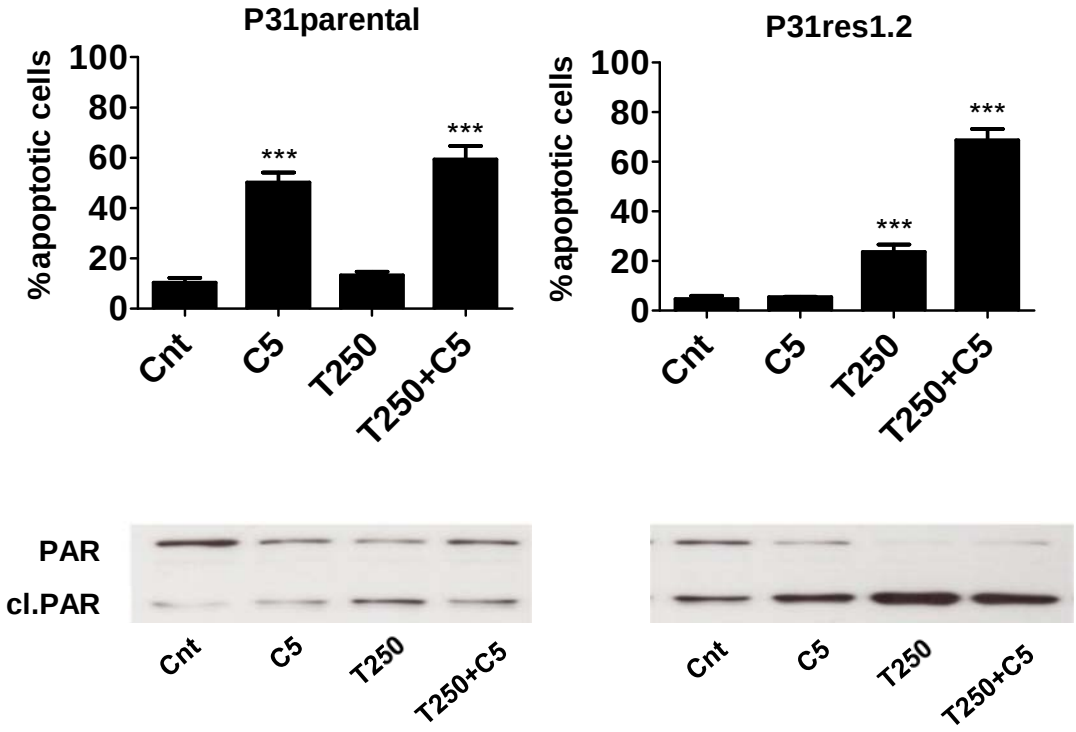



Figure 53. *Quantitative analyzes of apoptotic rates were determined by fluorescence microscopic evaluation of Hoechst 33342-stained P31 and P31res1.2 cells after 48h drug exposure (upper panel). Data show the mean percentage of apoptotic cells of at least 3 independent experiments (bars, SD; *, $p < 0.05$, ***, $p \leq 0.0005$). Lower panel demonstrates cleavage (cl. PARP) of the caspase-substrate PARP as an indicator of apoptotic cell death detected by immunoblot.*

### 5.4.3 Impact on cell cycle distribution

To identify alterations in cell cycle distribution induced by the combination of mTOR inhibition and chemotherapy, MPM cell lines were treated with temsirolimus and cisplatin at different concentrations for 48h before performing cell cycle analyses by PI staining and flow cytometry (Fig. 54). Cisplatin as single agent induced a severe and almost complete G2/M arrest in SPC111, SPC212 and P31parental already at 2.5 µM, while P31res1.2 which represents the cell line with highest cisplatin resistance showed approximate results only at the maximum concentration (5µM). Interestingly, in responsive cell models (SPC111, P31), G2/M arrest was less pronounced at 5 µM and

paralleled by an increase of cells arrested in S-phase or G0/G1-phase in SPC212 respectively. As described before (s.ch. 4.3.3), temsirolimus as single agent showed rather moderate effects on cell cycle alterations but in combination with chemotherapy distinctly inhibited G2/M cell cycle arrest by cisplatin leading to an enhanced accumulation of cells in S-phase and/or G0/G1-phase. Only in the cisplatin-resistance subline P31res1.2 this effect was less distinct.

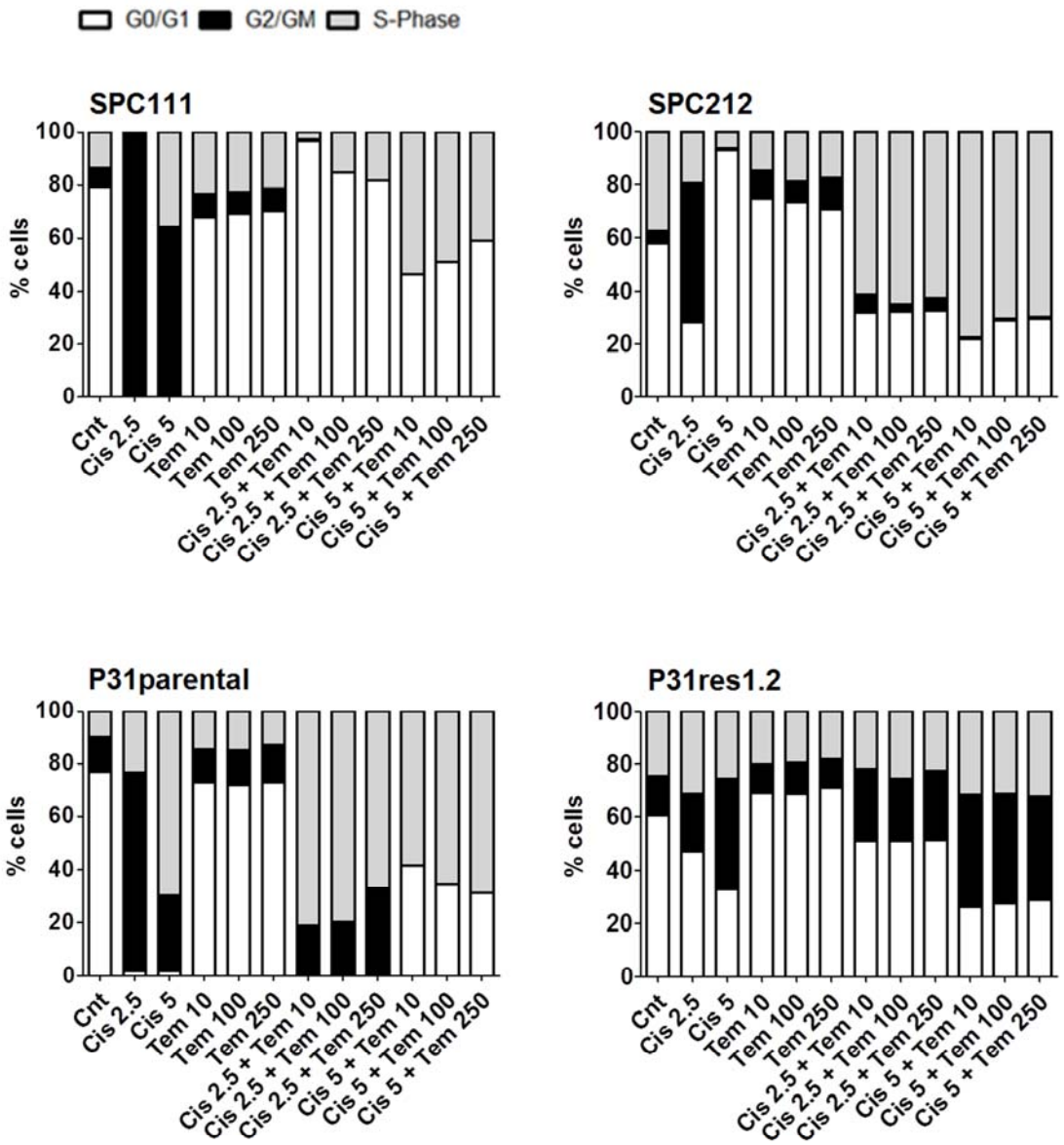



Figure 54. *Influence of temsirolimus and cisplatin treatment, both as single agent and in combination at the indicated concentrations, on cycle progression in HMM cells was determined by FACS analyses after 48h continuous drug exposure.*

### 5.4.4 Impact on molecular changes

In order to elucidate the molecular basis of the observed synergisms between mTOR inhibition and chemotherapy, the impact of temsirolimus and cisplatin alone and in combination on the activation of mTOR and downstream signal mediators was investigated in HMM cell lines by immunoblot (Fig. 55). Temsirolimus induced a distinct downregulation of p-mTOR but also total mTOR which was especially pronounced in temsirolimus-sensitive cell lines (SPC212, P31res1.2). Accordingly, phosphorylation of the downstream signal mediators was reduced significantly (p-P70S6K, p-4EBP1) or almost completely (p-S6). Cisplatin enhanced blockade of mTOR activation and downstream signals by temsirolimus especially at the level of 4EBP1. This synergistic effect was notably apparent in the temsirolimus-resistant cell lines (SPC111, P31parental).

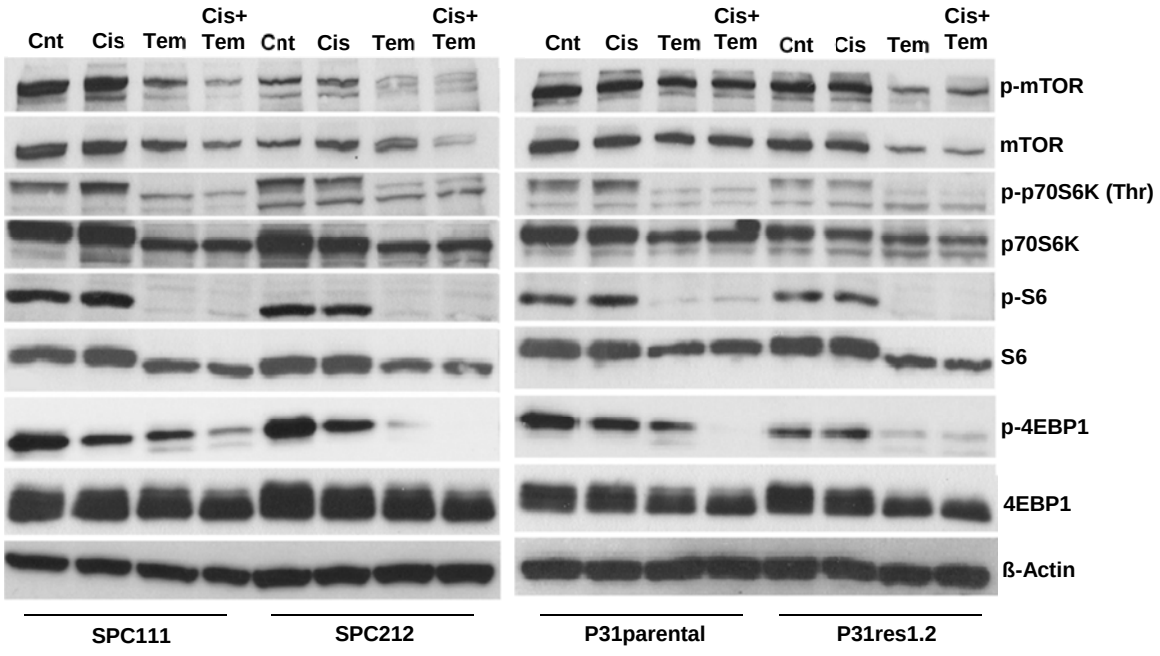



Figure 55. *Expression and phosphorylation state were analyzed for mTOR and its downstream mediators as indicated by Western blotting. β-actin antibody served as loading control.*

## 5.5 In vivo experiments

### 5.5.1 Effect of temsirolimus as single agent on HMM xenografts in SCID mice

To examine whether the in vitro activity of temsirolimus against MPM cells translates into in vivo anticancer effects, HMM cells were xenografted intraperitoneally in SCID mice. SPC111 and P31parental were tumorigenic leading to intraperitoneal tumor seed and hence used for subsequent experiments. Two weeks after tumor cell inoculation mice were randomized into two treatment groups receiving either solvent or temsirolimus as described in the *Material and Methods*. Drug treatment was well tolerated by all mice with no notable toxicity through the entire experiment. In the first experiment accomplished with SPC111, tumor weight was measured in all animals 38 days after transplantation when the first control animal had to be sacrificed. Temsirolimus led to a noticeable decrease of the intraperitoneal tumor burden (Fig. 56A) demonstrated by a significant weight reduction of harvested tumor nodes compared to the untreated control (Fig. 56B).

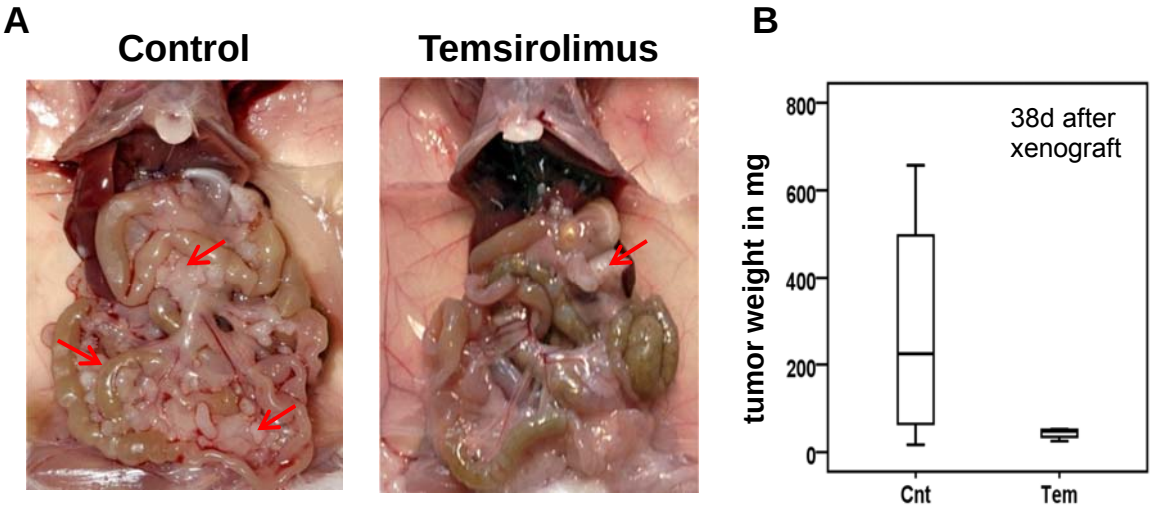



Figure 56. *(A) Impact of temsirolimus in vivo as single agent demonstrated by representative photographs of SCID mice peritoneum (arrows show tumor nodules).(B) Intraperitoneal tumor growth was determined 38 d following engraftment. Peritoneal tumor growth in a representative control (Cnt) and treated (Tem) animal (A) is opposed to quantification of the tumor weight in all animals given by box-plot (median tumor weight: 225 mg (control) vs. 48 mg (temsirolimus).*

In order to verify whether this remarkable anticancer effect prolongs survival, consecutive experiments using both xenograft models SPC111 and P31parental were performed and terminated not until enhanced tumor progression (for details see *Material and Methods*). The results demonstrated a significant prolonged survival of both models treated with temsirolimus (Fig. 57, left panel) paralleled by a dramatically tumor weight reduction (Fig 57, right panel).

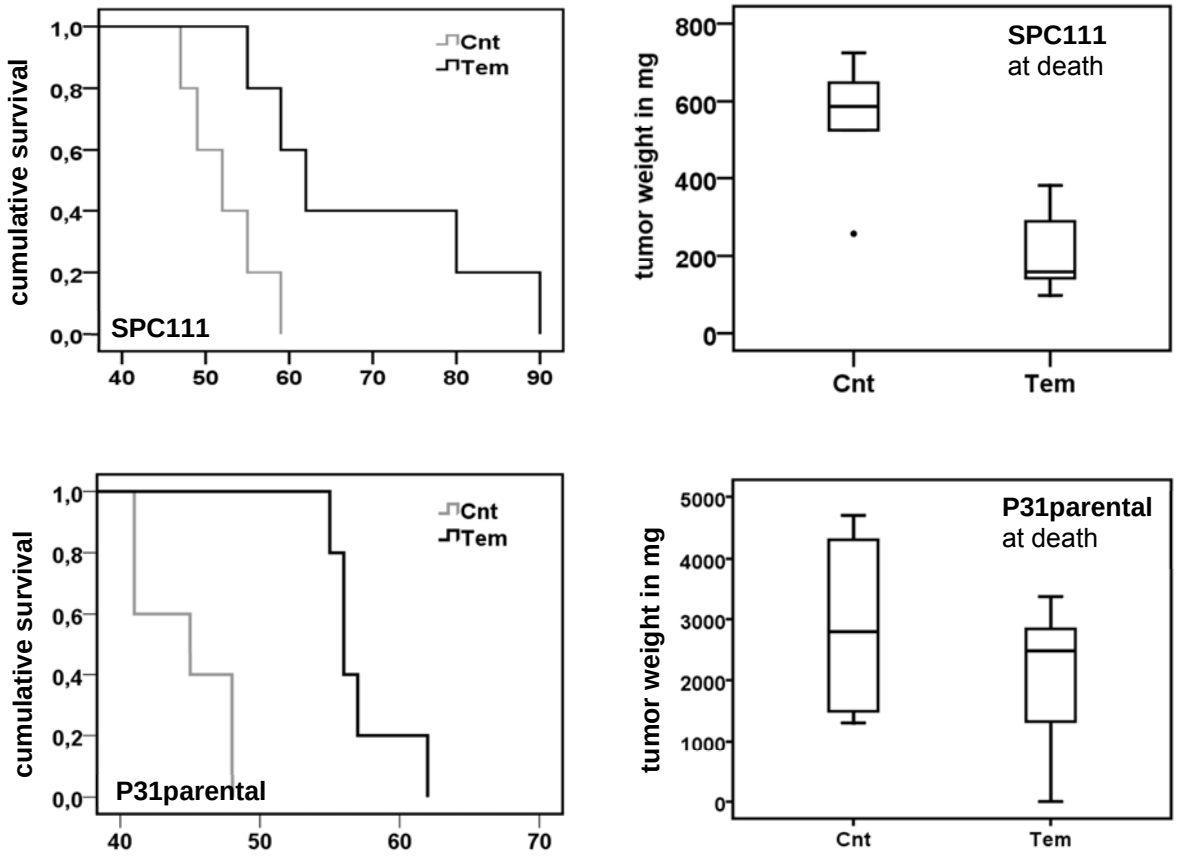



Figure 57. *Impact of temsirolimus treatment on overall survival of SCID mice is shown as Kaplan Meier curves (mean overall survival for SPC111: 52days [control] vs. 62 days [temsirolimus]; log rank test: p = 0.018; mean overall survival for P31: 45 days [control] vs. 57 days [temsirolimus]; log rank test: p = 0.002). Box-plots (right panel) show tumor weight at death.*

### 5.5.2 Molecular effects of temsirolimus as single agent on tumors in HMM xenografted SCID mice

To prove target interference, protein extract of control and temsirolimus-treated tumors were prepared 48h after the last treatment and analyzed for activation of mTOR and respective downstream signal mediators. As shown in Fig. 58, phosphorylation of mTOR and its downstream targets was severely reduced or completely inhibited by application of temsirolimus *in vivo*.

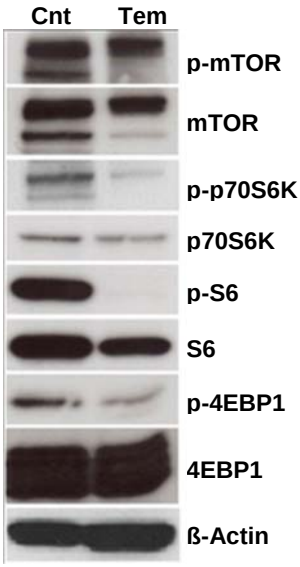
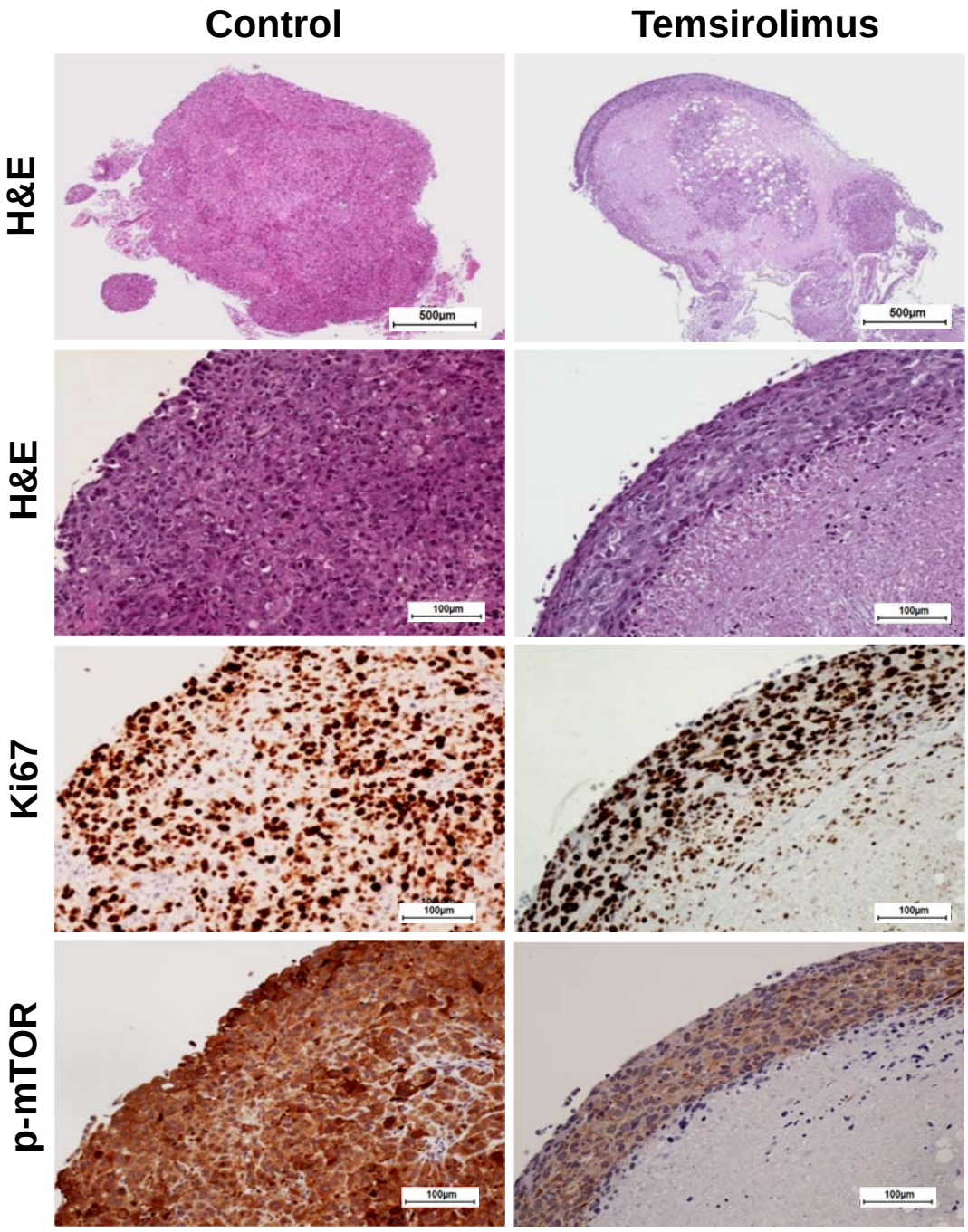



Figure 58. *impact of temsirolimus treatment on activation of mTOR and several downstream effectors in the HMM xenograft tissue (two days after the last treatment) was analyzed by Western blot.*

In order to perform histological analyses, SPC111 xenograft tumors were harvested from cumulative survival experiment (s. 4.5.1) and processed by routine methods (s. Materials and Methods). Even at this setting when treated mice had to be sacrificed due to tumor progression (several weeks after the last temsirolimus treatment), phosphorylation of mTOR was distinctly attenuated as compared to the control tumor and restricted to the proliferating outer cell layer (Fig.59, lower panels). As obvious from both H&E and Ki67 proliferation marker staining (Fig. 59, upper three panels), temsirolimus treatment resulted in dramatic induction of necrotic areas in the center of tumor nodules frequently leaving only a narrow rim of vital tissue. Especially at the border of vital to necrotic areas massive cell death was confirmed by TUNEL staining which was widely absent even in the center of larger tumor nodules of solvent-treated control mice (Fig. 60).



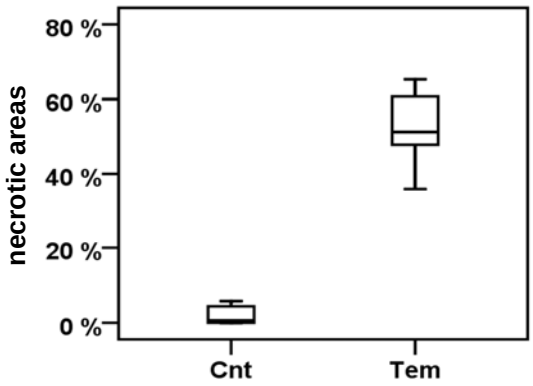




Figure 59. *Histological evaluation was performed for SPC111 HMM tumors grown in SCID mice and representative temsirolimus (Tem)- or solvent-treated control (Cnt) cases are shown. H&E stainings at two different magnifications are opposed to iummunostainings for Ki67 (proliferation marker) and phosphorylation of mTOR using consecutive sections.*

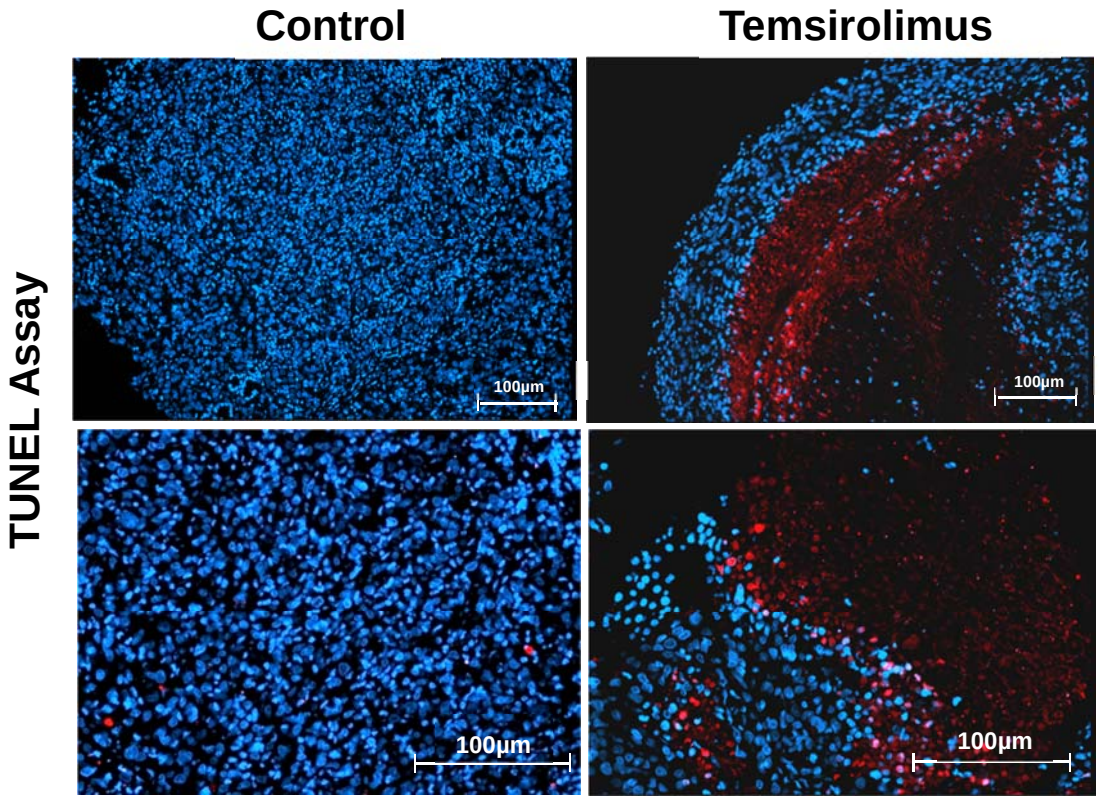



Figure 60. *TUNEL staining (cells with red nuclei) was performed on sections as under Fig. 59 to indicate programmed cell death in HMM xenograft tissues. (Blue, nuclear counter stain by DAPI).*

### 5.5.3 Effect of temsirolimus in combination with cisplatin on HMM xenografts in SCID mice

Finally, we investigated the activity of temsirolimus in combination with cisplatin against the SPC111 MPM xenograft model (Fig. 61). Both overall survival of mice (Fig.61, left panel) and the reduction of tumor weight (Fig.61, right panel) were significant for both single agent therapies but further enhanced in the combination setting.

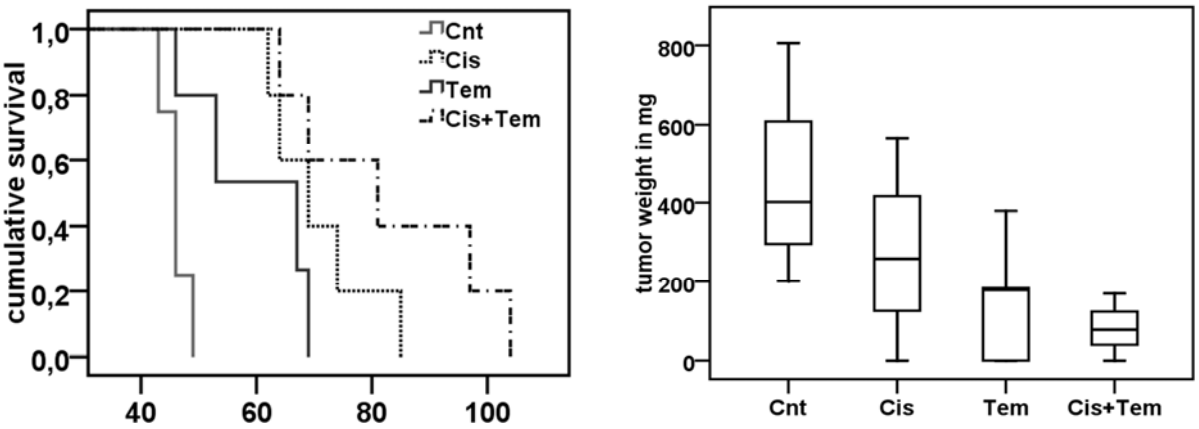



Figure 61. *Anticancer activity of temsirolimus and cisplatin as single agents or in combination was determined against the SPC111 xenograft in SCID mice and survival curves (left panel) (mean overall survival: 46 days [control] vs. 60 days [temsirolimus] vs. 71 days [cisplatin] vs. 83 days [temsirolimus/cisplatin]; log rank: p = 0.000) are opposed to tumor weight (right panel) (median tumor weight: 402 mg [control] vs. 259 mg [cisplatin] vs. 178 mg [temsirolimus] vs. 123 mg [temsirolimus/cisplatin]) at time when animals had to be sacrificed due to tumor progression.*

# 6. DISCUSSION

Human malignant mesothelioma (HMM) is a highly aggressive type of cancer that affects the lining of certain serosal body cavities, particularly the pleura and peritoneum but in fewer cases also the pericardium and tunica vaginalis. HMM is strongly associated with asbestos exposure and shows a 20 – 50 year latency period. Because of the extensive asbestos usage in the last century primarily in western industrial countries, an increasing incidence is prognosticated for the next decades. As current standard HMM chemotherapy regimen the combination of the platinum-based compound cisplatin and the multi-targeted anti-folate pemetrexed is used attaining a median survival for treated patients of nine to twelve months [171]. This prospect demonstrates that current therapy options only show modest benefits. Consequently, the development of novel approaches to fight this devastating malignancy is urgently needed.

In this study we demonstrate that 1) a distinct phosphorylation of the major oncogenic signal transducing factor mTOR is present in the majority of HMM tumor specimens and in investigated cell models 2) gene dose alterations of tumor suppressor genes and proto-oncogenes, as p53 and PTEN as well as several types of growth factor receptors, respectively, may only play a secondary role in the pathogenesis of at least in this study investigated HMM cell lines 3) inhibition of mTOR by the small-molecule compound temsirolimus as a single agent distinctly attenuated HMM cell growth in vitro and tumor formation in vivo; 4) mTOR inhibition was particularly effective against HMM cells with intrinsic or acquired resistance against both cisplatin and pemetrexed, components of the current standard HMM chemotherapy regimen [172] and 5) temsirolimus synergized with cisplatin against HMM models in vitro and in vivo.

## 6.1 The role of mTOR in HMM

Immunohistochemical analyses of HMM clinical specimen (N=70) showed a significant hyper-phosphorylation of mTOR in the tumor compartment but not in the surrounding stromal tissues, thus indicating an interrelation between aberrant mTOR signalling and

HMM development. This conclusion is emphasized by similar findings reported in two other independent series (N=26; N=41) of mesothelioma samples [173, 174].

However, our analysis revealed that mTOR hyper-activation cannot be associated with every histological subtype of HMM and demonstrated distinct differences. In our HMM panel, p-mTOR expression was clearly more pronounced in lesions with epitheloid as compared to sarcomatoid histology. In biphasic tumors again the immunoreactivity for activated mTOR was mainly restricted to the areas with epitheloid differentiation. To our knowledge, an association of mTOR activation and histological subtype of mesothelioma has not been reported before.

Nevertheless, it is still not clear which underlying causes are actual responsible for the development of sarcomatoid or mixed phenotype of tumor cells in a mesothelial tissue as the pleura. Due to sarcomatoid cancer cells share cytological and/or molecular properties of both epithelial and mesenchymal tumors, epithelial-to-mesenchymal transition (EMT) seems to be a process obviously involved in this histological transformation. Indeed, in some extent this may also explain the even poorer prognosis of patients suffering from sarcomatoid tumors, as EMT is characterized by loss of cell adhesion, repression of E-cadherin expression and increased cell motility [175, 176]. All these factors are known to confer aggressive tumors high invasiveness and chemoresistance. Actually it has been reported that EMT seems to play an important role in the development of asbestos induced lung diseases as fibrosis and mesothelioma [177]. Further biphasic HMM tumors may be considered as a true intermediate, as they are thought to contain cell types which have undergone EMT and/or the reverse mesenchymal-to-epithelial transition (MET) in the identical tissue [178]. Interestingly, it has been shown in diverse systems that the Pi3/AKT/mTOR pathway is a decisive factor in the EMT process. Our data regarding almost general restriction of mTOR phosphorylation to cases/regions with epithelial differentiation suggest that this pathway either might inhibit EMT or promote MET of transformed mesothelial cells. Accordingly, promotion of both epithelial differentiation and proliferation by mTOR signalling was demonstrated in mammary epithelial cells [179]. In contrast several other studies have indicated an essential role of the AKT/mTOR signal pathway in the execution of EMT in diverse cell types including murine mammary

epithelial cells [180] and even peritoneal mesothelial cells [181]. Consequently, the specific role of mTOR in (de)differentiation process during HMM progression needs to be determined in further studies.

Besides epitheloid differentiation, mTOR activation was particularly high in early-stage disease but negative in non-malignant tissues. This indicates that activation of mTOR might be a relatively early oncogenic event during the progression of HMM as also observed in early stage lung adenocarcinoma [182]. This assumption is also supported by other studies demonstrating that the mTOR upstream signal mediator AKT is activated in mouse mesotheliomas induced by intraperitoneal crocidolite asbestos injection [173] and early in asbestos-induced malignant transformation of human mesothelium [76]. Altogether, both factors, epitheliod subtype and early-stage disease, which seem to be strongly associated with mTOR hyper-activation, provide good prerequisites for a toehold in mTOR targeting. The majority of HMM patients are diagnosed with this histological type and a multitude of different studies have reported that generally targeted compounds, as the mTOR inhibitor temsirolimus, show best results applied in early tumor progression [183, 184].

With regard to our further in vitro analyses, all investigated HMM cell models showed a constitutively active mTOR pathway, as previously also found in other mesothelioma cell models [185]. This leads to the assumption that mTOR has not only a high relevance in early HMM development but also plays an important role in the continuous progression of this tumor. So what could this role be? The mTOR pathway is generally known to be centrally involved in the control of tumor cell metabolism, growth and proliferation in many different types of cancer [186]. Nevertheless, differing results from studies investigating mTOR as molecular target in cancer therapy and data from clinical trials with current inhibitors reflect the complexity of the mTOR signalling network. The two functionally distinct mTOR complexes, parallel regulatory pathways, and several feedback loops contribute to variable functions of this pathway and make it difficult to clearly determine its significance for each type of cancer. However, it is assumed that the PI3K/AKT/mTOR axis confers a significant survival benefit for HMM cells, helping to survive cytotoxic damage caused by asbestos fibers, which are considered to be the main cause of mesothelioma [187]. In that study both PI3K/AKT and mTOR signaling

have been demonstrated to be, in an apparently independent manner, important for the survival of mesothelial cells treated with asbestos. Elevated levels of calretinin, also known as a useful biomarker for mesothelioma, seem to obtain cytoprotective effects mediated via hyper-activation of the PI3K/AKT pathway. However, also mTOR has been shown to mediate a survival benefit but independent of the present calretinin level. Concerning the competence of tumor cells to metastasize, mesothelioma cells could be demonstrated to be highly dependent on mTOR in terms of spreading capability [185]. It is suggested that adhesion and spreading of mesothelioma cells on the extracellular matrix (ECM) require the continuous translation of pre-synthesized mRNAs, controlled by the mTOR pathway. Also a direct cytoskeletal remodeling by mTORC1 may be responsible for increased cell motility.

## 6.2 Genomic and molecular aberrations in HMM

In this study we investigated genomic alterations of HMM cell lines via array comparative genomic hybridization (aCGH) and focused on both, genes encoding for key players and regulators of the mTOR pathway and frequently mutated loci generally associated with cancer pathology. Analyses with regard to the mTOR pathway showed neither significant gains nor losses of the involved genes. This is not surprising considering the fact, that oncogenic mTOR signaling in HMM has not yet been reported in association with altered gene dose of the directly involved mediators such as S6K, S6 and 4EBP1. More likely considered are dysfunctioning regulatory mechanisms such as the PI3K/AKT pathway and key factors involved in energy metabolism, to be responsible for mTOR hyper-activation. However, also these networking molecules showed no significant gene copy alterations in the investigated cell lines in our study, indicating that genomic aberrations are rather no major players behind deregulated mTOR control mechanisms. Indeed, this is in accordance with several other studies, which have reported that at least genomic mutations of the mTOR upstream key mediators AKT and PI3K are rather an uncommon event in HMM pathology [188] (). Data on genomic alterations in the negatively regulator of these molecules, PTEN, which is known as one of the most commonly lost tumor suppressors in human cancer, are conflicting [189, 190]. None of our analyzed cell lines showed a deletion of the PTEN

locus. Nevertheless, several observations have suggested that the Pi3K/AKT/mTOR pathway might be hyper-activated in mesothelioma cells at least in vitro either at the level of mTOR or the upstream signal mediator AKT [76, 150, 173, 191, 192]. Accordingly, p-mTOR expression correlated significantly with activation of AKT in human and murine mesothelioma cell lines [173]. In that study both hepatocyte growth factor/cMet signalling and inactivation of the negative AKT regulator PTEN were suggested as mediators of AKT hyper-phosphorylation. Further it has been reported, that expression of PTEN was a strong predictor of mesothelioma patient survival [190] and adenoviral overexpression of PTEN in HMM cells induced massive apoptosis [193]. At this point, it is worth noting, that independently of the genomic pattern, altered PTEN expression may be a consequence of other associated dysfunctioning regulatory networks. Actually, suppressed PTEN expression by a deregulated balance between Notch-1 and -2 and consequently upregulation of AKT phosphorylation were suggested to contribute to the malignant phenotype of mesothelioma cells especially under hypoxic conditions [194]. Moreover, there is evidence, that AKT hyper-activity is not necessarily inhibited in the presence of PTEN. In a series of diffuse malignant peritoneal mesothelioma tumor specimens (n=20), 95% showed significant phosphorylation of AKT, mTOR and its downstream mediators, although PTEN showed a normal disomic pattern and was expressed in almost all samples [195]. In addition to oncogenic signal pathways, also a direct activation of AKT by SV-40 was suggested to be involved in asbestos-induced mesothelial cell transformation [76].

Regardless of the interplay between AKT and mTOR phosphorylation, we investigated for other potential genomic aberrations which may contribute to the constitutive activation of mTOR in HMM. Given that mTOR is highly regulated by the activity of multiple receptor tyrosine kinases (RTKs) such as EGFR, HGFR (MET), IGF1R and their associated ligands (data not shown) we analysed the genomic pattern of these major growth factors. Also in this case, no significant gains of the involved genes were detected in any cell line. The role of EGFR generally in the development of HMM seems to be highly controversial. Some studies reported that there was no relationship between EGFR over-expression and outcome in patients with mesothelioma [196-198], while others have reported that EGFR over-expression is associated even with

improved prognosis [199, 200]. Anyway, two phase II clinical trials based on EGFR tyrosine kinase inhibitor (gefitinib) therapy have not shown promise in mesothelioma therapy so far [201, 202], although the majority of patients exhibited EGFR overexpression. It is hypothesized that resistance to gefitinib may be caused by to the over-expression of alternative RTKs which compensates for the loss of EGFR function [167]. Interestingly, downstream proteins within the EGFR signaling pathway, such as AKT and mTOR, have been shown to be utilized by HGFR and IGF1R in case of EGFR inhibition. Even though our study could not reveal at least genomic alterations of HGFR and IGF1R, several research groups have suggested that both growth factor receptors take a key position in HMM pathology [203, 204]. With regard to the possibility that downstream mTOR activation plays an essential role in the oncogenic signaling of all three RTKs and that the inhibition of one only leads to the activation of compensatory "by-pass" mechanisms, we suggest that mTOR inhibition could strikingly diminish this escape axis.

In order to investigate frequently observed genomic alterations highly associated with HMM, we focused on NF2 (Neurofibromin 2 or Merlin) and CDKN2A (Cyclin-dependent kinase inhibitor 2A/ $p14^{ARF}$ and $p16^{INK4A}$). Mutations of the tumor suppressor gene Merlin (22q12.2) occur in approximately half of HMM cases [162] and are thought to be potentially linked with asbestos exposure [205]. Although chromosome 22 is known to be recurrently lost in HMM [206] we could not detect any genetic loss in this case and no significant aberrations of the NF2 loci in the analyzed cell models. Interestingly it has been reported that Merlin regulates mTORC1 activity in a PI3K/AKT and MAPK/TSC2 independent manner in human meningioma [207] and also HMM [208]. Furthermore, both studies demonstrated that Merlin-deficient cell lines exhibited constitutive mTORC1 activation and showed increased sensitivity to mTOR inhibition. Although in our study we could not reveal a relation between NF2 loss and the observed constitutive activation of mTOR, the mentioned studies again reflect that mTOR targeting may provide promising effects in the treatment of HMM.

The tumor suppressor gene CDKN2A, which is deleted in the vast majority of HMM cases [162, 209, 210], provides the two alternative reading frame (ARF) products $p14^{ARF}$ and $p16^{INK4A}$. Both are cyclin-dependent kinase (CDK) inhibitors and involved in cell

cycle regulation. The frequently observed mutations absolutely correlated with our study, which showed significant losses of CDKN2A in 5 of 6 cell models, one with an obviously heterozygous deletion. As $p14^{ARF}$ normally inhibits the p53 negative regulator MDM2 and therefore regulates the protein stability of p53, loss of $p14^{ARF}$ consequently prevents p53 expression. This is significant considering that TP53 mutations have been detected in a multitude of tumors, but they are not a very prominent feature in HMM [162]. Indeed, also the cell models investigated in this study showed no aberrations of the TP53 locus. Apparently, loss of p53 constitutes no essential event in the development of HMM, as in most cases p53 expression is inhibited anyway based on the loss of $p14^{ARF}$. If carcinogenesis is considered to underlie "Darwinian mechanisms" [7], this instance would reflect the absence of selective pressure.

## 6.3 Targeting mTOR – in vitro

Regardless of the upstream mechanism of the pathway activation, our data suggest that mTOR is an ideal point for therapeutic intervention. First, mTOR inhibitors like temsirolimus are already approved for therapy of other solid tumors like renal cell cancer [149]. Additionally, we demonstrate in this study that temsirolimus does not only target HMM cells in adherent monolayer cultures but also as non-adherent spheroids believed to be enriched in "cancer stem cells" with enhanced tumor-initiating capacity [9]. This cell compartment is frequently responsible for systemic therapy resistance of solid tumors [211]. Temsirolimus led to growth inhibition and disintegration of HMM spheroids. In a series of publications it was demonstrated that growth of human mesothelioma cells as tumor fragment-derived spheroids [212] or multicellular non-adherent spheres leads to resistance against drug-induced apoptosis [150]. This therapy insensitivity was based at least in part on hyper-activation of mTOR interfering with mitochondrial apoptotic pathways activation [76]. Together with our observations these data suggest that mTOR inhibition should not only lead to HMM growth inhibition even in the "cancer stem cell compartment" but also to reversal of intrinsic and/or acquired resistance to chemotherapy.

Indeed, we detected a distinct synergism between mTOR inhibition and the routine HMM chemotherapeutic agent cisplatin in vitro an in vivo. With regard to the in vitro

situation, comparable observations have been published recently by Hartman et al. demonstrating that sirolimus enhances cisplatin-induced cell death in HMM cells [192]). Also in several other cancer types such as ovarian, endometrial, neuroectodermal and lung cancers [213-216], mTOR inhibitors showed efficient chemosensitizing properties by increasing cisplatin-induced apoptosis. Accordingly, hyper-activation of the PI3K/AKT/mTOR signal pathway were indicated to be responsible for cisplatin resistance induced by e.g. the adenoviral E1a gene [217] and insulin-like growth factor receptor I (IGFR-I) [218]. In our study, both enhanced apoptosis induction and altered interference with cell cycle progression was underlying the synergism between cisplatin and temsirolimus. With regard to the inhibition of downstream signals, a synergistic blockade of 4EBP1 phosphorylation by the combination approach was especially prevalent. Accordingly, regulation of eIF4E by 4EBP1 phosphorylation and related cap-dependent translation was demonstrated to be a key factor in mTOR-mediated pro-oncogenic and anti-apoptotic signals [219].

In addition to the synergism between chemotherapy and mTOR inhibition, we found that intrinsic as well as acquired cisplatin resistance is generally accompanied by hyper-sensitivity against mTOR inhibition. In a cell model containing a sensitive parental and a cisplatin-resistant cell (sub)line, we demonstrate that temsirolimus hyper-susceptibility is based on potent apoptosis induction in the resistant cell model. Comparable sensitation against mTOR inhibition as a consequence of cisplatin resistance was also observed in ovarian (19) and lung cancer [220] in this cases based on upregulation of mTOR phosphorylation or AKT gene amplification, respectively. Interestingly, we did not observe a forced upregulation neither of the mTOR or AKT pathway (data of the latter not shown) activity in the cisplatin-resistant cell models suggesting that enhancement of these pathways is not directly responsible for the chemotherapy resistance. Our data rather suggest that cisplatin-resistant cell lines develop forced “addiction” to continuous mTOR signals. Accordingly, temsirolimus as single agent resulted in an almost complete dephosphorylation of S6 in all cell lines whereas the decrease of the p-4EBP1 level was significantly stronger in cisplatin-resistant phenotypes. As mentioned previously, it is thought that hyper-activation of the IGFR-I signalling pathway is an important event for cisplatin resistance [218] and also associated with an increase in both mesothelioma

cell proliferation and motility [204]. Interestingly it has been demonstrated, that stimulation with IGF-I in HMM cell lines directly correlates with increased phosphorylation of 4E-BP1 which was highly dependent on AKT/mTOR signalling [221]. Blockade of cap-dependent translation by transfection with constitutively active 4EBP1 inhibited IGF-I mediated proliferation and motility. If indeed the cisplatin resistant phenotype is mediated at least in part of IGFR-I hyper-activation, these data would contribute to our assumption of a forced “addiction” to continuous mTOR signals. The molecular mechanisms underlying this vulnerability of mTOR downstream survival signals in cisplatin-resistant cell models need to be investigated in further experimental approaches.

With regard to the impact of pemetrexed on HMM cell lines, we observed that cytotoxic effects were significantly reduced in those with cisplatin resistance. This got obvious especially comparing P31parental and its cisplatin-resistant subline. These results should give cause for concern, considering that the combination of cisplatin and pemetrexed presents the HMM first-line therapy. However, the underlying molecular background responsible for this multi-drug resistance is still unclear, but our ongoing studies have focused on this issue.

## 6.4 Targeting mTOR – in vivo

To the best of our knowledge this is the first report that mTOR inhibition distinctly attenuates human HMM growth in vivo as xenografts in SCID mice. Both, significantly reduced tumor mass and prolonged overall survival were observed accompanied by massive tumor necrosis. The inhibition of the molecular target of temsirolimus in vivo, namely downregulation of tumor-associated mTOR phosphorylation, was demonstrated immediately after therapy but still persisted for several weeks following the last temsirolimus administration. The profound reduction of tumor load might be based on massive tumor cell death demonstrated by TUNEL staining mainly at the junction to the necrotic areas. Moreover, temsirolimus is known to exert potent antiangiogenic activities [222, 223], which might support the development of central tumor necrosis. As discussed, spreading of mesothelioma cells is believed to be highly dependent on mTOR signalling, indicating that the significantly prolonged survival of temsirolimus

treated mice was at least in part caused by an effective inhibition of tumor metastases. Both aspects, abolished angiogenesis and suppressed cell motility, are obviously important factors contributing to a forced response to mTOR inhibition in vivo of cells which were rather temsirolimus insensitive in in vitro experiments.

In addition to the distinct activity of temsirolimus as a single agent against HMM xenografts, we also have performed an initial experiment for combination with cisplatin. As expected from the in vitro analyses, the combination approach exhibited synergistic anti-cancer effects against HMM xenografts. These experiments were done so far only in one combination setting using concurrent applications of cisplatin and temsirolimus. Currently, experiments are underway testing different settings involving on the one hand also pemetrexed and on the other hand applying temsirolimus after therapy failure with cisplatin.

In summary we present in this study strong evidence that temsirolimus is active against human HMM in vitro and in vivo, shows hyperactivity against cisplatin-resistant cells, and synergises with platinum-based chemotherapy. This suggests further investigation of mTOR inhibition as a promising treatment strategy for human HMM either in combination with chemotherapy to avoid resistance development or as second-line treatment following chemotherapy failure.

# 7. CONCLUSION

Due to the very poor clinical response and rising incidence of HMM, the past years have seen a considerable increase in the clinical and epidemiologic significance of this devastating malignancy. Although the usage of asbestos has been continuously restricted since the late 1980s, it is predicted that the peak in incidence is reached not before the next two or three decades. Main reasons are the long latency period after asbestos exposure and the proceeding usage of asbestos in developing countries. Hence, further research is urgently needed to advance therapeutic strategies and the understanding of HMM pathology.

In this study, the cytogenetic analyses underline the complexity and heterogeneity of chromosomal aberrations in HMM, most notably with regard to the PTEN locus. In contrast to findings of other research groups, we could not detect a deletion of PTEN in any investigated cell models. On the other hand, the frequently reported association of HMM with loss of CDK2A, has been confirmed in this study, indicating a reasonable explanation for the lacking incidences of p53 deletions in HMM. For this reason, we suggest that a possible implementation of CDKN2A as predictive biomarker for HMM should be considered.

Furthermore, we show that mTOR is constitutively activated in HMM tumor specimen and cell lines, highlighting the important role of this oncogenic mediator in HMM pathology. Also there is strong evidence that inhibition of mTOR with the targeted compound temsirolimus leads to striking anticancer effects against HMM in vitro and in vivo, shows hyperactivity against cisplatin-resistant HMM cells, and synergises with platinum-based chemotherapy. This suggests that acquired and intrinsic resistance of HMM against cisplatin is very complex and not primarily based on transporter-mediated mechanisms. With regard to the observed reduced cytotoxic effects of pemetrexed in cisplatin resistant cell models, we also assume that the combination of these agents, which constitutes the present standard therapy regimen, may be counterproductive, at least in particular cases.

Taken together we assume that mTOR inhibition may be a promising treatment strategy for human malignant mesothelioma either in combination with chemotherapy to avoid resistance development or as second-line treatment following chemotherapy failure.

# 8. ABBREVATIONS

ABC …… ATP-binding cassette

ATP …… Adenosine triphosphate

BSA …… Bovin serum albumin

CDK …… Cyclin-dependent kinase

Cisplatin …… cis-diamminedichloroplatinum(II)

DAPI …… 4',6-diamidino-2-phenylindole

dATP …… Deoxyadenosine triphosphate

DMSO …… Dimethyl sulfoxide

DNA …… Deoxyribonucleic acid

EGFR …… Epidermal growth factor receptor

FACS …… Fluorescence-activated cell sortiment ABBREVATIONS

FCS …… Fetal calf serum

HGF …… Hepatocyte growth factor

HIF-1..... Hypoxia inducible factor-1

HMM …… Human malignant mesothelioma

IGF …… Insulin-like growth factor

MAPK …… Mitogen activated protein kinase

MPM …… Malignant pleural mesothelioma

MTT …… Dimethyl thiazolyl diphenyl tetrazolium salt

NF $\kappa$ B …… Nuclear factor $\kappa$ B

NSLC …… Non-small lung carcinoma

PBS …….. Phosphate buffered saline

PDGF …….. Platelet-derived growth factor

PI …… Propidium iodide

PI3K …… Phosphotidyl-inositol-3-OH kinase

PMSF …… Phenylmethanesulfonylfluoride

PVDF …….…… Polyvinylidene fluoride

Rb ……..…….. Retinoblastoma protein

RCC ……..……..Renal cell carcinoma

ROS …… Reactive oxygen species

RTK …….. Tyrosine kinase receptor

SDS-PAGE …… Sodium dodecyl sulfate polyacrylamide gel electrophoresis

TAM …….. Tumor-associated macrophage

TBST …..…… Tris-buffered saline with Tween

TF $\beta$ …….. Tumor growth factor $\beta$

TNF $\alpha$ ……..……Tumor necrosis factor $\alpha$

VEGF ……..…….. Vascular endothelial growth factor

# 9. PUBLICATIONS

ORIGINAL ARTICLE

## Temsirolimus Inhibits Malignant Pleural Mesothelioma Growth In Vitro and In Vivo

### *Synergism with Chemotherapy*

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**Introduction:** Human malignant pleural mesothelioma (MPM) is an asbestos-related malignancy characterized by frequent resistance to chemotherapy and radiotherapy. Here, we investigated the feasibility of mammalian target of rapamycin (mTOR) inhibition by temsirolimus as an antimesothelioma strategy.

**Methods:** Phosphorylation of mTOR (p-mTOR) was assessed by immunohistochemistry in MPM surgical specimens ($n = 70$). Activation of mTOR and impact of mTOR inhibition by temsirolimus was determined in MPM cell lines in vitro ($n = 6$) and in vivo as xenografts in severe combined immunodeficiency mice ($n = 2$) either as single agent or in combination with cisplatin.

**Results:** Strong immunoreactivity for p-mTOR was predominantly detected in epitheloid and biphasic but not sarcomatoid MPM specimens while adjacent normal tissues remained widely unstained. Accordingly, all mesothelioma cell lines harbored activated mTOR, which was further confirmed by hyperphosphorylation of the downstream targets pS6K, S6, and 4EBP1. Temsirolimus potently blocked mTOR-mediated signals and exerted a cytostatic effect on mesothelioma cell lines in vitro cultured both as adherent monolayers and as nonadherent spheroids. Mesothelioma cells with intrinsic or acquired cisplatin resistance exhibited hypersensitivity against temsirolimus. Accordingly, cisplatin and temsirolimus exerted synergistic inhibition of the mTOR downstream signals and enhanced growth inhibition and/or apoptosis induction in mesothelioma cell lines. Finally, temsirolimus was highly active against MPM xenograft models in severe combined immunodeficiency mice both as a single agent and in combination with cisplatin.

**Conclusion:** The mTOR inhibitor temsirolimus is active against mesothelioma in vitro and in vivo and synergizes with chemotherapy. These data suggest mTOR inhibition as a promising novel therapeutic strategy against MPM.

**Key Words:** Mesothelioma, mTOR, Temsirolimus, Chemotherapy, Cisplatin resistance.

(*J Thorac Oncol.* 2011;6: 000–000)

Malignant pleural mesothelioma (MPM), a highly lethal malignancy affecting the serosal lining of the pleural cavity, is thought to develop from superficial mesothelial cells.[1] MPM is strongly associated with asbestos exposure and shows a 20- to 40-year latency period.[2,3] The expected median survival of patients suffering from MPM is, despite extensive surgery combined with chemotherapy and radiation,[4] still poor, ranging from 4 to 12 months from diagnosis.[5–7]

The most successful chemotherapy regimen in current use (pemetrexed/cisplatin) prolongs median survival only by a few months (from 9 to 12 months).[8] One major problem for systemic cancer therapy in general and cisplatin in particular concerns the development of acquired drug resistance of tumor cells leading to therapy failure.[9,10] Consequently, the development of new therapeutic concepts for mesothelioma with higher efficiency and defined cellular targets is of exceeding interest.[6,9]

One of the most important oncogenic signals is mediated by the phosphoinositol-3-kinase (PI3K)/AKT pathway via the downstream effector mammalian target of rapamycin (mTOR).[11,12] Its major downstream targets S6-Kinase (p70S6K) and 4EBP1 are for example involved in the translation of potent proto-oncogenes such as c-MYC and cyclin D.[11,13] Accordingly, pathologically activated mTOR mediates multiple oncogenic functions and supports chemotherapy resistance including the one against platinum compounds. Consequently, mTOR blockade exerts chemosensitizing activity in several cancer types.[14–18] Rapamycin, also known as sirolimus, and its analogs such as

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Disclosure: ●●●.

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ISSN: 1556-0864/11/0604-0001

temsirolimus (CCI-779) and everolimus (RAD001) are to date the best-investigated mTOR inhibitors.[11–13] Such mTOR inhibitors are already approved for the treatment of renal cell carcinoma and mantle cell lymphoma.[19] Several previous studies suggested hyperactivation of the PI3K/AKT/mTOR signal pathway in mesothelioma cells by different mechanisms.[20–24] Based on in vitro and in vivo experiments, we demonstrate in this study that mTOR is a feasible therapy target in human MPM.

## MATERIALS AND METHODS

### Clinical Samples

Formalin-fixed, paraffin-embedded tumor tissue was available from 70 patients suffering from histologically confirmed MPM. All patients underwent resection between January 1993 and January 2010 at the Medical University of Vienna, Department of Thoracic Surgery. Patients were staged according to the International Mesothelioma Interest Group staging system and tumor node metastasis stages I and II summarized as early-stage disease while higher tumor node metastasis stage as late-stage disease. Blocks were embedded during pathological routine diagnostic work-up. Informed consent was obtained from all patients.

### Cell Culture

SPC111and SPC212 cells (from biphasic MPM) were kindly provided by Prof. R. Stahel (Zurich, Switzerland), and the cell lines NCI-H28 and NCI-H2015 were obtained from American Type Culture Collection. The cisplatin-resistant subline P31res1.2 was established from the parental P31 by in vitro cisplatin selection[25] (kindly donated by Prof. K. Grankvist, Umea, Sweden). MPM cell lines were maintained in RPMI 1640 or M10 with 10% fetal calf serum (PAA Laboratories, Pasching, Austria). Cell lines were authenticated by array comparative genomic hybridization. The identical origin of the P31parent and P31res1.2 was additionally proven by DNA finger printing.

### Intraperitoneal Xenograft Models

All procedures involving animals and their care were approved by the Ethical Review Board and performed following FELASA guidelines. $1 \times 10^6$ SPC111 or P31 cells were injected in 200 $\mu$l serum-free medium intraperitoneally into 5- to 7-week-old female severe combined immunodeficiency (SCID) mice (in total $n = 80$). Two weeks after inoculation, mice were assigned into treatment groups, each consisting of 5 or 10 mice: (1) treated with solvent; (2) temsirolimus (10 mg/kg) daily × 5 for two cycles with 2 days off; (3) cisplatin (1 mg/kg) once a week for 2 weeks; combined temsirolimus and cisplatin as aforementioned. All drugs were applied intraperitoneally. Performance status and body weight were checked every second day. The first experiment was terminated after 38 days from transplantation when the first animal had to be killed due to weight loss. To establish the impact on survival, in another experiment, all mice were kept until individual tumor progression with weight loss and/or ascites development. Intraperitoneal tumors were harvested, weighted, and processed for histological examination.

### Immunohistochemistry and TUNEL Assay

Briefly, tissue sections were deparaffinized and rehydrated. After heating for 10 minutes in 10 mM citrate buffer (pH 6.0), the tissue sections were incubated overnight at 4°C with primary antibodies (p-mTOR Ser2448; Cell Signaling; Ki67, Dako; dilutions 1:100). Antibody binding was detected by means of the UltraVision LP detection system (Laboratory Vision Corporation). p-mTOR immunostaining was independently evaluated by two authors (B.G. and U.S.) in at least five representative areas per section. Interpretation of the results was limited to proven tumor tissue, and only cytoplasmic staining was considered positive. Staining intensity was scored as strong = 3, moderate = 2, and weak = 1. Intensity scores were multiplied by the respective percentages of cells and divided by 3, resulting in an H-score from 0 to 100. TUNEL assays were performed on histological sections using In Situ Cell Death Detection Kit, TMR red, following the instructions of the manufacturer (Roche Diagnostics, Nutley, NJ).

### Drugs

Temsirolimus was obtained from Wyeth Pharmaceuticals Inc., Cambridge, MA, and cisplatin from Sigma, St. Louis, MO. For in vitro experiments, temsirolimus was dissolved in DMSO at 25 ng/$\mu$l and cisplatin in PMF at 20 mM stock, and both were further diluted in culture medium. For in vivo experiments, temsirolimus was diluted in 5% PEG/5% Tween and cisplatin in serum-free culture medium. DMSO concentrations were always less than 1% in vitro and proven to be ineffective at those concentrations in all cases.

### Chemosensitivity Assays (MTT)

All MM cell lines were seeded in 96-well plates at a density of $2 \times 10^3$/100 $\mu$l/well 24 hours before drug treatment. Drug exposure time was 72 hours. Subsequently, cell viability was tested by an MTT-based survival assay (EZ4U, Biomedics, Vienna, Austria) as published.[26] Interactions between drugs were tested based on calculating the combination index (CI) according to Chou and Talalay with CalcuSyn software (Biosoft, Ferguson, MO). CI $<0.9$, CI $= 0.9–1.1$, or CI $>1.1$ represent synergism, additive effects, and antagonism of the two investigated substances, respectively.

### Clonogenic Assays

Cells were seeded into six-well plates at densities of $2 \times 10^3$ to $5 \times 10^3$ cells/well 24 hours before treatment. After ~7 days, the drug-containing medium was removed and cell clones were washed, fixed, and stained as published.[27]

### Western Blot Analysis

For protein extraction, cells were exposed to drugs for 24 hours, collected, lyzed, and proteins were extracted and processed for sodium dodecyl sulfate polyacrylamide gel electrophoresis and immunoblotting as published.[26,28] The following primary antibodies were used: mTOR, p-mTOR, S6, p-S6, S6K, p-S6K, 4EBP1, p-4EBP1, PARP (Cell Signaling Technologies), and $\beta$-actin (Sigma) at 1:1000 dilutions in phosphate-buffered saline with 3% bovine serum albumin. Horseradish peroxidase-coupled antirabbit/antimouse antibodies (3% bovine serum albumin, Santa Cruz Biotechnology) were used at 1:10000 dilu-

tions and developed by Western Blotting Luminol Reagent Kit (Santa Cruz Biotechnology).

### Cell Cycle Analysis

After 48 hours drug exposure, cells were trypsinized, pelleted, and processed for cell cycle analyses by propidium iodide staining and flow cytometry using FACS-Calibur (Becton Dickinson, Paolo Alto, CA) according to standard protocols as published.[26,28] Results were quantified using CellQuest Pro software (Becton Dickinson and Company, New York, NY).

### $^3$H-Thymidine Incorporation

Cells were seeded in 96-well microtiter plates at a density of $2 \times 10^4$ cells/well 24 hours before drug treatment. After 24 hours exposure time, the medium was removed and 100 $\mu$l of a 2 nM solution of [$^3$H]-thymidine (specific activity: 25 Ci/mM) was added. After incubation for 2 hours at 37°C, cells were washed twice with ice-cold phosphate-buffered saline, lyzed in 100 $\mu$l lysis buffer, and transferred into scintillator tubes. After the addition of 5 ml liquid scintillator (Zinsser Analytic, Frankfurt, Germany), probes were measured using a liquid scintillation counter (Tri-Carb 1900TR, Packard).

### Mesothelioma Cell Spheroid Growth Assay

To establish spheroid cultures from MPM cell lines, $5 \times 10^3$ cells were seeded in triplicate in DMEM/Ham's F-12 medium (PAA Laboratories) supplemented with 20 ng/ml basic fibroblast growth factor (Eubio, Vienna, Austria), 20 ng/ml epidermal growth factor (Sigma), and 2% B27 supplement (PAA Laboratories) in ultra-low attachment 24-well plates (Corning, NY). Temsirolimus was added at 100 ng/ml. Ninty-six hours after plating, all spheroids of each well were photographed. Only the spheres with a diameter more than 100 $\mu$m were counted and their diameter measured on the digital photographs by ImageJ software. To assess the self-renewal capacity of spheroid-derived cells, the spheres were treated for 10 minutes with trypsin (0.1%) and EDTA (0.01%). Cells were resuspended in DMEM/FBS medium, washed once in serum-free medium, and passed through a 70-$\mu$m filter (BD Biosciences, Bedford, MA). Single cell suspension was replated under the aforementioned conditions.

### Statistical Analysis

All the data were expressed as mean ± SD or median from at least three individual experiments. Statistical significance of differences observed in vitro and in vivo was determined by using an unpaired *t* test, Mann-Whitney *U* test, and one-way or two-way analysis of variance applying the appropriate statistical methods. *p* values less than 0.05 were considered significant. Survival analysis was performed using Kaplan-Meier curves, the log-rank test, and the Cox regression model using the SPSS 17 software package.

## RESULTS

### Phosphorylation of mTOR in Surgical Specimens of MPM Patients

Of the 70 mesothelioma specimens analyzed, 43 (61.4%) exhibited epitheloid, 19 (27.1%) biphasic type, and 4 (5.7%) sarcomatoid histology. In addition, four patients with pseudomesotheliomatous adenocarcinomas were investigated in this study (5.7%). Figures 1*A–C* shows expression of phosphorylated mTOR (p-mTOR) in representative cases of the different MPM histological subtypes. Strong immunoreactivity was found predominantly in the epitheloid-type tumors (Figure 1*A*), while sarcomatoid cases were widely p-mTOR negative (Figure 1*C*). A comparable restriction of p-mTOR immunoreactivity to the epitheloid histology was observed in the biphasic tumors (Figure 1*B*). Quantitative evaluation confirmed a strong phosphorylation of mTOR in mesotheliomas harboring epitheloid compared with the sarcomatoid histology (Figure 1*D*). Interestingly, p-mTOR levels were particularly high in early-stage MPM and tended to moderately decline in late-stage MPM (Figure 1*E*). Overall patient survival did not correlate significantly with p-mTOR staining intensity but tended to be higher in p-mTOR-positive cases (Cox regression: $p = 0.097$). This is not surprising considering the generally strong overexpression of p-mTOR in the epithelial subtype and in early-stage disease, both characterized by better prognosis.[5,29] In none of the patient subgroups, mTOR staining exhibited a significant association with patient survival.

### mTOR Pathway is Activated in MPM Cell Lines

To determine whether MPM cell cultures retain activation of mTOR, five MPM cell models (SPC111, SPC212, NCI-H28, NCI-H2052, and P31) and a cisplatin-resistant P31 subline (P31res1.2) were investigated for activation of several mTOR pathway mediators by immunoblot (Figure 1*F*). Generally, all investigated mesothelioma cell lines exhibited strong phosphorylation of mTOR corresponding to intense activation of the downstream effectors p70S6K, S6, and 4EBP1. mTOR activation was also present in two cell lines derived from biphasic mesothelioma (SPC111 and SPC212), indicating that the in vitro cell cultures predominantly reflect the epitheloid histology. In the cisplatin-resistant subline P31res1.2, phosphorylation of all investigated mTOR pathway mediators was equal or tended to be slightly reduced as compared with the parental cell line (Figure 1*F*).

### In Vitro Growth-Inhibitory Effects of Temsirolimus Against MPM Cell Lines

Responsiveness of the investigated MPM cell models to temsirolimus (Figure 2*A*, *left panels*) was compared with cisplatin clinically used for MPM chemotherapy (Figure 2*A*, *right panels*). Temsirolimus at low nanomolar concentrations led to a moderate to distinct but in all cases significant reduction of viable MPM cells as measured by MTT assay after 72 hours drug exposure. When comparing temsirolimus sensitivity with responsiveness to cisplatin (Figure 2*A*) and pemetrexed (data not shown), it turned out that the intrinsically chemoresistant MPM cell lines SPC212 exhibited dis-

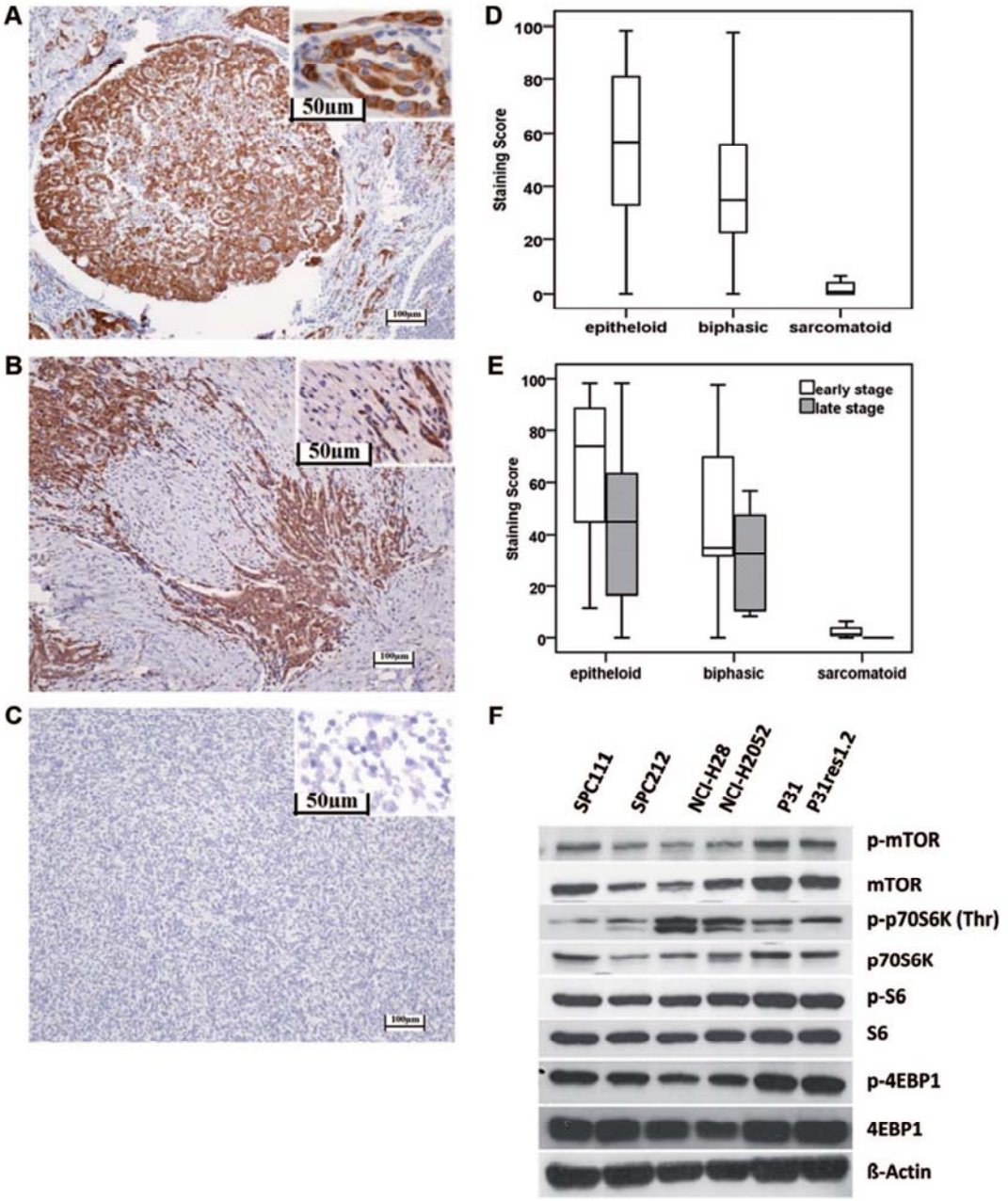


**FIGURE 1.** mTOR phosphorylation in human MPM. *A–C*, 70 human paraffin-embedded MPM surgical specimens of different histology (*A*, epitheloid; *B*, biphasic; *C*, sarcomatoid) were immunostained for phosphorylated mTOR (*brown*) and counterstained with hematoxylin (nuclei in *blue*) and (*D*, *E*) evaluation was performed by H-score as described under Materials and Methods section. Hyperphosphorylation of mTOR was predominantly detected in tumors with epitheloid differentiation (mean staining score: 55 ± 30 [epitheloid] versus 2 ± 3 [sarcomatoid]; $p < 0.0001$; unpaired *t* test; *D*) and in early-stage epitheloid MPM (mean staining score: 66 ± 27 [early-stage epitheloid] versus 46 ± 31 [late-stage epitheloid]; $p = 0.031$; *E*). *F*, Phosphorylation of mTOR and several downstream effectors as indicated in the investigated MPM cell lines by immunoblot. mTOR, Mammalian target of rapamycin; MPM, malignant pleural mesothelioma.

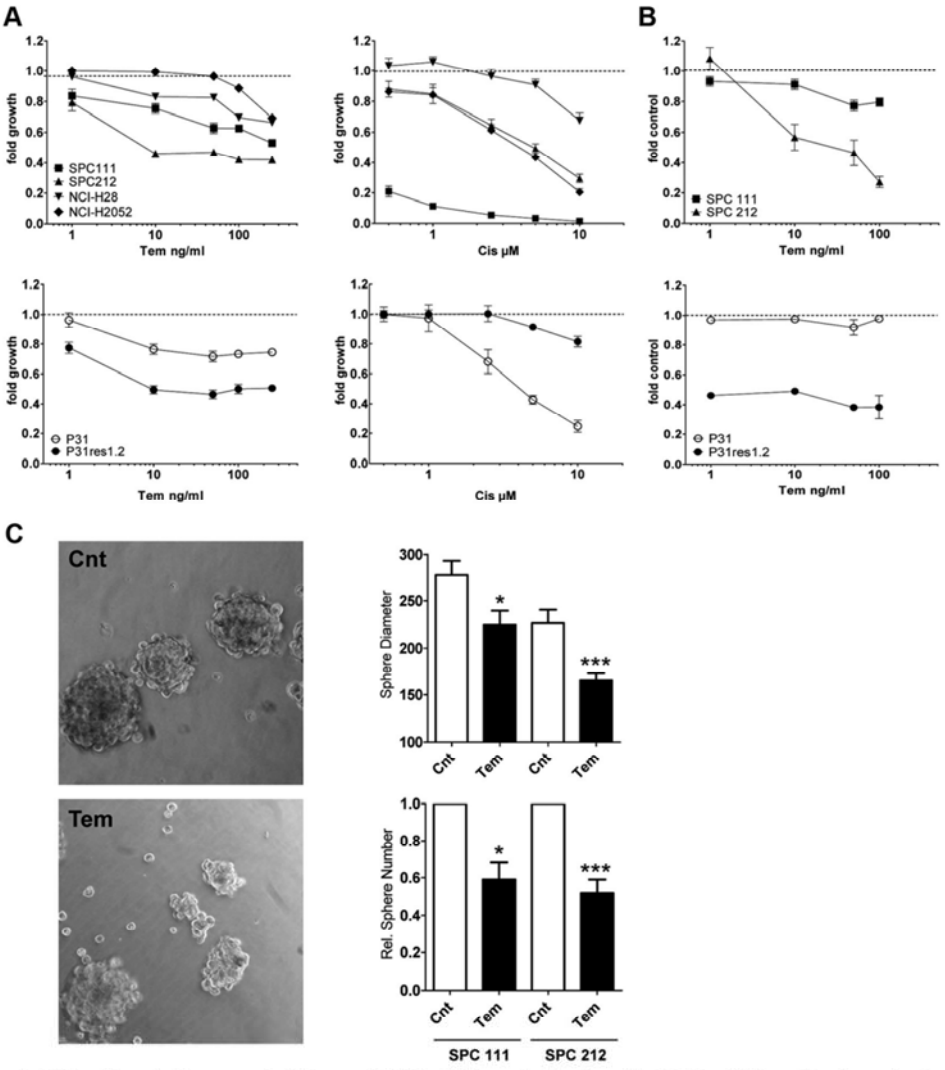


**FIGURE 2.** Activity of temsirolimus against human MPM cell lines relating to cell viability, DNA synthesis, and spheroid formation. *A*, Temsirolimus (Tem) and cisplatin (Cis) dose-response curves as indicated were established in four unrelated MPM cell lines (*upper panel*) and the cisplatin-resistant MPM cell model P31 (*lower panel*). Viability after 72 hours continuous drug exposure were measured by MTT assay and normalized to the untreated controls (Cnt). *B*, Effects of 24 hours drug temsirolimus exposure on DNA synthesis determined by [$^3$H]-thymidine incorporation assay are given normalized to untreated controls. DNA synthesis inhibition was significantly stronger in P31res1.2 as compared with P31 cells ($p < 0.0001$, two-way analysis of variance). *C*, Effects of temsirolimus exposure (100 ng/ml) on nonadherent spheroid formation of MPM cells was determined after 96 hours drug exposure. Representative photomicrographs of SPC111 cells are shown in the *left panel*. Impacts on spheroid size and sphere number are indicated in the *right panels*. In all cases, data are means of at least three independent experiments; bars, SD; *$p < 0.05$, ***$p < 0.0005$. MPM, malignant pleural mesothelioma.

tinct sensitivity to mTOR inhibition while the highly cisplatin-sensitive SPC111 cells were comparably temsirolimus-resistant. Surprisingly, significant temsirolimus hypersensitivity was also observed in P31res1.2 characterized by acquired cisplatin resistance (Figure 2*A*, *lower panels*).

To gain more insight into the growth-inhibiting activity of temsirolimus, impact on DNA synthesis was measured by $^3$H-thymidine incorporation. DNA synthesis in both cisplatin-sensitive cell lines analyzed (SPC111 and P31) was only marginally affected by 24 hours temsirolimus exposure (Fig-

 

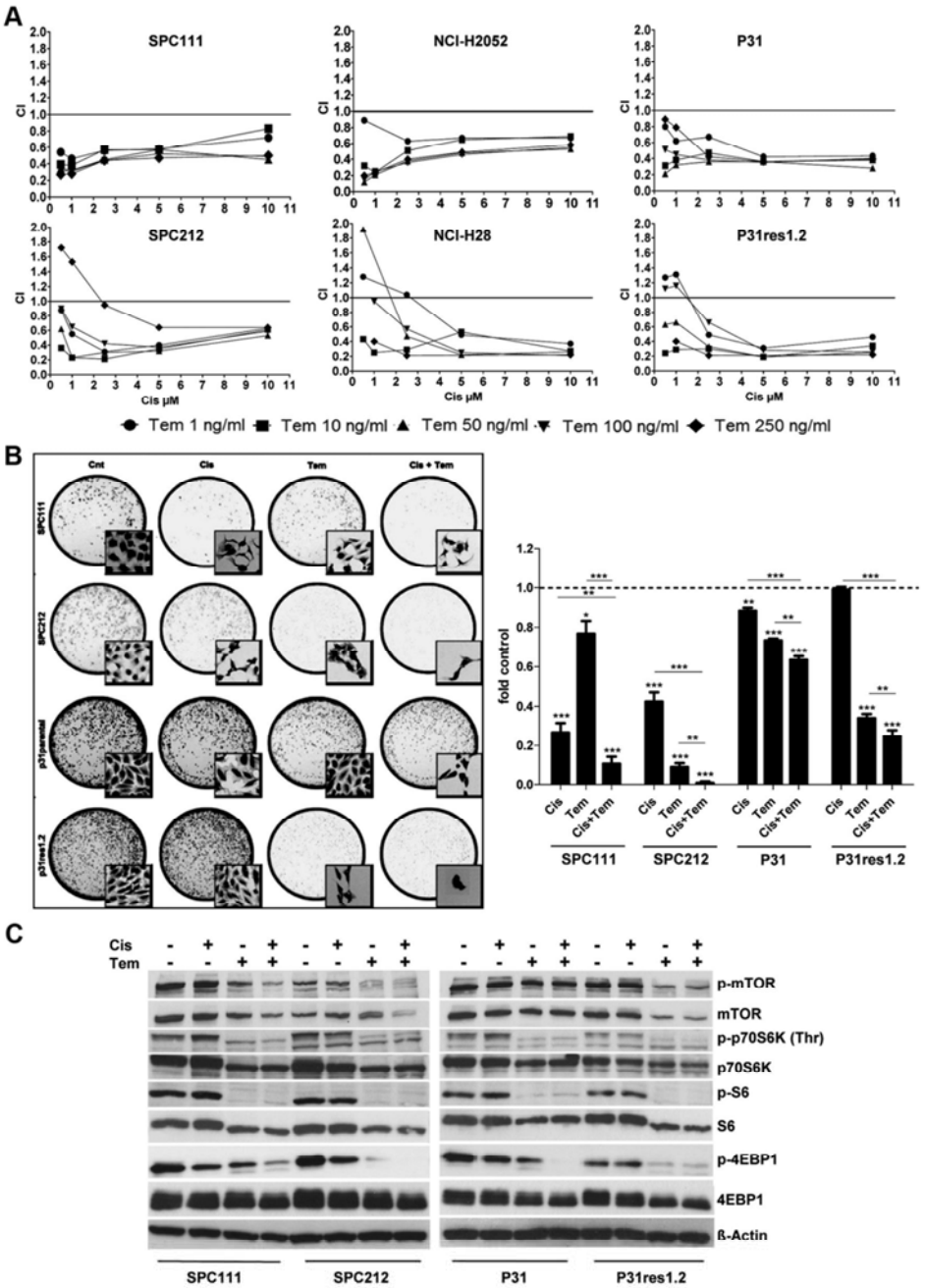


**FIGURE 3.** Activity of temsirolimus (Tem) combined with cisplatin (Cis) against human MPM cell lines. *A*, MPM cell lines were continuously exposed for 72 hours to the indicated doses of temsirolimus combined with cisplatin. Subsequently, changes in viability were measured by MTT assay and data are given as combination indices (CI) calculated using CalcuSyn Software (see Materials and Methods section). *B*, Effects of long-term exposure to temsirolimus without and with cisplatin on colony formation of the indicated cell lines were established. Cell lines sensitive to cisplatin (SPC111 and P31res1.2) were

ure 2*B*). By contrast, $^{3}$H-thymidine incorporation showed a significantly stronger decrease in both cisplatin-resistant cell lines (SPC212 and P31res1.2).

### Impact of Temsirolimus on Nonadherent, Multicellular MPM Spheroid Formation

To investigate whether inhibition of mTOR by temsirolimus affects MPM stem cell-like cell populations,[30] we generated spheroid cultures from two mesothelioma cell lines (Figure 2*C*). Importantly, temsirolimus treatment caused progressive loss of MPM spheroid integrity (SPC111 in Figure 2*C*, *left panel*) and a significantly decreased number as well as mean diameter of spheroids (Figure 2*C*, *right panels*). Moreover, self-renewal of spheroids was widely blocked in the presence of temsirolimus (data not shown). Considering all these parameters, it is apparent that the temsirolimus treatment has a distinct impact on this possibly tumor-initiating cell compartment of MPM cell lines.

### Combination of Temsirolimus with Cisplatin Results in Synergistic Anticancer Effects

We investigated whether inhibition of mTOR by temsirolimus in combination with cisplatin might exert synergistic antiproliferative effects under short-term (72 hours MTT survival assays) and long-term (~7 days clonogenic assays) drug exposure (Figure 3). Results for MTT assays (Figure 3*A*) are given as CIs (see Materials and Methods section). In all investigated MPM cell lines, predominantly synergistic (CI <0.9) anticancer effects were observed independent of the responsiveness of the MPM cell lines to both single agents (compare Figure 2). Antagonistic effects (CI >1.1) were only occasionally observed at high temsirolimus concentrations in the more sensitive cell lines, which might be explained by a strong cell cycle arrest by mTOR inhibition. Clonogenic survival assays confirmed both the inverse relation between temsirolimus and cisplatin responsiveness and the enhancement of temsirolimus sensitivity by induction of cisplatin resistance. In addition, the effect of the combined treatment in all MPM cell models was stronger than that of cisplatin or temsirolimus alone (Figure 3*B*).

The impact of temsirolimus and cisplatin alone and in combination on the activation of mTOR and downstream signal mediators was investigated in MPM cell lines by immunoblot (Figure 3*C*). Especially in sensitive cell lines (SPC212 and P31res1.2), temsirolimus induced a distinct downregulation of phosphorylated as well as total mTOR. Accordingly, phosphorylation of the downstream signal mediators was reduced significantly (p-P70S6K and p-4EBP1) or almost completely (p-S6). Blockade of mTOR activation and downstream signals by temsirolimus was enhanced by cisplatin, especially at the level of 4EBP1. This synergistic effect was notably apparent in the temsirolimus-resistant cell lines (SPC111 and P31).

### Combination of Temsirolimus with Cisplatin Induces Apoptosis Predominantly in Cisplatin-Resistant Cell Models

To investigate apoptosis induction, the proportion of DAPI-stained cells with condensed chromatin was counted (the P31 resistance model is representatively shown in Figure 4*A*). As expected, cisplatin at 5 $\mu$M induced strong G2/M cell cycle arrest (large nuclei in the DAPI staining) in both cell models, but it induced apoptosis predominantly in the parental cell line (quantification in Figure 4*A*, *lower panel*). Temsirolimus (250 ng/ml) induced a significant increase of apoptotic cells only in the cisplatin-resistant subline, again confirming the inverse relationship of chemotherapy and mTOR inhibition response. Apoptosis induction was dramatically enhanced especially in P31res1.2 by combination of both agents inducing ~75% apoptotic nuclei. This was also reflected by cleavage of the caspase substrate PARP (inset in the *lower panel* of Figure 4*A*, lanes corresponding to the four experimental groups). Comparable data were obtained in the SPC111 and SPC212 cell models (data not shown).

### Combination of Temsirolimus with Cisplatin Results in Altered Cell Cycle Distribution

The impact of mTOR inhibition on cell cycle distribution either alone or in combination with cisplatin was determined by FACS analysis (Figure 4*B*). The effect of temsirolimus as a single agent on cell cycle distribution in all MPM cell models was moderate with a slight increase in G0/G1 phase cells in the more sensitive cell lines SPC212 and P31res1.2. Cisplatin alone induced a severe and almost complete G2/M arrest in responsive cell models at 2.5 $\mu$M. Interestingly, G2/M arrest was less pronounced at 5 $\mu$M paralleled by an increase of cells arrested in S-phase (SPC111 and P31) and/or G0/G1-phase (SPC212). Combination with temsirolimus distinctly inhibited G2/M cell cycle arrest by cisplatin, leading to an enhanced accumulation of cells in S-phase and/or G0/G1-phase (Figure 4*B*) as well as sub-G1 indicative for apoptosis (data not shown). This effect was less distinct only in the cisplatin-resistant subline P31res1.2.

### Effect of Temsirolimus as a Single Agent on MPM Xenografts in SCID Mice

Intraperitoneal injection of SPC111 and P31 leads to tumor seed (SPC111 representatively shown in Figure 5*A*). Two weeks after tumor cell inoculation, mice were randomized into two treatment groups receiving either solvent or temsirolimus (compare Materials and Methods section). Drug treatment was well tolerated with no notable toxicity through

**FIGURE 3.** (*Continued*) treated with 1 $\mu$M and the more resistant ones (SPC212 and P31res1.2) with 2.5 $\mu$M cisplatin. Temsirolimus was used at 100 ng/ml. After 7 days of exposure, cells were stained with crystal violet, representative photomicrographs were taken (*left*), and colonies were counted. Data from three experiments are given normalized to controls (*right*). $^{**}p < 0.05$, $^{***}p < 0.0005$, significantly different from nontreated cells. Significance levels immediately above the SD bars represent comparisons with the untreated control. C, MPM cells were cultured in the presence of cisplatin (5 $\mu$M) and temsirolimus (250 ng/ml) alone or in combination for 48 hours. Expression and phosphorylation state were analyzed by Western blotting for mTOR and its downstream mediators as indicated. mTOR, Mammalian target of rapamycin; MPM, malignant pleural mesothelioma.

 

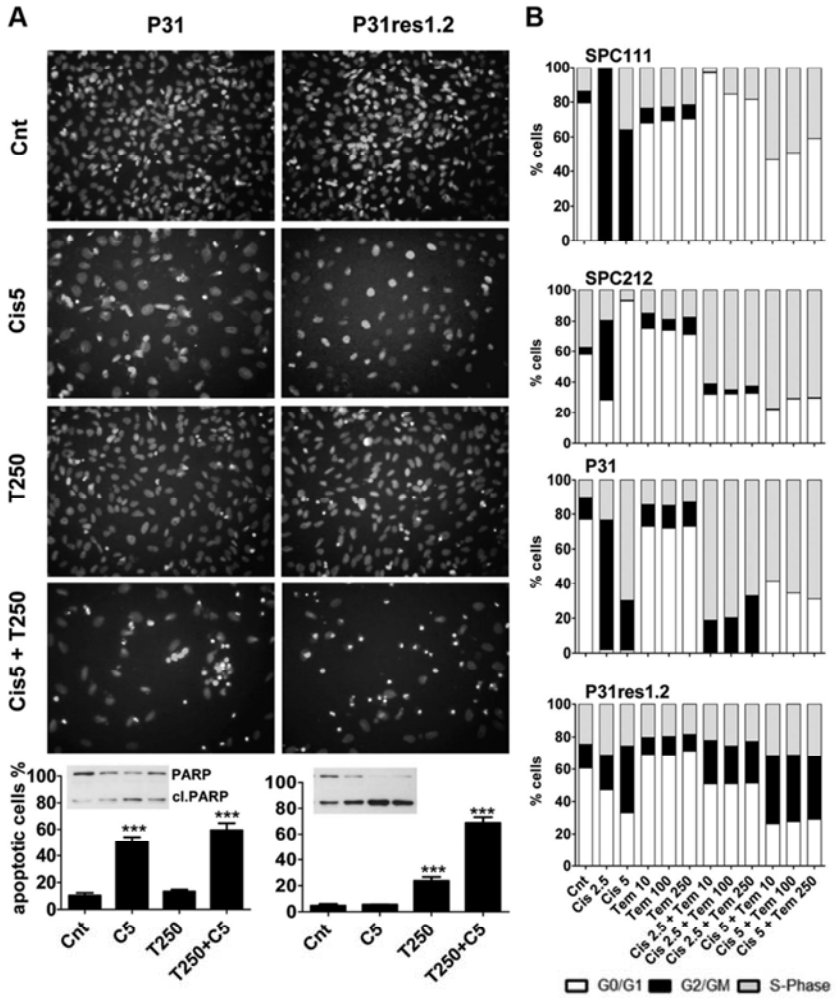


**FIGURE 4.** Impact of temsirolimus combined with cisplatin on apoptosis induction and cell cycle progression. *A*, Apoptosis induction by a 48 hours exposure to temsirolimus (250 ng/ml) and cisplatin (5 $\mu$M) as a single agent and in combination were determined by fluorescence microscopic evaluation of Hoechst 33342-stained P31 and P31res1.2 cells. Representative photomicrographs were taken (*upper panel*) and the mean percentage of apoptotic cells of at least three independent experiments was determined; bars, SD; $*p < 0.05$, $***p < 0.0005$ (*lower panel*). Insets show cleavage of the caspase substrate PARP detected by immunoblot. The four lanes correspond to the indicated experimental groups. *B*, Influence of temsirolimus and cisplatin treatment, both as a single agent and in combination at the indicated concentrations, on cycle progression in MPM cells was determined by FACS analyses after 48 hours continuous drug exposure. One representative experiment out of three delivering comparable results is shown. MPM, malignant pleural mesothelioma.

the entire experiment. In the first experiment, tumor weight was measured in all animals 38 days after transplantation when the first control animal had to be killed. Temsirolimus led to a noticeable decrease of the intraperitoneal tumor burden (Figures 5*A*, *B*). This corresponded to a significant prolongation of survival in both xenograft models analyzed in consecutive experiments (Figure 5*C* and tumor weight at the time of experiment termination due to tumor progression in Figure 5*E*, *left panel*). To prove target interference, tumor protein extracts were prepared 48 hours after the last treatment and analyzed for activation of mTOR and respective downstream signal mediators. As shown in Figure 5*D*, phosphorylation of mTOR and its downstream targets was distinctly reduced or completely inhibited by temsirolimus in vivo.

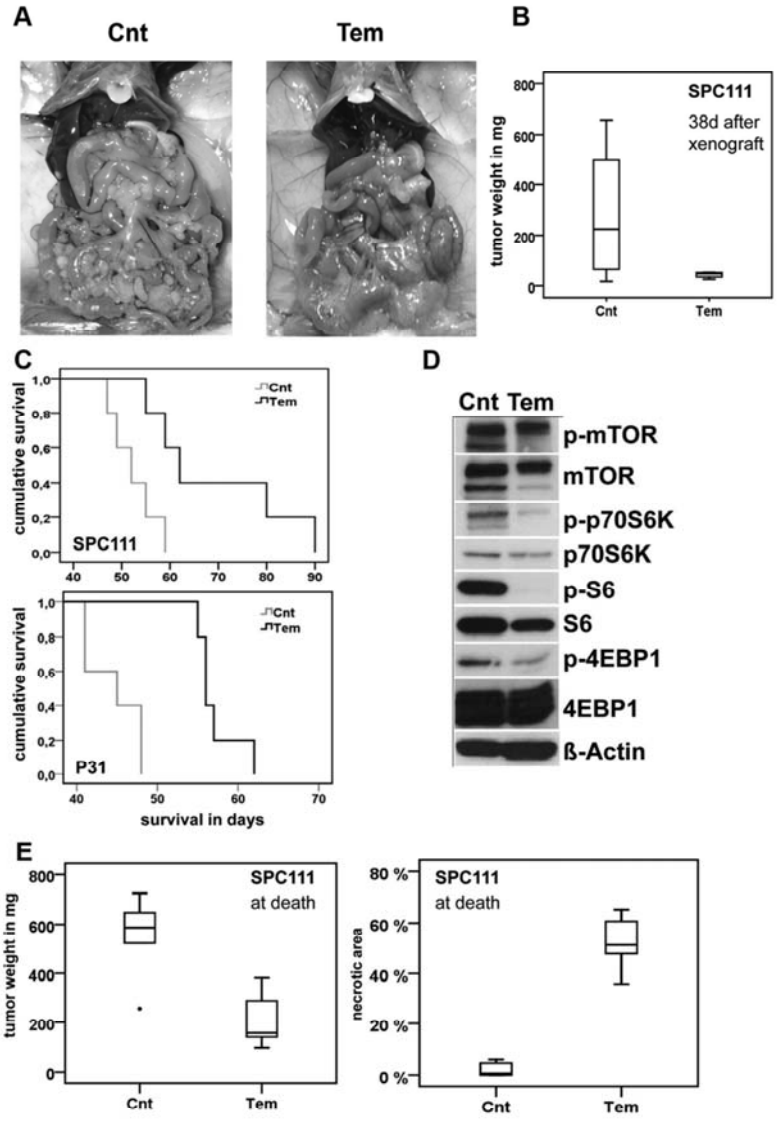


**FIGURE 5.** Temsirolimus inhibits MPM tumor growth in vivo. *A* and *B*, Intraperitoneal tumor growth was determined 38 days after engraftment. Tumor growth in a representative control (Cnt) and treated (Tem) animal (*A*) is opposed to quantification of the tumor weight in all animals (median tumor weight: 225 mg [control] versus 48 mg [temsirolimus]) (*B*). *C*, Overall survival of severe combined immunodeficiency mice is shown as Kaplan-Meier curves (mean overall survival for SPC111: 52 days [control] versus 62 days [temsirolimus]; $p = 0.018$; mean overall survival for P31: 45 days [control] versus 57 days [temsirolimus]; $p = 0.002$). *D*, Impact of temsirolimus treatment on activation of mTOR and downstream effectors in the MPM xenograft tissue (48 hours after the last treatment) was analyzed by Western blot. *E*, Impact of temsirolimus on tumor weight (*left panel*) (median tumor weight: 587 mg [control] versus 156 mg [temsirolimus]; $p = 0.032$) and amount of tumor necrosis (*right panel*) at tumor progression and necessity for experiment termination (median tumor necrotic area: 1% [control] versus 51% [temsirolimus]; $p < 0.0001$). mTOR, Mammalian target of rapamycin; MPM, malignant pleural mesothelioma.

## Temsirolimus Induces MPM Necrosis In Vivo and Synergizes with Cisplatin

In a second experiment, SPC111 xenograft tumors were harvested when mice had to be killed due to tumor progression (Figures 6*A*, *B*). This setting indicated that even several weeks after treatment, phosphorylation of mTOR in temsirolimus-treated tumors was distinctly attenuated and restricted to the proliferating outer cell layer (Figure 6*A*, *lower panel*). Accordingly, the tumor mass was still significantly reduced as compared with the control group (compare Figure 5*E*, *left panel*). As obvious from both H&E and Ki67 proliferation marker stainings (Figure 6*A*, *upper three panels*), temsirolimus treatment resulted in dramatic increase of necrotic areas in the center of tumor nodules (quantification of tumor necrosis in Figure 5*E*, *right panel*). Especially at the border of vital to necrotic areas, massive cell death was confirmed by TUNEL staining, which was widely absent even in larger tumor nodules of solvent-treated control mice (Figure 6*B*). Finally, we investigated the activity of temsirolimus in combination with cisplatin against the SPC111 MPM xenograft model (Figure 6*C*). Both overall survival of mice (Figure 6*C*, *left panel*) and the reduction of tumor weight

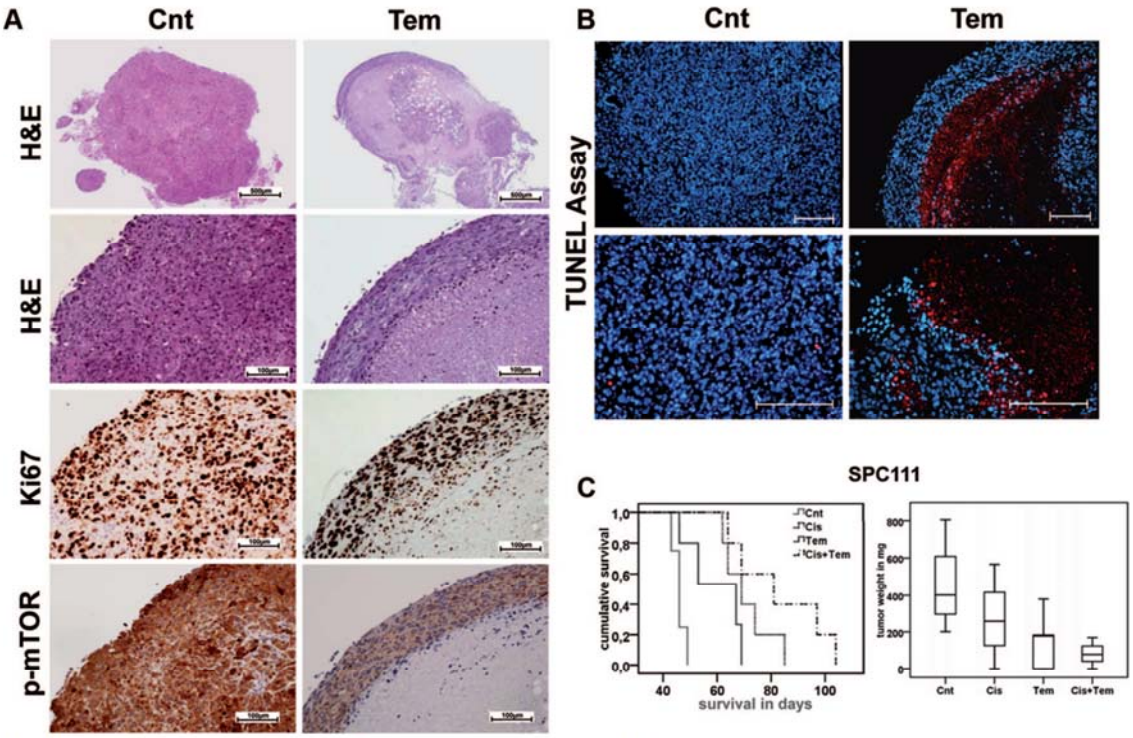


**FIGURE 6.** Activity of temsirolimus combined with cisplatin against MPM in vivo. *A*, Representative temsirolimus (Tem)- or solvent-treated (Cnt) SPC111 MPM tumors are shown. H&E stainings at two different magnifications are opposed to immunostainings for Ki67 and phosphorylated mTOR using consecutive sections. *B*, TUNEL stainings (red nuclei) were performed on sections as detailed in (*A*) to indicate programmed cell death in MPM xenograft tissues. *Blue*, nuclear counter stain by DAPI. Arrow bars, 100 μm. *C*, Anticancer activity of temsirolimus and cisplatin as single agents or in combination was determined against the SPC111 xenograft and survival curves (*left panel*) (mean overall survival: 46 days [control] versus 60 days [temsirolimus] versus 71 days [cisplatin] versus 83 days [temsirolimus/cisplatin]; $p < 0.0001$) are opposed to tumor weight (*right panel*) (median tumor weight: 402 mg [control] versus 259 mg [cisplatin] versus 178 mg [temsirolimus] versus 123 mg [temsirolimus/cisplatin]) at the time when animals had to be killed due to tumor progression. mTOR, Mammalian target of rapamycin; MPM, malignant pleural mesothelioma.

(Figure 6*C*, *right panel*) were significant for both single agents but further enhanced in the combination setting.

## DISCUSSION

In this preclinical study, we demonstrate that (1) a distinct phosphorylation of the major oncogenic signal-transducing factor mTOR is present in the majority of MPM tumor specimens and cell models; (2) inhibition of mTOR by the small-molecule compound temsirolimus as a single agent distinctly attenuated MPM cell growth in vitro and tumor formation in vivo; (3) mTOR inhibition was particularly effective against MPM cells with intrinsic or acquired resistance to cisplatin, a component of the current standard MPM chemotherapy regimen[31]; (4) temsirolimus synergized with cisplatin against MPM models in vitro; (5) addition of temsirolimus enhanced the anticancer effect of cisplatin also in vivo.

Hyperphosphorylation of mTOR selectively in MPM but not surrounding stromal tissues has also been reported in another series ($n = 26$) of mesothelioma samples.[20] In addition, based on microarray analyses, Varghese et al.[32] suggested hyperactivation of the PI3K/mTOR pathway in highly aggressive peritoneal mesotheliomas. In our study, p-mTOR expression in an extended panel of MPM clinical specimens ($n = 70$) was not associated with altered patient survival but more pronounced in lesions with epitheloid as compared with sarcomatoid histology. In biphasic tumors, activated mTOR was again restricted to the areas with epitheloid differentiation. It has been shown in diverse systems that the PI3K/AKT/mTOR pathway is a decisive factor in processes such as epithelial-to-mesenchymal transition (EMT). Mesothelioma is here of specific interest, as biphasic tumors are thought to contain cell types that have undergone EMT and/or the

reverse mesenchymal-to-epithelial transition (MET).[33] Our data regarding restriction of mTOR phosphorylation to epithelial differentiation suggest that this pathway is regulated by EMT/MET processes. Accordingly, promotion of both epithelial differentiation and proliferation by mTOR signals was demonstrated in mammary epithelial cells.[34] By contrast, several other studies have indicated an essential role of the AKT/mTOR signal pathway in the execution of EMT in diverse cell types including murine mammary epithelial cells[35] and peritoneal mesothelial cells.[36]

mTOR activation was particularly high in early-stage disease but negative in nonmalignant tissues. This indicates that activation of mTOR might be a relatively early oncogenic event during MPM development as also observed in early-stage lung adenocarcinoma.[37] This assumption is supported by other studies demonstrating that the mTOR upstream signal mediator AKT is activated in mouse mesotheliomas induced by asbestos injection[20] and in early phase of asbestos-induced malignant transformation of human mesothelium.[22]

Several observations have suggested that the PI3K/AKT/mTOR pathway might be hyperactivated in mesothelioma cells at least in vitro either at the level of mTOR or the upstream signal mediator AKT.[20–24,38] Accordingly, p-mTOR expression correlated significantly with activation of AKT in human and murine mesothelioma cell lines.[20] In that study, both hepatocyte growth factor/c-Met signaling and deletion of the negative regulator PTEN were suggested as mediators of AKT hyperphosphorylation. Expression of PTEN was a strong predictor of mesothelioma patient survival,[39] and adenoviral PTEN overexpression in human MPM cells induced massive apoptosis.[40] Also, a direct activation of AKT by SV-40 was suggested to be involved in asbestos-induced mesothelial cell transformation.[22] Recently, loss of the tumor-suppressor NF2/merlin, mutated in ~50% of MPM cases,[1] was demonstrated to activate mTOR signaling via an AKT-independent, integrin-mediated mechanism. As a consequence, NF2-negative mesothelioma cells were hypersensitive to mTOR inhibition.[38]

Regardless of the upstream mechanism of pathway activation, our data suggest that mTOR is an ideal point for therapeutic intervention. First, mTOR inhibitors such as temsirolimus are already approved for therapy for other solid tumors.[19] In addition, we demonstrate in this study that temsirolimus also targets MPM nonadherent spheroids believed to be enriched in "cancer stem cells" with enhanced tumor-initiating capacity.[30] This cell type is frequently responsible for systemic therapy resistance of solid tumors.[41] Accordingly, it was demonstrated that growth of human mesothelioma cells as tumor fragment-derived spheroids[42] or multicellular nonadherent spheres leads to resistance to drug-induced apoptosis.[24] This therapy insensitivity was based at least in part on hyperactivation of mTOR interfering with mitochondrial apoptosis activation.[21] Together with our observations, these data suggest that mTOR inhibition should not only target the mesothelioma stem cell population but also reverse intrinsic and/or acquired resistance to chemotherapy.

Indeed, we detected a distinct synergism between mTOR inhibition and cisplatin in vitro and in vivo. With regard to the in vitro situation, comparable observations have been published recently by Hartman et al.,[23] demonstrating that sirolimus enhances cisplatin-induced cell death in MPM cells. Also, in several other cancer types such as ovarian, endometrial, neuroectodermal, and lung cancers,[14,16,17,43] mTOR inhibitors showed efficient chemosensitizing properties by increasing cisplatin-induced apoptosis. Accordingly, hyperactivation of the PI3K/AKT/mTOR signal pathway was indicated to be responsible for cisplatin resistance induced by the adenoviral E1a gene[44] and insulin-like growth factor receptor I.[45] In addition to the synergism between chemotherapy and mTOR inhibition, we found that intrinsic as well as acquired cisplatin resistance is generally accompanied by hypersensitivity to temsirolimus. Comparable sensitization against mTOR inhibition as a consequence of cisplatin resistance was also observed in ovarian[18] and lung cancers.[46] Interestingly, we did not observe an upregulation of the mTOR pathway activity in the cisplatin-resistant cell models, suggesting that hyperactivation is not directly responsible for the chemotherapy resistance. Consequently, our data rather suggest that cisplatin-resistant cell lines develop forced "addiction" to continuous mTOR signals. The molecular mechanisms underlying this vulnerability of mTOR downstream survival signals in cisplatin-resistant cell models need to be investigated by further experimental approaches.

To the best of our knowledge, this is the first report that mTOR inhibition distinctly attenuates human MPM growth in vivo as xenografts in SCID mice. The inhibition of the molecular target of temsirolimus in vivo, namely downregulation of tumor-associated mTOR phosphorylation, was demonstrated immediately after therapy but still persisted for several weeks after temsirolimus administration. The profound reduction of tumor load might be based on massive tumor cell death demonstrated by TUNEL staining mainly at the junction to the necrotic areas. Moreover, temsirolimus is known to exert potent antiangiogenic activities,[47,48] which might support the development of central tumor necrosis. In addition to the distinct activity of temsirolimus as a single agent against MPM xenografts, we also performed an initial experiment for combination with cisplatin. As expected from the in vitro analyses, the combination approach exhibited the strongest anticancer effect against MPM xenografts.

In summary, we present in this study strong evidence that temsirolimus is active against human MPM in vitro and in vivo, shows hyperactivity against cisplatin-resistant MPM cells, and synergizes with platinum-based chemotherapy. This suggests further investigation of mTOR inhibition as a promising treatment strategy for human MPM either in combination with chemotherapy or as second-line treatment after chemotherapy failure.

## ACKNOWLEDGMENTS

*The authors thank Vera Bachinger, Christian Balcarek, and Elisabeth Gal for assistance. Sabine El Gazzar, Elisabeth Rabensteiner, and Anita Brandstetter are kindly acknowledged for help with immunohistochemistry analyses.*

 

*Supported by Medical Scientific Fund of the Mayor of the City of Vienna, Initiative Krebsforschung of the Medical University Vienna, Wyeth/Pfizer research grant.*

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# Curriculum vitae

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## Publications

A. Hoda, **A. Mohamed**, B. Ghanim, M. Filipits, B. Hegedus, M. Tamura, J. Berta, B. Kubista, B. Dome, M. Grusch, U. Setinek, M. Micksche, W. Klepetko, W. Berger. *Temsirolimus Inhibits Malignant Pleural Mesothelioma Growth In Vitro and In Vivo.* Journal of Thorac Oncol. 2011. Supplied by publisher.

T. Falta, P. Heffeter, **A. Mohamed**, W. Berger, S. Hann, G. Koellensperger. *Quantitative determination of intact free cisplatin 1 in cell models by LC-ICP-MS.* J. Anal. At. Spectrom., 2011, 26, 109-115.

## Oral Presentations

**A. Mohamed**, M. A. Hoda, C. Pirker, P. Heffeter, B. Ghanim, B. Hegedus, W. Klepetko, W. Berger (Austria). *Effects of mTOR Inhibition in malignant mesothelioma.* Annual congress of the European Respiratory Society, Barcelona, 18-22 Sept. 2010.