New Molecular Strategies for the Therapy of Human Malignant Pleural Mesothelioma

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1. Introduction

Human Malignant Pleural Mesothelioma (MPM), first described in 1870 and diagnosed in 1947 , is an aggressive neoplasm arising from the mesothelial cells lining the pleura.

In the United States, MPM affects approximately 2500 persons per year while in Western Europe the number of patients is almost doubled and the overall incidence is increasing worldwide (Morinaga, Kishimoto et al. 2001; Leigh and Driscoll 2003).

Asbestos fibers are the major carcinogen associated with MPM (Mossman, Bignon et al. 1990; Mossman 1996; Mossman and Churg 1998; Robledo and Mossman 1999) and the first link between an occupational or incidental exposure to asbestos fibers was reported in South Africa in 1960 (Wagner, Sleggs et al. 1960). Because of its extraordinary insulating and fire-resistant properties, asbestos was largely used in the construction industry between the 1940s and early 1980s until its use was curtailed by health agencies in Europe and in the United States, where now the ban has been reverted to a restricted use under the terms of Regulated Asbestos-Containing Material (RACM) legislation. Asbestos has also been used for the production of automotive parts, roads, school playgrounds, as a roofing material and even in professional clothing. During the decades of its use, a large percentage of the workforce and of the populations surrounding industrialized areas was exposed to asbestos fibers.

Although its use has been largely eliminated in the industrialized world, asbestos fibers are still present as flame retardant or insulating materials in many buildings. A recent concern in asbestos contamination occurred during the World Trade Center disaster on September 11 2001 where dust containing asbestos fibers engulfed the air of New York, NY, exposing residents and workers of the

area to possible long term carcinogenic effects of these fibers (Nolan, Ross et al. 2005).

Based on World Health Organization reports, MPM rates show large differences between gender and country of origin. Virtually worldwide, male rates are much higher than female rates, rising from the sporadic background rate of around 1 per million to over 40 per million in some countries. Industrialized countries have much higher incidence rates than their non-industrialized counterparts, reflecting the past production and use of asbestos in the industry.

In Italy, the incidence of MPM has taken on almost epidemic proportions. Examples are seen in Casale Monferrato, in the northwest, where Eternit remained in operation until 1985, in Monfalcone in the northeast, where naval dockyards and related activities created pollution, and in Biancavilla (Sicily) because of natural presence of amphibole fluoro-edenitic fibers in the environment (Degiovanni, Pesce et al. 2004).

Few common cancers have such a direct causal relation with an exposure to a defined carcinogen as mesothelioma has with asbestos fibers exposure. Indeed, the future occurrence of mesothelioma can be predicted from the pattern of asbestos use around the world.

Although genetic predisposition has been proposed to play a strong role in the etiology of MPM (Dogan, Baris et al. 2006), an intriguing and highly controversial co-factor linked to mesothelioma development in patients is the presence of the tumorigenic virus SV40 (Simian Virus 40), a contaminant of the early batches of poliomyelitis vaccines. MPM is usually not diagnosed until two or three months after the onset of symptoms, often insidious and non-specific; the non-specific symptoms are one of the main obstacles to an early diagnosis, highly important since a surgical approach can be considered feasible in the early stages.

Complete surgical resection with histologically negative margins is very difficult due to the diffuse spread of the tumor in the mediastinal pleura and the diaphragm; hence surgeons normally adopt a technique known as “cytoreduction” which implies resection of a vast bulk of the tumor, generally leaving microscopic tumor tissue behind.

Although metastatic deposits of mesothelioma are fairly common at post mortem, these deposits rarely manifest clinically (Lumb and Suvarna 2004). The most common sites of spread are the hilar, mediastinal, internal mammary, and supraclavicular lymph nodes. Local invasion involves other contiguous organs such as the spinal cord (resulting in back pain and paralysis), the pericardium (resulting in pericardial effusions and tamponade), and the contra lateral lung. Metastasis occurs in major organs, such as bone, and occasionally, miliary spread is apparent (Musk 1995).

The most common diagnostic problem is distinguishing MPM from adenocarcinoma, especially when the tumor has invaded the pleura. Both diseases present similar symptoms and they both invade the pleura therefore a multimodal diagnostic approach is often needed. Currently utilized diagnostic tools include: Computed Chest Tomography (CT) that can highlight pleural effusions, enlarged lymph nodes and the presence of pleural masses, Positron Emission Tomography (PET) that may confirm the high metabolic activity characteristic of the tumor to help in staging the tumor, and Video Assisted Thoracoscopic Surgery (VATS) that allows directed pleural biopsy and drainage of the effusions.

Standard histology of the biopsies taken from the patients can help to distinguish MPM from adenocarcinoma, because calretinin and vimentin are present in 88% and 50% of patients respectively. A newly discovered serum marker called Soluble Mesothelin-related Peptide (SMRP) (Robinson, Creaney et al. 2003), may improve early diagnosis and will provide a valuable tool to monitor treatment responses.

Median survival of patients from presentation is 9–12 months and the most common clinical presentation is a progressive dyspnea, mostly accompanied by a dry cough and a constant steady chest wall pain, normally a result of a large pleural effusion and a reduced lung capacity. Other symptoms include weight loss and fatigue but these generally appear later in the course of the disease and are associated with a poor prognosis (Baris, Simonato et al. 1987).

In the past few years, there have been several major improvements in the management of MPM, especially the development of more effective therapies plus new discoveries, which could improve mesothelioma diagnosis and provide new insights into the pathobiology of the disease (Kindler 2000; Treasure and Sedrakyan 2004). These discoveries are producing new approaches to diagnosis and prognosis, such as DNA microarray patterns to predict outcome, and genetic information to develop novel therapies such as gene therapy (Nelson, Robinson et al. 2005).

Besides surgery, chemotherapy is the most common treatment for MPM. Until recently, no chemotherapy regimen has increased survival rates. With the advent of anti-metabolite therapy with multi-targeted folate antagonists (i.e. Alimta or pemetrexed), survival has been shown to be increased from 9 months (cisplatin alone) to 12 months (cisplatin plus Alimta) (Vogelzang, Rusthoven et al. 2003). Unfortunately, no current chemotherapy regimen for MPM has proven to be curative, but several regimes are valuable for palliation. Most available chemotherapy drugs have been tested in malignant mesothelioma as single agents. In general, single-agent response rates are under 20%, and no survival advantage for single-agent chemotherapy has ever been clearly demonstrated. Most of the studies using either single agents or combination regimens have been small, unrandomized phase II trials. Patients are usually heterogeneous between studies with regard to stage and prognostic factors. These variations make comparisons between studies difficult and potentially misleading. The introduction of standardized criteria and the adoption of uni-dimensional measurement standards as outlined by the Response Evaluation Criteria In Solid Tumors (RECIST) guidelines, will make tumor response in MPM studies more meaningful in the future and improve the consistency of reported response rates. (Therasse, Arbuck et al. 2000; Byrne and Nowak 2004).

Radiotherapy has been studied in MPM for many years and overall, the results have been largely unsuccessful (Baldini 2004). Local radiotherapy directed to surgical sites prevents the seeding of tumor and can also provide palliative relief

of somatic chest-wall, but the diffuse nature of the tumor, which often covers most of the lung and the interlobular fissures, is the principal limitation to this approach.

Failure of single modality treatments to increase MPM patients survival rates has led to the use of multimodality approaches (Neragi-Miandoab 2006), including intrapleural chemotherapy, photodynamic therapy, immunotherapy and vaccination that have demonstrated some benefits, but have yet to be evaluated for their efficacy (Nowak, Lake et al. 2002).

Photodynamic therapy generates toxic oxygen radicals when light converts a sensitizing targeted drug in the presence of oxygen. These radicals damage mesothelioma cells and induce cellular necrosis. Intracavitary treatment of MPM with this form of therapy involves the administration of a sensitizing drug followed by intrapleural or intra-abdominal delivery of light to the tumor by dye lasers. A light-scattering medium is used to ensure even distribution of the treatment. This approach is laborious and time-consuming but has been shown to be an effective method of cytoreduction (Pass and Donington 1995; Pass 2002). As with other approaches, increased survival has not been demonstrated.

Immunotherapy is an additional promising novel treatment, since mouse models have been extensively studied and a large body of preclinical information is available. Patients with MPM mount a weak anti-mesothelioma immune response, but are unable to fight the tumor (Robinson, Callow et al. 2000). The goal of immunotherapy is to amplify the weak host immune response and thereby induce tumor regressions. Trials of interferon alpha (IFN-α), intrapleural interleukin-2 (IL-2), and intratumoral granulocyte–macrophage colony-stimulating factor (GM-CSF) have shown some tumor regression, but not to the magnitude which would warrant the widespread use of these agents (Webster, Cochrane et al. 1982; Davidson, Musk et al. 1998; Castagneto, Zai et al. 2001; Powell, Creaney et al. 2006).

1.1. Asbestos fibers

Asbestos is a naturally occurring family of minerals, composed of long thin crystal fibers, mainly divided into two groups: serpentine (chrysothile – white asbestos) and amphyboles (crocidolite – blue asbestos and amosite – brown asbestos), the latter being more friable in the fibrous form thus being more hazardous for the respiratory system. Chrysotile asbestos is also classified as a sheet silicate, which means it forms flat sheets of long, thin fibers and is more easily woven into cloth than other types of asbestos and amphiboles are classified as chain silicates, since they have a chain-like structure of fibers.

These crystal structures, when in a submicron size having a greater than three to one length to diameter ratio, can penetrate into the deep lung and their long persistence in the respiratory tract (when inhaled the fibers are too large to be phagocytized by macrophages) and their high iron content, which catalyzes the production of reactive oxygen radicals (Broaddus V.C. 1996), determines a chronic inflammatory state of the lungs that may end with the involvement of the serosal surfaces of the pleura, pericardium and the peritoneum. Asbestos fibers can also induce other clinical conditions like benign pleural plaques, pleural fibrosis, lung cancer and asbestosis (Niklinski, Niklinska et al. 2004; Hessel, Gamble et al. 2005).

Morphology, size, geometry, chemical composition and surface charge of various asbestos types play important roles in interactions with cells that lead to cell injury and disease (Kamp and Weitzman 1999)

Asbestos may irritate the pleural mesothelial lining and the fibers' shape and length-to-width ratio is an important physical attribute that determines how deeply into the lung the fibers are inhaled and whether they will have the capacity to penetrate the lung epithelium and enter the pleural space (Sebastien, Janson et al.

1980; Pott, Ziem et al. 1987). The most dangerous fibres are long and thin and can more easily penetrate lungs and smaller airways.

Mesothelial cells normally facilitate the free movement of the pleural surfaces during respiration by secretion of lubricating glycoproteins (Andrews and Porter 1973), providing a slippery, non-adhesive and protective surface. The mesothelial lining also has an antigen presenting role, mediates inflammation and tissue repair. These cells readily proliferate in response to injury and growth factors (Pelin, Hirvonen et al. 1994; Herrick and Mutsaers 2004) and an impaired healing and cell transformation can lead to the formation of serosal adhesions and malignant mesothelioma, respectively.

Fibers also have the capability of interfering with the mitotic process, severing the mitotic spindle, disrupting mitosis, potentially causing aneuploidy and the other forms of chromosome damage that characterize mesothelioma (Ault, Cole et al. 1995), like chromosomal aberrations (Wang 1987), aneuploidy and chromosomal breaks (Levresse, Renier et al. 2000).

Their ability to induce DNA damage (Liu 2000) is thought to be mediated by reactive oxygen species (Weitzman and Graceffa 1984) which induce DNA damage and strand breaks (Kamp, Israbian et al. 1995). These properties are strictly related to the content of iron and magnesium of the minerals, conferring a specific surface charge and different chemical reactivity (Light and Wei 1977).

Asbestos fibers, in contrast to nonpathogenic glass fibers and particles, selectively induce ERK 1/2 phosphorylation and activity in mesothelial cells, leading to apoptosis and/or cell proliferation (Goldberg, Zanella et al. 1997; Jimenez, Zanella et al. 1997). The initiation of the ERK cascade in mesothelial cells by asbestos is due to auto-phosphorylation of the EGF receptor (EGFR) (Zanella, Posada et al. 1996). Aggregation of the EGFR by long crocidolite fibers may initiate cell signaling cascades important for asbestos-induced mitogenesis and mesothelioma (Pache, Janssen et al. 1998). PDGF-α and TGF-β gene expression by amosite asbestos is also ERK-dependent (Dai and Churg 2001) In addition to ERK, p38 kinase also is activated by asbestos via oxidative stress

(Swain, O'Byrne et al. 2004), modulating the DNA-binding activity of transcription factors like AP-1 and NF-κB.

The transcription factor NF-κB is well established as a regulator of genes encoding cytokines, cytokine receptors, and cell adhesion molecules that drive immune and inflammatory responses (Ghosh, May et al. 1998) and more recently, its activation has been connected with multiple aspects of oncogenesis, including the control of apoptosis, the cell cycle, differentiation, and cell migration (Baldwin 1996; Baldwin 2001). Noteworthy, it has also been recently elucidated how asbestos induces the secretion of Tumor Necrosis Factor-α (TNF-α) and the expression of TNF- α receptor, via activation of NF-κB, in human mesothelial cells, providing a mechanistic rationale for the ability of mesothelial cells to survive the cytotoxic effects of asbestos fibers (Yang, Bocchetta et al. 2006).

Other molecular activities triggered by asbestos include modulation of intracellular calcium and activation of isoforms of protein kinase C (PKC) (Perderiset, Marsh et al. 1991), probably involved in oxygen radical generation (Lim, Kim et al. 1997) and induction of IL-8 production.

Another mineral, a natural fibrous zeolite named erionite, has also been proposed to be a strong environmental pollutant leading to the pathogenesis of mesothelioma as revealed by the epidemiological data collected in three specific villages (Karain, Tuzkoy and Sarihidir) in central Turkey (Baris, Saracci et al. 1981; Baris 1991) and in Oregon, USA (Poole, Brown et al. 1983). Erionite is considered a more potent fibrous carcinogen than asbestos for mesothelioma and the median survival of patients associated with erionite exposure after diagnosis was significantly shorter than those associated with asbestos exposure (Selcuk, Coplu et al. 1992).

Recent data reveal that erionite fibers have higher intrinsic transformation ability than asbestos fibers (Bertino, Marconi et al. 2007). While amosite asbestos induces relevant cytotoxicity in normal mesothelial cells, possibly because of their longer fibers or high iron content (Kamp, Graceffa et al. 1992), erionite fibers induce lower cell death, spontaneous activation of survival pathways like NF-κB, Akt and MAPK and higher levels of HGFR/Met, EGFR and PDGFRb. These events

highly support mesothelial cells survival and favor the accumulation of DNA damages, promoting a transformed phenotype of the exposed cells.

These latest molecular insights on the zeolite fibers action are an explanation for the high incidence and malignancy of MM in Turkey villages, where SV40 virus infection of MM cells has never been detected (Emri, Kocagoz et al. 2000) and erionite may *per se* lead to transformation of mesothelial cells.

1.2. Simian Virus - 40

Simian Virus 40 (SV40) is a polyomavirus indigenous to the rhesus macaque. Cell cultures derived from these primates were used in the preparation of polio vaccine pools, inadvertently allowing the virus to be included in the vaccine. The first reported presence of SV40 in the Salk vaccine was in 1960 (Sweet and Hilleman 1960), 5 years after the release of its license in the United States and some vaccines prepared in Eastern Europe contained infectious SV40 until the 1970s (Cutrone, Lednicky et al. 2005).

In its natural host, rhesus macaque, SV40 infection is asymptomatic and harmless but it becomes oncogenic in other organisms. The oncogenicity of SV40 can be seen by its capacity to induce tumors when administered to newborn rodents in high doses, and by its capacity to transform rodent and human cells in vitro. SV40 viral gene sequences have also been identified in a variety of malignancies (Klein, Powers et al. 2002; Shah 2007). Importantly it has been recently demonstrated that SV40 has the ability to promote mesothelial cell survival after asbestos exposure, facilitating the accumulation of genetic damage and the transformation of these cells (Cacciotti, Barbone et al. 2005).

The transforming capabilities of SV40 mainly reside in the large and small t antigens (Tag and tag), early protein expressed in the host cells that cause genetic modifications and cell signaling events, most notably the induction of cell survival pathways and activation of cell surface receptors (Rundell and Parakati 2001; Saenz-Robles, Sullivan et al. 2001). Other viral proteins such as middle T antigen (Gottlieb and Villarreal 2001) contribute to the growth and establishment of cancer cells as well as their resistance to chemotherapy.

SV40-associated mesothelial cell transformation has been attributed to the ability of Tag to inactivate the tumor suppressors, p53 (Carbone, Rizzo et al. 1997)

and the p-retinoblastoma (pRb) families of tumor suppressors proteins (De Luca, Baldi et al. 1997). SV40 Tag inactivation of pRb and p53 has indirect effects on p16 and p21 cyclin D–dependent kinases which appear to be depleted in mesothelioma cells as a result of loss of heterozygosity and through the reduction of p53-dependent transcription, respectively (Hara, Smith et al. 1996; Murthy and Testa 1999). Tag activity also results in the rearrangement of cyclin-dependent kinases subunits (Xiong, Zhang et al. 1993), promoting transformation of cells via alteration of the cell cycle (Testa and Giordano 2001). Other known targets of Tag are the transcriptional co-activator p300/CBP (Avantaggiati, Carbone et al. 1996; Cho, Tian et al. 2001), essential for virus growth and transcription/replication of viral DNA (Alexiadis, Halmer et al. 1997), and p130, a member of the family of Rb proteins (Lin and DeCaprio 2003).

The SV40 small t-antigen (tag) enhances the activity of telomerase (Foddis, De Rienzo et al. 2002), Extracellular Signal-Regulated Kinases (ERKs), Activator Protein-1 (AP-1) (Carbone, Rizzo et al. 1997) and NF-κB. These molecular alterations are predominantly caused by inhibition of the activity of Protein Phosphatase 2A (PP2A), altering cellular behavior and promoting cell growth and transformation (Sontag, Sontag et al. 1997).

Sequences of SV40 have been shown in samples from 60% mesothelioma patients (Carbone, Pass et al. 1994), however these results have become highly controversial as many reports showed the high incidence of false-positives due to the presence of SV40 sequences in the promoter regions of many plasmids routinely used as protein-expression vectors (Lopez-Rios, Illei et al. 2004). These studies have concluded that primers targeting sequences in the 4,100–4,713 region of SV40 are at high-risk for providing false-positive data as a result of plasmid contamination. Although it is not known to what extent plasmid contamination contributed to positive data in other studies, it is noteworthy that almost all the PCR studies to date for SV40 DNA have employed high-risk primers.

The debate on the contribution of SV40 to the development of mesothelioma has been recently discussed in a dedicated and animated session during the

recent IMIG (International Mesothelioma Interest Group) meeting in Chicago, IL (2006).

At the present time, the ability of SV40 to induce cellular transformation cannot be disputed but the direct epidemiologic association between malignant mesothelioma and SV40 infection is still unclear, providing an argument of discussion and further research.

1.3. Chromosomal alterations

Mesothelioma also results from the accumulation of numerous somatic genetic events (Dianzani, Gibello et al. 2006). The occurrence of multiple, recurrent cytogenetic deletions suggest that loss and/or inactivation of tumor suppressor genes are critical to the development and progression of mesothelioma (Lee and Testa 1999). Deletions of specific regions in the short (p) arms of chromosomes 1, 3, and 9 and long (q) arms of 6, 13, 15, and 22 are repeatedly observed in MM, and loss of a copy of chromosome 22 is the most common numerical change seen (Murthy and Testa 1999). However, relatively little is known about critical genetic changes in the genesis of mesothelioma. Of the known cytogenetic changes, the most frequent is loss of CDKN2A/ARF, encoding the tumor suppressors p16INK4a and p14ARF at 9p21 (by homozygous deletion) (Hirao, Bueno et al. 2002; Altomare, You et al. 2005), adversely affecting both Rb and p53 pathways, respectively.

NF2 (Merlin), a tumor suppressor located at 22q12 (by an inactivating mutation coupled with allelic loss) is also frequently altered in mesotheliomas (Cheng, Lee et al. 1999) and its re-expression inhibits mesothelioma invasiveness (Poulikakos, Xiao et al. 2006). Other conventional proto-oncogenes and tumor suppressor genes have been investigated including NRAS (Papp, Schipper et al. 2001), HRAS and KRAS (Kitamura, Araki et al. 2002), and TP53, which encodes

the tumor suppressor p53 (Mayall, Jacobson et al. 1999) but no consistently frequent mutations are found.

1.4. Chemotherapy

Currently there is no widely accepted treatment for the cure of MPM, the response rates for single-agent chemotherapy are under 20% and no survival advantage has ever been clearly demonstrated. A number of multi-center studies are now under way and several new therapeutic regimens appear to be successful, however MPM cells display a profound chemoresistance (Fennell and Rudd 2004) and a standard of care is still missing.

Doxorubicin has been one of the most studied treatments but the response rate has been reported to be only 14% (Lerner, Schoenfeld et al. 1983).

Platinum analogues have been studied both as single agents and in combined regimens. High concentrations of cisplatin achieved a response rate of 36%, but with significant discontinuations (34%) due to its toxic effects. Carboplatin, an analogue of cisplatin which is better tolerated and easier to administer, produces response rates similar to conventional doses of cisplatin (7–16%) in phase II studies (Raghavan, Gianoutsos et al. 1990; Vogelzang, Goutsou et al. 1990; Planting, Schellens et al. 1994).

The association of cisplatin with pemetrexed (Alimta®) demonstrated a statistically significant survival advantage of the patients in phase III trial (Vogelzang, Rusthoven et al. 2003) and is currently clinically used as a first-line therapeutic agent. Cisplatin exerts its antitumor activity by inducing DNA damage, apoptosis and necrosis (Rosenberg 1985; Cepeda, Fuertes et al. 2007) while pemetrexed, an anti-folate drug (Hanauske, Chen et al. 2001), targets and inhibits three enzymes used in purine and pyrimidine synthesis: thymidylate synthase (TS), dihydrofolate reductase (DHFR), and glycinamide ribonucleotide formyl transferase (GARFT).

The alpha-folate receptor gene is overexpressed in up to 72% of MPM tumors, encouraging a successful role for antifolate drugs among therapeutic strategies (Bueno, Appasani et al. 2001). Raltitrexed, a thymidylate synthase inhibitor, is currently being evaluated in combination with oxaliplatin and cisplatin in two phase III trials (Fizazi, Caliandro et al. 2000; van Meerbeeck, Gaafar et al. 2005). Interestingly, unlike most newer antifolates tested , 5-FU and its oral derivative capecitabine, showed little clinical benefit (Otterson, Herndon et al. 2004).

Other compounds that have been considered for the treatment of mesothelioma as single agents with generally poor results include gemcitabine, mitomycin D, cyclophosphamide, vinorelbine and temozolomide (Sorensen, Bach et al. 1985; Bajorin, Kelsen et al. 1987; Kindler and van Meerbeeck 2002; van Meerbeeck, Baas et al. 2002; Tomek and Manegold 2004). Different and improved clinical results, associated with lower collateral effects, may be obtained when a second drug is added to the normal chemotherapy regimen. Many of the combinations shown in **Table 1** are currently being evaluated in clinical trials.

Regimen	Response rate
Doxorubicin + cyclophosphamide	12%
Doxorubicin + cisplatin	25%
Gemcitabine + cisplatin	16–48%
Pemetrexed + cisplatin	41%
Raltitrexed + cisplatin	23%
Oxaliplatin + raltitrexed	30–35%
Oxaliplatin + gemcitabine	40%
Oxaliplatin + vinorelbine	23%
Vinorelbine (weekly)	24%
CPT-11 + docetaxel	0%
CPT-11 + cisplatin	27%
Methotrexate + leucovorin + alpha-interferon + gamma-interferon	29%

Table 1: Average response rates of patients treated with selected chemotherapy regimens for malignant mesothelioma. Pemetrexed plus cisplatin and gemcitabine plus cisplatin or oxaliplatin are the most effective chemotherapeutic combinations currently available. Adapted from (Steele and Klabatsa 2005).

Promising advances have also been achieved in the development of new drug delivery technologies. Strategies that have already proved to be successful are the liposome-entrapped cisplatin analogs (L-NDDP) (Lu, Perez-Soler et al. 2005) and the recently reported acid-prepared mesoporous spheres (APMS). When administered intranasally and intrapleurally in a murine system, these new devices are non-immunogenic and non-toxic, suggesting their potential role in a more efficient treatment of lung and pleural diseases (Blumen, Cheng et al. 2007).

One novel agent for the treatment of malignant mesothelioma is Ranpirnase (Onconase®), an RNAse derived from eggs and early embryos of the leopard frog (Rana pipens), that has shown activity against a variety of human tumors in vitro and in vivo (Leland and Raines 2001). Onconase is an homologue of RNAse A, which preferentially degrades tRNA, and inhibits cell growth and proliferation, and induces apoptosis through protein synthesis inhibition-dependent and inhibition-independent mechanisms (Iordanov, Ryabinina et al. 2000). It as been tested in phase I and phase II human clinical trials for treatment of numerous solid tumors, including MPM (Mikulski, Costanzi et al. 2002).

The growing knowledge in the biology of mesothelioma is documented by an increasing number of tumor molecular characters which make the assessment of targeted agents particularly interesting. It is now known, for example, that vascular endothelial growth factor (VEGF) and platelet derived growth factor (PDGF) are autocrine growth factors involved in MPM (Langerak, De Laat et al. 1996; Strizzi, Catalano et al. 2001) and epidermal growth factor receptor (EGFR) is also highly overexpressed (Destro, Ceresoli et al. 2006; Edwards, Swinson et al. 2006). Cytotoxic drugs which target these molecular pathways offer fresh potential for the treatment of MPM and other solid tumors (Arora and Scholar 2005).

1.5. Growth factors and signalling pathways

1.5.1. Vascular Endothelial Growth Factor

Vascular Endothelial Growth Factor (VEGF) is a known potent inducer of angiogenesis and plays a critical role in tumor progression (Hanahan and Folkman 1996). MPM patients express significantly higher VEGF levels compared to patients with any other types of solid tumors (Linder, Linder et al. 1998). High VEGF expression is associated with high microvessel density, increased permeability and pleural effusions and normally correlates with poor survival (Ohta, Shridhar et al. 1999).

Another potent angiogenic factor involved in MPM is interleukin-8 (IL-8) (Leung, Cachianes et al. 1989; Galffy, Mohammed et al. 1999). Secretion of VEGF and IL-8 is a widespread phenomenon in tumors, however, expression of their cognate receptors has generally been thought to be limited to endothelial cells. MPM is one of a growing list of neoplasms where receptor expression (VEGF-C, VEGFR-1/-2/-3) and a functional autocrine pathway has been shown for VEGF and IL-8 (Galffy, Mohammed et al. 1999; Ohta, Shridhar et al. 1999; Konig, Tolnay et al. 2000; Strizzi, Catalano et al. 2001). Increased levels of VEGF have also been found in mesothelial cells transfected with the whole SV40 genome sequences (Cacciotti, Strizzi et al. 2002). These cells are able to spread SV40 infection to co-cultured cells and exhibit an Rb dependent autocrine HGF/Met loop (Cacciotti, Libener et al. 2001), that has been reported to regulate VEGF and VEGF receptor expression (Wojta, Kaun et al. 1999).

The development of strategies to control tumor angiogenesis will ultimately lead to the management of MPM progression. Recent studies have shown than antisense oligonucleotides simultaneously directed against VEGF and VEGF-C, specifically inhibits mesothelioma cell growth. Similarly, antibodies targeted against to the VEGF receptor (VEGFR-2) and VEGF-C receptor (VEGFR-3) are synergistic in inhibiting mesothelioma cell growth. (Masood, Kundra et al. 2003).

Other therapeutic tools to reduce angiogenesis and to reduce the progression of MPM are the VEGF inhibitors SU5416 and bevacizumab.

SU5416 (semaxanib, SUGEN, Inc.) is a selective inhibitor of the tyrosine kinase activity of the VEGF receptor flk-1/KDR (Fong, Shawver et al. 1999). Other remarkable effects of this compound are the inhibition of Met receptor in hepatoma cells and the reduction of VEGF and HIF-1α levels through the PI3K-AKT/p70S6K pathway (Wang, Chen et al. 2004; Zhong, Zheng et al. 2004).

Bevacizumab (Avastin, rhuMAbVEGF, Genentech), a recombinant humanized anti-VEGF monoclonal antibody, blocks the binding of VEGF to its receptor and is now under evaluation in association with gemcitabine and cisplatin a multi-center randomized phase II trial (Kindler 2001).

1.5.2. Platelet Derived Growth Factor

Platelet derived growth factor (PDGF) has been reported to be an autocrine growth factor for mesothelioma (Gerwin, Lechner et al. 1987; Langerak, De Laat et al. 1996). PDGF is a potent mitogen for connective tissue cells and, in vitro, mesothelial cells proliferate in a dose-dependent manner in the presence of exogenous PDGF (Mutsaers, Bishop et al. 1997). PDGF receptors are differentially expressed in mesothelioma cells compared with normal mesothelium. While mesothelioma cell lines express PDGF-β receptors, normal mesothelial cells express PDGF-α receptors (Versnel, Claesson-Welsh et al. 1991).

Two PDGF inhibitors are currently evaluated in clinical trials in MPM patients. Gleevec (imatinib mesylate, STI-571, Novartis Pharmaceuticals) is an oral, selective inhibitor of the tyrosine kinases associated with the PDGF receptor β, c-kit (stem cell factor receptor) and Bcr-Abl (Buchdunger, Cioffi et al. 2000). Unfortunately, a recent report from a pilot study, using Gleevec as a single therapeutic agent, highlighted a very low toxic response from patients but overall unsatisfactory results, according to previous experiences (Mathy, Baas et al. 2005).

PTK787 (vatalanib, Novartis Pharmaceuticals) is an orally available drug that inhibits the tyrosine kinases associated with the receptors for PDGF, VEGF and c-kit (Wood, Bold et al. 2000). A phase II trial on 40 mesothelioma patients is currently ongoing.

1.5.3. Epidermal Growth Factor

Epidermal growth factor receptor (EGFR), a transmembrane glycoprotein, is overexpressed in many malignancies, including MPM. Ligand binding activates the intracellular domain of EGFR, triggering cell growth.

ZD1839 (gefitinib, Iressa®, AstraZeneca), an oral highly selective inhibitor of the EGFR tyrosine kinase is active against a broad range of human tumor xenografts, and has demonstrated clinical activity in non-small cell lung cancer (NSCLC) and other malignancies (Ciardiello, Caputo et al. 2000). Recent findings demonstrate that, in vitro, ZD1839 is as effective or more effective against mesothelioma cell line growth as it is against the NSCLC cell line A549, suggesting that it may be an effective therapeutic option (Janne, Taffaro et al. 2002).

Most patients with non-small-cell lung cancer show no response to gefitinib, however, about 10% of patients have a rapid and often dramatic clinical response. It has been recently highlighted how activating mutations in the EGFR underlie the

responsiveness of non-small-cell lung cancer to gefitinib (Lynch, Bell et al. 2004). Furthermore, most patients who initially respond subsequently experience disease progression while still on treatment. Part of this "acquired resistance" is attributable to a secondary mutation (Kosaka, Yatabe et al. 2006). These mutations are not prevalent in MPM (Cortese, Gowda et al. 2006) and the results of a recent phase II trial showed that although 97% of patients with mesothelioma had EGFR overexpression, gefitinib did not show any efficacy. (Govindan, Kratzke et al. 2005).

Since the majority of MPMs aberrantly express the epidermal growth factor receptor ErbB1, new compounds like GW572016 (lapatinib), a dual inhibitor of ErbB1/ErbB2, have been tested in vitro (Mukohara, Civiello et al. 2005). Results from this study revealed that there is no relationship between the presence or the amount of ErbB1, phospho-ErbB1, phospho-ErbB2, ErbB3, ErbB4 and activation of transducing signals like phospho-Akt and the ability of lapatinib to inhibit phospho-ErbB1, in sensitive cells compared to those that did not respond. The combination of lapatinib with transduction inhibitors resulted in increased growth inhibition and this approach is now being extensively considered in treatment strategies.

1.5.4. Insulin Growth Factor

Insulin growth factor-I and -II (IGF-I and IGF-II), single chain peptides of approximately 70 and 67 amino acids, respectively, was initially characterized as possessing both mitogenic and insulin-like activities in adipose and muscle tissues (Rinderknecht and Humbel 1976). In addition to protecting hemopoietic cells and flbroblasts from apoptosis, IGF-I has been shown to be anti-apoptotic in many other cell systems. IGF can behave in an autocrine fashion to stimulate tumor growth or in paracrine fashion to stimulate growth of adjacent tissues. The presence of IGF can also have stimulatory effects on extracellular matrix

development (Syrokou, Tzanakakis et al. 1999). Interestingly, the presence of a functioning IGF receptor is necessary for SV40 induced transformation of mesothelial cells arguing for the importance of the IGF axis in the development of MPM (Porcu, Ferber et al. 1992). Not surprisingly, expression of the two tyrosine kinase IGF receptors, IGF-R1 and IGF-R2, has been found to be present in all MPM cell lines (Liu and Klominek 2003).

Whitson and colleagues recently evaluated the effect of a novel IGF-I receptor inhibitor, NVP-AEW541, on cell growth and IGF associated pathways (Whitson, Jacobson et al. 2006). Their results demonstrated that NVP-AEW541 has a concentration-dependent inhibitory effect on mesothelioma cells in culture, decreasing their viability, suggesting an interesting role for IGFR inhibition in a multimodal treatment of MPM.

1.5.5. Hepatocyte Growth Factor

Hepatocyte growth factor / scatter factor (HGF/SF), the natural ligand for cMet receptor, was originally identified as an oncogene activated in vitro in a human osteogenic sarcoma (HOS) (Cooper, Park et al. 1984).

Signaling via the Met–HGF/SF pathway has been shown to lead to an array of cellular responses including proliferation (mitosis), scattering (motility), and branching morphogenesis. The cellular responses to c-Met stimulation by HGF/SF are important in mediating a wide range of biological activities including embryological development, wound healing, tissue regeneration, angiogenesis, growth, invasion, and morphogenic differentiation (Stella and Comoglio 1999; Furge, Zhang et al. 2000; van der Voort, Taher et al. 2000).

Aberrant c-Met signaling has been described in a variety of human cancers (Longati, Comoglio et al. 2001). Mutations in the tyrosine kinase domain, over-expression or increased co-expression of c-Met and HGF/SF by the same cell can

all contribute to tumorigenesis (Jeffers, Schmidt et al. 1997; Jeffers, Fiscella et al. 1998; Jeffers, Koochekpour et al. 1998).

An high percentage of MPM specimens stain positively for cMet and HGF (Harvey, Warn et al. 1996), suggesting an important role in the development of this malignancy. Furthermore, HGF autocrine and paracrine signalling induces proliferation and migration of mesothelial cells (Warn, Harvey et al. 2001) during mesothelium healing and of mesothelioma cells (Klominek, Baskin et al. 1998). As previously described, the presence of SV40 genome sequence in mesothelial cells is able to induce an HGF/Met autocrine loop (Cacciotti, Strizzi et al. 2002), mediated by large T antigen, which can sustain cell transformation addressing normal mesothelial cells toward the neoplastic transformation.

PHA-665752, a specific small-molecule inhibitor of the Met receptor tyrosine kinase, has been recently tested in a panel of 10 MPM cell lines, to evaluate a potential therapeutic involvement of this signalling pathway (Mukohara, Civiello et al. 2005). Interestingly, only cell lines that exhibited a Met/HGF autocrine loop were sensitive to the inhibitor, showing a marked reduction of growth and in vitro invasion and motility. Other interesting results have been obtained with the small-molecule c-Met inhibitor SU11274 (Sattler, Pride et al. 2003), able to reduce mesothelioma cells growth in a dose-dependent fashion, more selectively on cells carrying a T1010I mutation in the juxtamembrane domain (Jagadeeswaran, Ma et al. 2006), whose clinical significance is not known but has been shown to increase the receptor activity in tumorigenic assays (Lee, Han et al. 2000).

These preliminary results provide evidence as to how this pathway may be an important target in MPM therapy and the preclinical testing of compounds targeting Met receptor are expected to give encouraging results.

1.5.6. Wingless - Int

The Wnt signal transduction pathway plays a critical role in cell fate determination, proliferation and patterning during embryogenesis. Although Wnt is essential for normal developmental processes, its aberrant activation has been closely associated with tumorigenesis. The binding of Wnt ligands to the frizzled transmembrane receptors, leads to the stabilization and accumulation in the cytoplasm of β-catenin. Subsequently, β-catenin translocates into the nucleus and interacts with transcription factors, promoting oncogenes like c-myc and cyclinD. Multiple recent studies have highlighted the abnormal activation of the Wnt/β catenin pathway in MPM (Abutaily, Collins et al. 2003; Dai, Bedrossian et al. 2005). Dysregulation of genes encoding proteins upstream in the Wnt pathway, like extracellular signalling components or disheveled proteins, may play an important role since mutations of β-catenin have not been identified in MPM (Uematsu, Kanazawa et al. 2003; Batra, Shi et al. 2006).

Monoclonal antibodies and siRNA duplexes directed against Wnt-1 and Wnt-2 receptors have been shown to induce apoptosis in cancer cells with activated Wnt pathway (He, You et al. 2004; Mazieres, You et al. 2005) suggesting an interesting future role of Wnt-targeted therapies for the treatment of MPM.

1.5.7. Phosphatidyl-Inositol 3-Kinase / Akt / mTOR

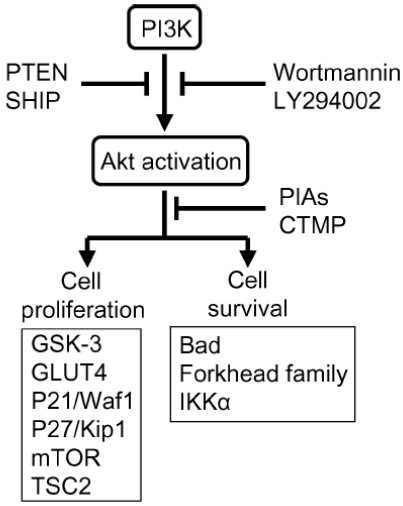
A growing body of evidence suggests that the phosphatidyl-inositol 3-kinase (PI3-K/AKT) pathway plays an important role in human cancers, and numerous Akt substrates have been implicated in tumorigenesis.

PI3K is responsible for the phosphorylation of 3 position of the inositol ring of $PI(4,5)P_2$, to generate $PI(3,4,5)\ P_3$, a potent second-messenger required for survival signaling. The effects of $PI(3,4,5)P_3$ on cells are mediated through specific

binding to at least two distinct protein-lipid binding domains, namely, the FYVE and plekstrin homology (PH) domains (Pawson and Nash 2000).

Akt, also called Protein Kinase B (PKB), is a serine/threonine kinase and the cellular homologue of the viral oncoprotein v-Akt. After the production of PI(3,4,5)P3 by PI3K on the inner side of the plasma membrane, Akt translocates and binds to the phospholipids. The interaction of the PH domain of Akt with PI(3,4,5)P3 is thought to provoke its conformational changes, resulting in the exposure of two main phosphorylation sites (Thr308 in the kinase domain and Ser473 in the C-terminal regulatory domain). Phosphorylation of Thr308 by phosphoinositide-dependent kinase 1 (PDK1), a nucleo-cytoplasmic shuttling protein kinase (Kikani, Dong et al. 2005), is a requirement for the activation of Akt, and phosphorylation of Ser473 by PDK2 is required for full activation of the kinase (Andjelkovic, Alessi et al. 1997; Alessi and Cohen 1998).

Figure 1: Regulation of cell proliferation and survival by PI3K/Akt signalling. Several factors are directly phosphorylated by Akt. The PI3K-Akt pathway is negatively regulated not only by PTEN but also compounds such as wortmannin, LY294002 and phosphatidyl-inositol ether lipid analogues (PIAs). It is also has been suggested that SHIP (SH2-containing inositol phosphatase) and CTMP (carboxy terminal modulating protein) inhibit PI3K-Akt signaling in vivo (Osaki, Oshimura et al. 2004).



Akt mainly regulates cell proliferation and cell survival by directly phosphorylating several substrates (**Figure 1**). Activation of Akt triggers anti-apoptotic mechanisms, positively influences NF-κB transcription, modulates angiogenesis, enhances telomerase activity, increases tumor invasion, and antagonizes cell-cycle arrest [Bellacosa et al.,2005]. Perturbations of the normal PI3K / Akt signalling pathway, either by increased expression of growth factor receptors (Blume-Jensen and Hunter 2001) or mutation of PTEN and SHIP (Simpson and Parsons 2001), always associate with a malignant phenotype (Osaki, Oshimura et al. 2004).

Besides being a key molecular pathway activated in mesothelial cells by SV40, granting them survival from asbestos-induced citotoxicity (Cacciotti,

Barbone et al. 2005), Akt has been shown to be involved in several aspects of mesothelioma biology, from cell-cycle progression to resistance to apoptosis (Altomare, You et al. 2005) and inhibition of the PI3K/Akt pathway is an emerging and promising therapeutic strategy (Mohiuddin, Cao et al. 2002; Ramos-Nino, Vianale et al. 2005; Cole, Alleva et al. 2006).

Mammalian target of rapamycin (mTOR) is a central regulator of ribosome biogenesis, protein synthesis and cell growth. The mTOR kinase controls the translation machinery, in response to amino acids and growth factors, via activation of p70 ribosomal S6 kinase (p70S6K) and inhibition of eIF-4E binding protein (4E-BP1). Although mutations of mTOR itself have not been reported, mutations in components of mTOR-related signaling pathways have frequently been described in human malignant diseases (Hay 2005).

mTOR signalling has important functions in the resistance to apoptosis: it is involved in the enzymatic cascade that, starting from damaged microtubules, induces downstream phosphorylation of the Bcl-2 protein (Asnaghi, Bruno et al. 2004) and p70S6K has been shown to inactivate Bad via phosphorylation (Harada, Andersen et al. 2001). Most notably, pharmacological inhibition of mTOR signalling is a promising therapeutic strategy for several tumors (Huang and Houghton 2003) and inhibitors such as rapamycin (Sirolimus®, Wyeth) and its derivatives CCI-779 (Temsirolimus®, Wyeth), RAD001 (Everolimus®, Novartis Pharma AG), and AP23573 (Ariad pharmaceuticals) have been extensively tested in clinical trial, being well tolerated and able to induce prolonged stable disease and tumor regressions in cancer patients (Dancey 2006).

1.5.8. Mitogen-Activated Protein Kinases

Mitogen-activated protein kinases (MAPK) represent an important family of regulatory signaling molecules that serve as integration points connecting

extracellular signals to the transcriptional programs of the cell. One important group of MAPKs is represented by the extracellular signal-regulated kinases (principally ERK-1 and -2), which are activated in response to growth factors and cytokines via cell surface receptors (Platanias 2003). Like many of the components of the MAPK pathways, ERKs can regulate cell proliferation both positively or negatively, depending on the cellular context in which activation takes place (Ishikawa and Kitamura 1999). MAPKs phosphorylate specific serines and threonines of target protein substrates and regulate cellular activities ranging from gene expression, mitosis, movement, metabolism, and programmed death.

MAPKs are part of a phosphorelay system composed of three sequentially activated kinases, and, like their substrates, they are regulated by phosphorylation. MAPKkinases (MKKs) catalyze phosphorylation and activation of MAPK while MAPK-phosphatases reverse the phosphorylation and return the MAPK to an inactive state. MKKs are highly selective in phosphorylating specific MAPKs. MAPK kinase kinases (MKKKs) are the third component of the phosphorelay system and phosphorylate and activate specific MKKs. MKKKs have distinct motifs in their sequences that selectively confer their activation in response to different stimuli since cells receive many different stimuli from their environment that influence their metabolic rate, interaction with other cells, survival and proliferative potential, and other cellular processes involved in homeostasis and health of the organism (Volmat and Pouyssegur 2001).

A recent study shows how phospho-ERK is significantly increased in mesothelioma specimens in comparison to normal lung tissue, indicating that activation of this pathway may constitute a relevant process in regulating mesothelioma cells proliferation (de Melo, Gerbase et al. 2006) and that inhibition of this pathway, i.e. inhibition of EGF receptor activity, may have therapeutic applications to the clinical setting. ERK activity may also be necessary for mesothelial cells transformation (Ramos-Nino, Timblin et al. 2002) although MAPKs expression and activation does not differentiate benign from malignant mesothelial cells (Vintman, Nielsen et al. 2005). Furthermore recent data (Zhong, Gencay et al. 2006) highlights how deregulated ERK and p38 signalling are

important for mesothelioma progression, driving increase expression of metallo-matrix proteases (MMP 1 and 2), which facilitate migration through an extracellular matrix (ECM) layer.

1.5.9. Nuclear Factor-κB

Rel/NF-κB transcription factors are critical players in the control of the apoptotic response to a variety of stimuli. They are best known for their pivotal roles in immune, inflammatory and acute phase responses, but they also modulate cell growth, apoptosis and oncogenesis (Silverman and Maniatis 2001; Karin and Lin 2002; Lin and Karin 2003).

Presently, there are five known members of the NF-κB family: p50/p105 (NF-κB1), p52/p100 (NF-κB2), c-Rel, RelB, and p65 (RelA), each distinguished by the Rel-homology domain, the portion of the protein that controls DNA binding, dimerization and interactions with inhibitory factors.

Rel family members activity is controlled by scaffold proteins, namely IκBs, that control their subcellular localization and inhibit their DNA-binding activity. Phosphorylation of IκB by the IκB-kinase complex (IKK), following a variety of stimuli, targets IκB for degradation via the ubiquitin–proteasome pathway and culminates in the nuclear translocation of active Rel/NF-κB dimers, their binding to DNA and the transcriptional activation of genes encoding anti-apoptotic proteins like Bcl-2 and inhibitor of apoptosis (IAP) family members. In turn, nuclear NF-κB factors trigger the resynthesis of IκBs, giving rise to an autoregulatory loop that terminates the activation process. NF-κB transcription factors has also been associated with increase in pro-apoptotic responses in the immune system, via the increase of key molecules like FasL and TRAIL and their receptors CD95 and DR4/DR5 (Kucharczak, Simmons et al. 2003).

Activation of NF-κB transcription in mesotheliomas has been correlated with the increase of IAP-1 levels (Gordon, Mani et al. 2007) and also with the reduction of asbestos induced citotoxicity on mesothelial cells (Yang, Bocchetta et al. 2006). Successful therapeutic results with proteasome inhibitors (Bortezomib is able to inhibit NF-κB nuclear translocation by accumulating IκBs in the cytoplasm) (Borczuk, Cappellini et al. 2007) and the pivotal role of the Rel family transcription factors downstream PI3K, MAPK and SAPK pathways in the control of the apoptotic process, open a new interesting field in the quest for new molecular targets in the therapy of malignant mesothelioma.

1.6. Pro- and anti-apoptotic proteins

Mesothelioma resistance to apoptosis is partly due to an impairment of the normal levels and functions of Bcl2 family proteins, a class of proteins that allow a fine control on the process of apoptotic death. It includes members that inhibit apoptosis, such as Bcl2, Bcl-X_L, Bcl-w, Mcl-1 and Bfl-1 or induce apoptosis, like Bax, Bad, Bim and Bid (Willis and Adams 2005). Interestingly it has been recently elucidated that Bcl-2 family members may also exhibit anti-proliferative properties (Zinkel, Gross et al. 2006).

Bcl2 family proteins possess conserved α-helices with sequence conservation clustered in Bcl2 homology (BH) domains. Antiapoptotic members exhibit the homology in all segments BH1 to 4, while pro-apoptotic molecules lack stringent sequence conservation of the first α-helical BH4 domain and can be further subdivided into ‘‘multidomain’’ and ‘‘BH3-only’’ proteins. Multidomain pro-apoptotic members such as Bax, Bak and Bok display sequence conservation in BH1-3 domains. BH3-only members like Bim, Bid, Bik and Bim display sequence conservation only in the amphipathic α-helical BH3 region (Gross, Yin et al. 1999).

Death signals trigger activation of the multidomain pro-apoptotic proteins by transcriptional regulation or post-translational modification, connecting death stimuli to the apoptotic machinery, resulting with release of cytochrome c from mitochondria, caspase activation and the release and activation of a series of other molecules, including SMAC, endonuclease G, and AIF, which augment cell death. (Gross, McDonnell et al. 1999).

Histological analyses show a lack of Bcl2 overexpression in most mesotheliomas (Segers, Ramael et al. 1994; Chilosi, Facchettti et al. 1997) while pro-apoptotic protein Bax is consistently found in mesothelioma cell lines (Narasimhan, Yang et al. 1998). Bcl2 is thought to exert its antiapoptotic function by heterodimerizing with Bax , therefore it is the ratio of Bcl2 to Bax that appears to direct the cell away from or toward apoptosis. Since Bcl2/Bax ratio has been proposed to be low in MPM (Falleni, Pellegrini et al. 2005), resistance to apoptosis of mesothelioma may lie in a mechanisms that counteract the function of Bax.

More recent studies on a broader panel of samples, identified overexpression of Bcl2 in 22 out of 54 samples (40%), Bcl-X_L 13/54 (24%) and Mcl-1 50/54 (92%), while loss of expression of Bad was recorded in 14 samples (14/54 - 24%), Bak 13/54 (24%), Bax 23/54 (42%), Bid 20/54 (37%) and Bim 10/54 (18%) (O'Kane, Pound et al. 2006). Significant differences in abnormal expression of apoptosis proteins were also found between epithelial and sarcomatoid subtypes but histological subtype was the only factor with significant association to patient prognosis. Little is known on the involvement of these modulator of the apoptotic process in the pathogenesis of mesothelioma and their cellular levels haven’t been associated with any particular phenotype.

In another study (Soini, Kinnula et al. 1999), elevated Bcl-X_L mRNA levels have been detected in all mesothelioma cell lines and tumor samples examined. Furthermore, pharmacologic inhibition of Bcl-X_L expression with sodium butyrate, has been shown to lead to apoptotic cell death *in vitro* (Cao, Mohuiddin et al. 2001), suggesting that Bcl-X_L may be necessary to neutralize Bax pro-apoptotic functions, thus being a possible oncogenic candidate in this tumor type.

Interestingly, modulation of Bcl2 family members can increase the chemo-sensitivity of mesothelioma cells (Hopkins-Donaldson, Cathomas et al. 2003; Broaddus, Dansen et al. 2005; Cao, Rodarte et al. 2007), engendering apoptotic cell death in vitro and in vivo. The use of antisense sequences or synthetic inhibitors against anti-apoptotic proteins in combination with other chemotherapeutics, thus represents a promising novel therapeutic strategy (Chawla-Sarkar, Bae et al. 2004).

Inhibitor of apoptosis 1 (IAP-1) is another well described anti-apoptotic factor involved in mesothelioma, member of a large family of genes that promote cell survival after apoptotic stimuli. IAP family members (i.e. livin, XIAP and survivin) generally inhibit apoptotic action of caspases either by preventing proteolytical cleavage pro-forms and inhibiting activated caspases directly (Deveraux, Roy et al. 1998; Deveraux and Reed 1999). TNFα, a cytokine that is induced by asbestos exposure, can increase mRNA and protein levels of IAP-1, IAP-2, and XIAP (Gordon, Mani et al. 2007) and attenuation of IAP-1 mRNA levels in mesothelioma cell lines increased their sensitivity to Cisplatin (Gordon, Appasani et al. 2002) by nearly 20-fold. These results further highlight the importance of the axis TNFα / NF-κB / IAP-1 / Bcl-X_L in mesothelioma and give a strong rationale for the effective use of proteasome inhibitors (like Bortezomib - Velcade®) in pre-clinical studies.

In the perspective of further improving the limited array of therapeutic tools for MPM, newer approaches are urgently needed and molecular studies of resistance to apoptosis of newer *in vitro* models, that can resemble more closely the real tumor structure, will provide precious insights of this malignancy.

Three dimensional cell cultures have been increasingly used in the study of resistance to chemotherapy (Mueller-Klieser 1997), since they mimic many morphological and physiological characteristics of their tumor counterparts.

1.7. Three-dimensional cell cultures

First studies on in vitro three-dimensional (3-D) structures were performed by Holtfreter (Holtfreter 1944) and then Moscona (Moscona 1952; Moscona 1957; Moscona 1957; Moscona 1961), contributed to the field analyzing the formation of “aggregates” of chicken and mouse embryonic cells. More recently, Sutherland (Inch, McCredie et al. 1970; Sutherland, Inch et al. 1971; Sutherland, McCredie et al. 1971; Sutherland 1988) inaugurated the cells spheroid model in the study of resistance of tumors to chemotherapy.

Nowadays spheroids represent a precious research tool and in the last five years huge progress has been made in the study of molecular biology of these 3-D structures that resemble an in vitro system of intermediate complexity between monolayer cultures in vitro and tumors in vivo.

In the three following decades the cell biology of spheroids and the knowledge of the mechanisms to be held responsible for their increased drug resistance, advanced considerably (Mueller-Klieser 1997). Increased resistance to ionizing radiation and later on to cytotoxic drugs of multi-cellular spheroids was identified by Durand and Sutherland, who created the term ‘contact effect’ for this phenomenon (Inch, McCredie et al. 1970) since previously identified mechanisms of resistance to chemotherapy are functional at the level of the isolated cell, and may therefore be termed ‘uni-cellular resistance’.

There are two sides from where research can approach the 3-D tumor biology and each direction has its own advantages and disadvantages. Essentially three-dimensional cell cultures require conditions such that the adhesive forces between the cells are greater than that for the substrate on which they are plated. In the most simple form, this may involve an overlay method, such as coating plastic tissue culture surfaces with a thin layer of agarose, or other substrate which

will prevent the deposition of a matrix. Under these conditions, many cell types, in particular tumor-derived lines, will often undergo spontaneous homotypic aggregation.

Growing 3-D *ex-vivo* cultures (tumor fragment spheroids – TFS), using minced mesothelioma samples, allows to maintain the cellular heterogeneity and the microenvironment of the original tumor, important characteristics from a clinical point of view but on the other side the system doesn't provide any control on the cell population and spheroids' size, doesn't allow certain assays and also, the availability of mesothelioma samples can be a limiting step.

Multi-cellular spheroids, homogeneous 3-D cultures from established and continuous cell lines, can be reproducibly obtained, having control on cell population and spheroids' size, allowing a more accurate detection of molecular mechanisms driving the acquired resistance to chemotherapy. The absence of the tumor microenvironment and different cell population is here a limitation, since the system lacks the complexity of the original tumor.

One of the advantages of using multi-cellular spheroids is their controlled and reproducible geometry, with a defined layered structure that may vary depending on the type and number of cells forming the aggregates:

- an external layer of proliferating cells that are metabolically active
- a second layer of quiescent cells
- a necro-apoptotic core

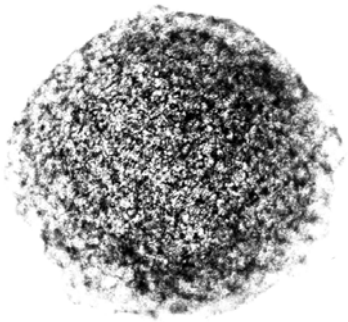


Figure 2: Photo of a MPM spheroid. A multi-cellular spheroid formed by 50.000 REN cells after 24 hours of culture using the polyHEMA method.

The importance of this layer classification relies on the different susceptibility to chemotherapy of cells with different metabolic conditions. Some generical biological differences that may alter the response to chemotherapeutic agents are:

- a decreasing inward nutrient gradient and an increasing inward gradient of CO_2 that may explain the presence of a necrotic core (a phenomenon that is directly related to the dimension and the age of spheroids). More realistically, since it has been shown that the diffusion of certain macromolecules and antibodies is not impaired (Fracasso and Colombatti 2000), the presence of a layered cell system creates an extracellular pH that may affect the diffusion of certain substances
- an increased production of extra-cellular matrix proteins compared to the respective monolayers, especially ialuronic acid and collagen
- an activation of specific transduction signals induced i.e. by cadherins and integrins, responsible for cell-cell junctions (although in some structures has been shown a reduced expression of certain integrin subunits like α6 and β4)
- a cytoskeleton remodeling, a better inter-cellular integration (social control) and a more dense chromatin structure that correlates with the reduction in metabolism and protein synthesis of the inner layers
- differences in the expression of genes like an increase of p21 and p27 (accumulation of cells in G_0/G_1 phase (Hamilton 1998; Bates, Edwards et al. 2000)) and HSP27 (a protein chaperone involved in ROS-induced cyto-protection) and a decrease of certain growth factor receptors like EGF (Mansbridge, Ausserer et al. 1994)

As an example of the consequences that these pattern may have on chemotherapy, it is known that the lower metabolic index, decreased protein synthesis, altered sub-cellular localization of topoisomerase II (Oloumi, MacPhail et al. 2000) and a more compact chromatin structure make spheroids more resistant to Etoposide, while different extra-cellular pH impairs the activity of Doxorubicin (Erlichman and Vidgen 1984).

Mesothelioma tumor fragment spheroids have already been studied and characterized (Kim, Wilson et al. 2005) and they have been shown to be resistant to TRAIL plus cycloheximide treatment. Interestingly, apoptotic resistance of

mesothelioma cells was significantly reduced by inhibiting either the PI3K / Akt pathway or the mTOR pathway with rapamycin.

Interestingly, many mixed-type mesothelioma continuos cell lines has been reported to form floating spheroids in culture (MSTO-211H and H2595) (Pass, Stevens et al. 1995) and it is well known how mesothelial cells have the ability to float and maintain viability in the pleural space (Mutsaers 2004), suggesting that the study of spheroids may be of clinical interest since they may resemble early tumor formations and provide a more relevant platform for the study of molecular mechanisms driving the poor response of MPM to chemotherapy.

2. Study aims

The present work aims to evaluate new approaches for the therapy of human malignant mesothelioma by the evaluation of new strategic synergisms i.e. the inhibition of specific survival pathways in association with a classical chemotherapeutic agent, and by studying molecular mechanisms supporting acquired resistance to apoptosis of three dimensional aggregates of MPM cells.

Imatinib mesylate (STI571, Gleevec®) is a selective inhibitor for a subset of tyrosine kinases, including bcr-abl, c-Kit, PDGFRβ and c-Fms (Dewar, Zannettino et al. 2005), currently used successfully in the treatment of chronic myeloid leukemia (CML). PDGFRβ is known to be overexpressed in MPM and has been identified as a therapeutic target in many solid tumors (George 2001). Recent reports show that inhibition of PDGF receptor signalling by Gleevec is able to sensitize solid tumors to radioimmunotherapy (Baranowska-Kortylewicz, Abe et al. 2005), gastric carcinoma to chemotherapy (Kim, Emi et al. 2005) and impair the increased growth rate of small cell lung cancer cell lines (Krystal, Honsawek et al. 2000). Furthermore, chemotherapeutic regimens based on the association of gemcitabine or pemetrexed with cisplatin are currently the first line choices for the treatment of MPM, hence we evaluated the possibile positive influence of Gleevec-mediated tyrosine kinase inhibition in association with chemotherapy treatment *in vitro*.

Recent data has highlighted the selective antineoplastic potential of bis(1,1-dioxoperhydro-1,2,4-thiodiazinyl-4)-methane (Taurolidine), an antibacterial drug, successfully used in the clinic as a safe antibiotic lavage (Gorman, McCafferty et al. 1987; Burri 1990). Taurolidine (Taurolin®, Geistlich Pharma, CH-6110

Wolhussen, Switzerland), a derivative of the aminoacid taurine, has been shown to enhance survival in an animal model for melanoma and to inhibit proliferation rate of rat metastatic colorectal tumor cells, *in vitro* and *in vivo* (McCourt, Wang et al. 2000; Da Costa, Redmond et al. 2001; Ribizzi, Darnowski et al. 2002; Stendel, Stoltenburg-Didinger et al. 2002; Shrayer, Lukoff et al. 2003; Stendel, Scheurer et al. 2003). Furthermore, Taurolidine has been shown to induce apoptosis by enhancing glioma cells sensitivity to Fas-ligand and via a mitochondrial cytochrome-c dependent mechanism in HL-60 cells (Sutherland 1988). Interestingly, a recent study demonstrated the ability of Taurolidine to trigger the apoptotic program in MPM cell lines and to reduce tumor spreading in a mouse model (Nici, Monfils et al. 2004). Our aim was to evaluate the mechanism of action of Taurolidine in MPM, and provide further molecular insights to warrant its potential clinical effectiveness.

Also, the study of three-dimensional cell structures is emerging as a critical model of apoptotic resistance in cancer research. Tumor spheroids not only express intrinsic resistance to cytotoxic drugs but also display 'multimodal resistance' to other agents such as radiation, immunotoxins, cytostatic cytokines and hyperthermia (St Croix and Kerbel 1997).

We hence aimed to study the resistance to chemotherapy of three-dimensional MPM cell structures compared to monolayers in order to evaluate molecular mechanisms of acquired apoptotic resistance, in a more clinically relevant three-dimensional model, as to better define the molecular patterns driving the poor response of MPM to therapy.

3. Materials and methods

3.1. Cell cultures

In this study, two human mesothelial non-neoplastic cell lines (HMC and MET5A), ten human malignant mesothelioma cell lines (MMPav, ISTMES2, REN, M28, VAMT, MMP, MMBO, MMCa, MMB and MMMA), human dermal fibroblasts (HDF), a human lung carcinoma cell line (A549) and T-lymphoblastic leukemia cells (CCRF) were used. Primary HMC were obtained from patients with congestive heart failure and cultured in Ham's F12 medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS, Life Technologies, Rockville, MD, USA). MET5A were purchased from ATCC and cultured in M-199 medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% FBS. MMPav, MMP, MMBO, MMCa, MMB and MMMA were derived from pleural effusions of malignant mesothelioma patients and cultured in Ham's F12 medium supplemented with 10% FBS. ISTMES2 cells were from the IST cell depository of Genoa and were cultured in Ham's F12 medium supplemented with 10% FBS. REN cells (a generous gift of Dr. Steven Albelda, University of Pennsylvania, Philadelphia, USA) were cultured in Ham's F12 medium supplemented with 10% FBS. M28 and VAMT cells, a gift of Dr. Courtney Broaddus, University of California San Francisco, were cultured in DMEM medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% FBS. HDF were obtained from an healthy donor and cultured in Ham's F12 medium supplemented with 10% FBS. A549 were purchased from ATCC and cultured in Ham's F12 medium supplemented with 10% FBS. CCRF cells were cultured in RPMI-1640 medium (Sigma-Aldrich, St. Louis,

MO, USA) supplemented with 10% fetal calf serum. Cells were grown at 37°C in a 5% CO_2-humified atmosphere.

Spheroids were cultured by seeding cells in round-bottomed 96 multi-well plates coated with a thin layer of poly (2-hydroxyethyl methacrylate) (polyHEMA - Sigma-Aldrich, St. Louis, MO, USA). Wells were coated with a 1:24 dilution in 95% ethanol of polyHEMA stock solution (120mg/ml in 95% ethanol) and dried at 37°C for 48 hours. Plates were UV sterilized for 30 minutes prior to use. Spheroids were allowed 24 hours of culture after which they were transferred to flat-bottomed 24 multi-well polyHEMA-coated plates for treatment.
Spheroids were also generated with a rotatory cell culture system (RCCS, Synthecon, Houston, TX, USA) by seeding $5x10^6$ cells in a RCCS chamber, allowing 14 days for full formation changing medium every 4 days.

3.2. RNAinterference: cell nucleofection

M28, REN or VAMT cells (5 x 10^6) were pelleted and resuspended in 100 µl of buffer (solution V, Amaxa Biosystems, Cologne, Germany) with 3 µg of the appropriate siRNA duplex. This suspension was transferred to a sterile cuvette and nucleofected using program T-20 on a Nucleofector II device (Amaxa Biosystems). Cells were recovered for 30 min in complete DMEM medium before being plated. Transfected cells were treated after 48 hours of cell culture. Used siRNA sequences were: **scramble** – ACG UGA CAC GUU CGG AGA AdTdT, **p70S6K** – CUG UUA GUU UCA CAU GAC CdTdT, **Bid** - UAU UCC GGA UGA UGU CUU CdTdT

3.3. Chemotherapeutics, chemicals and inhibitors

Imatinib mesylate was kindly provided by Novartis (Basel, Switzerland); gemcitabine and pemetrexed by Lilly (Indianapolis, IN, USA). Taurolidine (Taurolin®) was purchased from Geistlich Pharma AG (Wolhussen, Switzerland). Recombinant human TRAIL was from R&D (Minneapolis, MN, USA). glutathione reduced ethyl ester (GSH), N-Acetyl-L-cysteine (L-NAC), wortmannin, LY294002, MG-132, trichostatin A, sodium butyrate, anisomycin and rapamycin were purchased from Sigma-Aldrich (St. Louis, MO, USA). PI-103 was a kind gift of Dr. Kevan Shokat (UCSF, CA, USA).

3.4. Cytofluorimetric analysis of apoptosis

Monolayers or spheroids were exposed to the indicated treatments then resuspended in binding buffer (10 mM Hepes/NaOH pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$) stained in the dark for 10 minutes with 5 µl of FITC-labeled Annexin V (Alexis, Lausen, Switzerland), washed with binding buffer then stained with 1 µg/ml propidium iodide (Sigma-Aldrich, St. Louis, MO, USA). 5,000 events per sample were analyzed, and cells positive to Annexin V staining were considered apoptotic.

3.5. Immunohistochemistry

Spheroids were collected, fixed in 10% formalin, and embedded in paraffin. In brief, 8 µm sections were mounted on glass slides and processed as follows: after deparaffinization and rehydration, antigens were retrieved by boiling in sodium

citrate solution (pH 6.0) with 0.1% Tween-20 in a pressure cooker for 10 min. The slides were then cooled at room temperature for 20 min. Sections were blocked with hydrogen peroxide solution for 20 min to remove endogenous peroxidase. Primary antibodies, anti-phospho Akt (Ser 473) or anti-phospho p70S6K (Thr 389) (Cell Signaling Technology, Beverly, MA, USA) were applied overnight at 4°C. A secondary antibody conjugated to a horseradish peroxidase–labeled polymer was applied for 30 min and detected by the 3, 3-diaminobenzidine tetrahydrochloride (DAB) method. Biotinylated anti-human TRAIL (R&D system, Minneapolis, MN, USA) was applied overnight at 4°C and visualized with streptavidin-conjugated Oregon Green 488 (Molecular Probes, Eugene, OR, USA).

3.6. Hoechst staining

After the indicated treatments, monolayers and spheroids were disaggregated with trypsin (Gibco, Invitrogen, Carlsbad, CA, USA), pelleted at 2000 RPM for 10 minutes at 4°C, fixed with 2.5% glutaraldehyde (Sigma-Aldrich, St. Louis, MO, USA), then stained with 8 μg/ml of Hoechst 33342 (Molecular probes, Invitrogen, Carlsbad, CA, USA). Cells were then placed on slides and 100 cells were counted in triplicate, possibly in the same microscopic field, for each experiment. Cells with distinctive signs of nuclear condensation and blebbing were considered apoptotic.

3.7. Cell cycle analysis

Cells were synchronized by 0.1 μg/ml Colcemyd (Sigma-Aldrich, St. Louis, MO, USA) treatment for 24 h, and then kept in normal medium for 4 days prior to analysis. After treatment, cells were washed in PBS, fixed in 50% ethanol in PBS

and stained for 30 minutes at room temperature with 50 µg/ml Propidium Iodide (Sigma-Aldrich, St. Louis, MO, USA) in PBS containing 0.5 mg/ml RNAse (Sigma-Aldrich, St. Louis, MO, USA). 10,000 events per sample were analyzed by flow cytometry.

3.8. Cytotoxicity and DNA adducts

$4x10^3$ cells were seeded on multiwell plates and treated for different times and at different drug concentrations. Cytotoxicity was assessed by MTT assay, using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma-Aldrich, St. Louis, MO, USA) and performed in quadruplicate, as previously described (Mosmann 1983). Normalized cytotoxicity percentages were obtained according to the ratio: (A_{570} mean values of extracts from treated samples / A_{570} mean values of extract from control samples) x100. A_{570} values were corrected with background A_{670} absorbance values. Data are expressed as the mean of corrected A_{570} values ± S.E. DNA adducts were evaluated by HPLC on DNA extracts from treated cells. Data are expressed as amount of 8-OHdG per 10^5 dG, as previously described (Toyokuni and Sagripanti 1996).

3.9. Isobologram analysis

LC50 values, calculated using Origin software (Microcal Software, USA), were used to draw the theoretical additivity isobole, according to the “50% Isobologram” method (Tallarida 2001) Afterward, series of dose-response curves for each chemotherapeutic drug were generated as above, in the presence of several fixed

concentrations of imatinib. The resulting different LC50 values were plotted on the isobologram, for assessment of the hypothetical superadditive effect.

3.10. Nitrite production

Nitrite production was determined by the Griess reagent system (Promega, Madison, WI) after indicated treatments.

3.11. Immunoblotting

After treatment, monolayer or spheroids were lysed in boiling lysis buffer (2,5% SDS, Tris-HCl 250 mM pH 7.4). Total protein concentration of samples was evaluated with DC protein assay from BioRad (Hercules, CA, USA). 30-50 µg of total cell lysates were loaded in reducing conditions (0.2 M Tris, pH 6.8, 5% SDS, 3% glycerol, 0.01% bromophenol blue and 200mM DTT). After separation in SDS-PAGE (5 to 15% acrylamide) and transfer to nitrocellulose filter (Protran, Schleicher & Schuell, Dassel, Germany) or PVDF (Immobilon, Millipore, Billerica, MA USA), membranes were probed with antibodies diluted in appropriate buffer at 4°C overnight. Secondary antibodies were from Amersham (Piscataway, NJ, USA). The signal was detected by the enhanced SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL, USA).

Phospho-Akt (Ser473), Phospho-p70 S6 Kinase (Thr389), Phospho-p38 MAPK (Thr180/Tyr182), Phospho-SAPK/JNK (Thr183/Tyr185), Phospho-PTEN (Ser380/Thr382/383), Phospho-PP1α (Thr320), Akt, p70S6K, ERK 1/2, p38, PTEN, caspase-8 (1C12), cleaved caspase-8, Bcl2, Bcl-X_L, Mcl-1, Bax, Bad and survivin antibodies were from Cell Signaling Technology (Beverly, MA, USA).

Phospho-Erk 1/2 (Thr^{183}/Tyr^{185}), β-actin and α-tubulin antibodies were from Sigma-Aldrich (St. Louis, MO, USA). Phospho-PP2A (Tyr^{307}), PARP-1 (F2), $FLIP_S$, Met (C-28) and PDGFRα/β (958) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). BIM antibody was from Stressgen (Ann Arbor, Michigan, USA). Phospho-tyrosine (4G10) antibody was from Upstate Biotechnology (Lake Placid, NY, USA) and HSP-90 (clone 68) antibody was from BD Biosciences (San Jose, CA, USA).

3.12. Immunoprecipitation and co-immunoprecipitation

For immunoprecipitation, after drug treatment, total cellular proteins were extracted by RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.5% sodium deoxycholate, 1% Triton X-100, 0.1% SDS) containing protease inhibitors (10 µg/ml aprotinin, 10 µg/ml leupeptin, 10 µg/ml pepstatin, 1 mM phenylmethylsulfonyl fluoride) and phosphatase inhibitors (1 mM Na_3VO_4, 2 mM NaF). For co-immunoprecipitation, after drug treatment cells were lysed in solubilization buffer (20 mM Tris-HCl pH 7.4, 5 mM EDTA, 150 mM NaCl, 10% glycerol, 1% Triton X-100) with protease and phosphatase inhibitors. 500 µg aliquots of clarified cell lysates were incubated with 1 µg of antibody immobilized on protein-A-Sepharose-4B packed beads (GE Healthcare) for 2 hours at 4°C. After extensive washes with lysis buffer, precipitated proteins were loaded in reducing or non-reducing conditions (co-immunoprecipitation).

3.13. Statistical analysis

For cytotoxicity assay, we performed three separate experiments for each drug and combination in the different cell types. Data from each experiment are expressed as mean percentage ± Standard Error (S.E.) of eight determinations for every concentration point. All mean values from each of three experiments were used to calculate the best fit curve by the Origin software and to calculate the corresponding LC50 with confidence limits by regression analysis. These LC50 values were compared by t-Student test, with theoretically additive doses and their confidence intervals, calculated as described by (Tallarida 1992) For apoptosis, statistical differences were evaluated by t-Student test between theoretical additive effects of chemotherapeutics (gemcitabine or pemetrexed) plus imatinib, vs. the measured effects of imatinib/chemotherapeutic combinations. In all statistical evaluations the significance threshold was specified in the text.

4. Results

4.1. Association of imatinib mesylate with chemotherapy

At present time, no chemotherapeutic regimen for MPM has proven to be curative and only two drugs combinations have been shown to be of clinical value, the association of gemcitabine or pemetrexed with cisplatin. Gemcitabine is a false nucleotide that terminates DNA polymerization and inhibits DNA repair, and pemetrexed is a potent inhibitor of thymidilate synthase, which is required for DNA synthesis. Several multi-center trials determined an objective response rate of 48% for gemcitabine (Nowak, Byrne et al. 2002) and 48% for pemetrexed (Vogelzang, Rusthoven et al. 2003) in association with cisplatin.

Imatinib mesylate, a selective inhibitor of tyrosine kinases such as bcr-abl, c-Kit, c-Fms and PDGFRβ, represents the first-line treatment for chronic myeloid leukemia (CML), metastatic gastro-intestinal stromal tumor (GIST) (Schnadig and Blanke 2006), and is promising for the treatment of other solid tumors (Steeghs, Nortier et al. 2007). Knowledge of the role of PDGF signalling pathways in the etiology of MPM, has led to trials of imatinib mesylate (STI571, Gleevec®) used as single agent, unfortunately providing poor results (Mathy, Baas et al. 2005; Porta, Mutti et al. 2007).

Because of their positive and promising response rates in patients, we aimed to evaluate the potential synergism arising from the interference with PDGF signalling and the simultaneous treatment with the pyrimidine analogue gemcitabine or the multi-targeted antifolate pemetrexed.

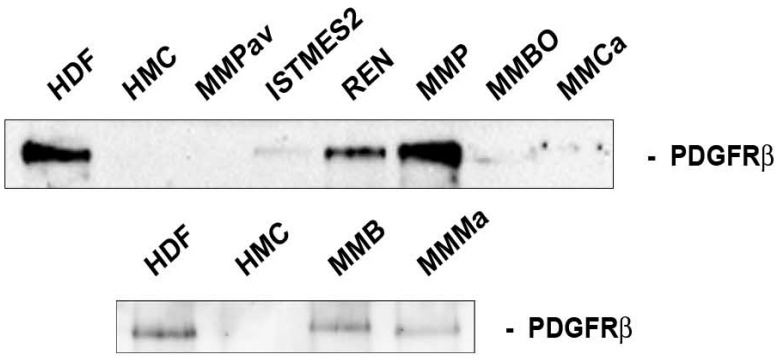


Figure 3: MPM cells differently express PDGFRβ. Immunoblotting with PDGFRβ antibodies of whole cell lysates from eight mesothelioma cell lines. Seven out of eight cells expressed PDGFRβ at different levels, while HMC did not express the receptor. Human dermal fibroblasts (HDF) were used as positive control.

A panel of eight mesothelioma cell lines (MMPav, ISTMES2, REN, MMP, MMBO, MMCa, MMB and MMMA) was tested for the presence of PDGFRα, PDGFRβ, c-Kit and c-Fms (**Figure 3**).

None of the cell lines expressed PDGFRα, only present in untransformed human mesothelial cells (HMC) as previously published (Langerak, De Laat et al. 1996). All the cell lines, with the exception of MMPav, expressed PDGFRβ at varying levels. The receptor for colony stimulating factor-1 c-Fms was faintly detected in all cell lines, whereas the stem cell factor receptor c-Kit was highly expressed by MMP cells, and at lower levels by REN and ISTMES2 cells. Because of the representative expression patterns of their receptors, MMP, REN and ISTMES2 cells were chosen for further experiments (**Figure 4**).

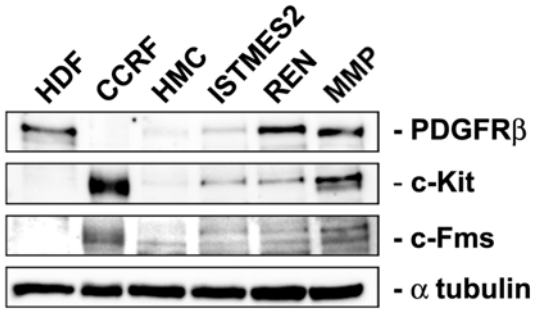


Figure 4: MPM cells express receptors sensitive to imatinib at different leves. Immunoblotting with PDGFRβ, c-Kit and c-Fms specific antibodies on whole cell lysates from mesothelioma cell lines. The three analyzed cell lines expressed the receptors at different levels. MMP showed the highest expression of both PDGFRβ and c-Kit. For positive control, human dermal fibroblasts (HDF) were used for PDGFRβ and human T-lymphoblastoid leukemia cells (CCRF) for c-Kit and c-Fms.

To asses receptor activation, the phosphorylation state of PDGFRβ was measured by immunoprecipitation and detection with phospho-tyrosine antibodies. Tyrosine phosphorylation of PDGFRβ was not detectable when cells were cultured in low serum, but was promptly induced when recombinant human PDGF was given to cells. The addition of imatinib to PDGF stimulated cells completely inhibited receptor tyrosine phosphorylation, confirming specific blockade of the receptor activation (**Figure 5 upper panel**). To further test the ability of imatinib to interfere with the intracellular signalling elicited by PDGFRβ, phosphorylation of Akt Ser473, essential for full Akt activity (Alessi and Cohen 1998), was measured in basal conditions and upon PDGF stimulation (**Figure 5 lower panel**).

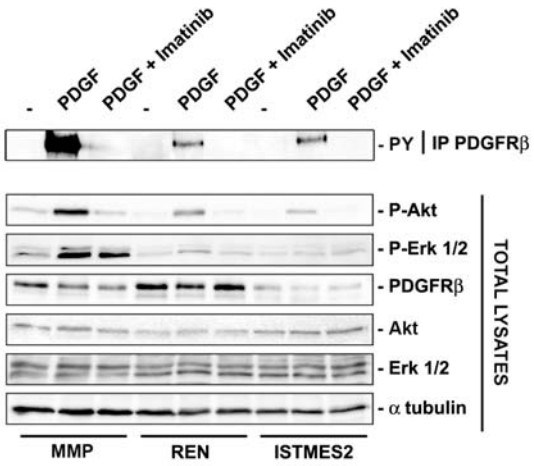


Figure 5: Imatinib is able to completely inhibit activation of PDGFRβ signalling pathways after PDGF stimulation. Immunoprecipitation with PDGFRβ antibodies followed by immunoblotting with phospho-tyrosine antibodies (**upper panel**). Immunoblotting with the indicated antibodies on whole lysates (**lower panel**). No basal level of tyrosine phosphorylation was detected in all low serum cultured cells whereas stimulation with recombinant PDGF (20 ng/ml) rapidly activated the receptor and Akt phosphorylation (30 minutes). In both panels, 90 minutes pre-treatment with imatinib (10 μM), completely inhibited PDGFRβ-mediated Akt phosphorylation. Erk1/2 phosphorylation was increased in MMP cells but was not inhibited by imatinib

Only MMP cells displayed spontaneous Akt activity, whereas PDGF stimulation induced prompt PDGFRβ tyrosine phosphorylation in all the cells tested, which was markedly inhibited with the addition of imatinib (10 μM). Conversely, PDGF stimulation induced ERK1/2 activity in MMP cells and, at a lesser extent, in REN cells, but was not affected by co-treatment with imatinib. This

demonstrates a selective blockade of the Akt pathway exerted by the inhibitor, comparable to the results obtained with wortmannin, a highly selective PI3K inhibitor (**Figure 6 upper panel**).

To further define the specific blockade of PDFGRβ-dependent Akt signalling by imatinib, HGFR/Met expressing MMP cells (Cacciotti, Libener et al. 2001) were stimulated with recombinant human hepatocyte growth factor (HGF) in the presence or absence of imatinib. Interestingly, Akt Ser473 phosphorylation was induced by HGF treatment but not inhibited by imatinib, indicating its selective inhibition of Akt phosphorylation downstream of PDGFRβ activation (**Figure 6 lower panel**).

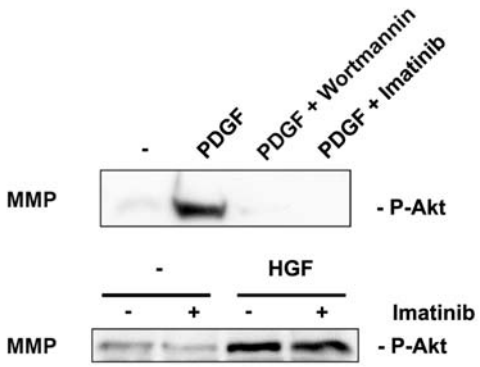


Figure 6: Imatinib is able to inhibit PDGFRβ-mediated Akt activation. Immunoblotting with phospho-Akt (phospho-Ser 473) antibodies of MMP cells stimulated by 20 ng/ml PDGF(**upper panel**) or with HGF (50 ng/ml) (**lower panel**) in the presence or absence of wortmannin (100 nM) or imatinib (10 μM) for 30 minutes. Imatinib was able to inhibit Akt activation upon PDGF stimulation, comparable to results obtained with a specific PI3K inhibitor. Also, imatinib was not able to reduce Akt phosphorylation following HGF stimulation of MMP cells, suggesting the specific blockade of Akt activation downstream of PDGFRβ receptor.

The Akt signal transduction pathway has been largely recognized as a pivotal survival pathway for mesothelial cells transformation and MPM progression (Cacciotti, Barbone et al. 2005; Ramos-Nino, Vianale et al. 2005). Hence, we assessed the ability of imatinib to affect PDGFRβ-positive mesothelioma cells viability by evaluating the LC50 values (lethal concentration for 50% of cell population) using the colorimetric MTT viability assay in the most responsive MMP cells (Mosmann 1983) (**Table 2**).

	LC50 (48 hours)
MMP	1.84×10^{-5}M
REN	1.89×10^{-5}M
ISTMES2	2.05×10^{-5}M

Table 2: Imatinib is cytotoxic to MPM cells. LC50 values for imatinib on selected cells after 48 hours of treatment, measured by MTT assay.

Gemcitabine and pemetrexed cytotoxicity was also tested, when used as single agents or in presence of increasing concentrations of imatinib. Gemcitabine and pemetrexed, caused death of MMP mesothelioma cells and the presence of imatinib lowered their LC50 values, thereby increasing the percentage of treatment-sensitive cells (**Figure 7**).

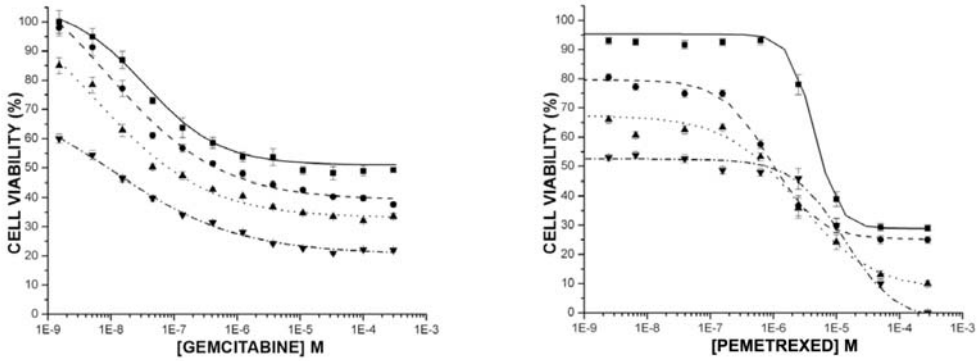


Figure 7: Imatinib sensitizes MMP cells to chemotherapy. Dose-response curves of cell viability for gemcitabine (**left**) and pemetrexed (**right**), in presence of different concentrations of imatinib. For the imatinib/gemcitabine combination: ●, $1x10^{-7}$ M; ▲, $2.5x10^{-6}$ M; ▼, $1x10^{-4}$ M. For imatinib/pemetrexed combination: ●, $3x10^{-7}$ M; ▲, $6x10^{-7}$ M; ▼, $1.5x10^{-6}$ M. Data is representative of three different experiments, conducted with eight determinations for each point. Points indicate percentage mean ± S.E. The presence of imatinit effectively reduced the viabiliy of cell lines exposed to the chemotherapeutic agents.

Recent studies have also proposed a mechanism in which gemcitabine-induced phosphorylation of EGFR tyrosine kinase may target the receptor for degradation, suggestive that the use of tyrosine kinase inhibitors may influence the effectiveness of gemcitabine treatment (Feng, Varambally et al. 2006).

We then analyzed PDGFRβ phosphorylation after treatment with gemcitabine or pemetrexed and did not observe any evidence of receptor activation (**Figure 8**).

Given the sensitization of PDGFRβ expressing mesothelioma cells to gemcitabine or pemetrexed treatment exerted by imatinib, we aimed to qualitatively assess the contribution of this tyrosine kinase inhibitor to treatment as a co-agent, using the isobologram method (Tallarida 2001). This method allows the detection of the amplitude of the reinforcement exerted by a co-agent with a specific treatment and graphically represents equally effective dose pairs (isoboles), referring to a defined cytotoxicity value.

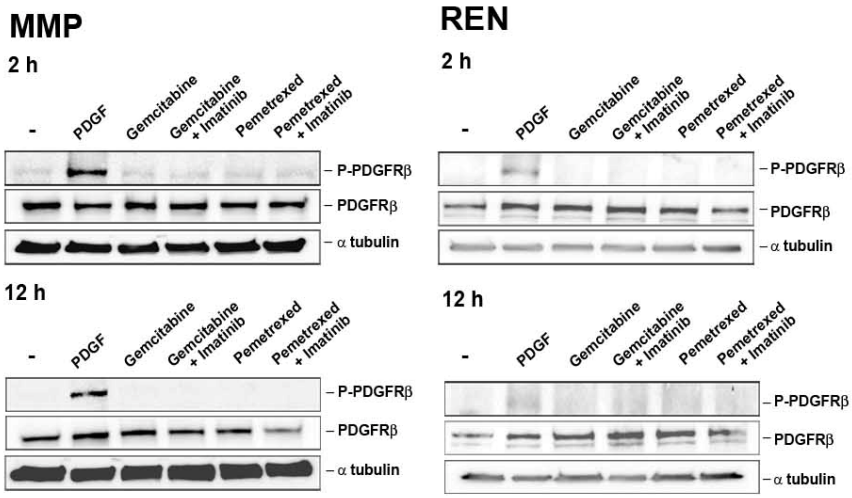


Figure 8: Neither gemcitabine nor pemetrexed induce PDGFRβ phosphorylation. Immunoblotting with antibodies recognizing PDGFRβ and its active phosphorylated form in MMP and REN whole cell lysates. Cells cultured in low serum conditions were stimulated with PDGF (20 ng/ml) or treated for 2 or 12 hours with LC50 concentrations of the indicated chemotherapeutics in presence or absence of imatinib (10 μM). No PDGFRβ phosphorylation was detected upon gemcitabine or pemetrexed treatment.

Specifically, a particular effect level is selected, such as 50% of the maximum (LC50 values in this study) and doses of drug A and drug B (each alone) whic produce this effect are plotted as axial points in a Cartesian plot. The line connecting the LC50 values of the single agents represents the dose pairs that will result in an additive effect, while dose pairs lining above and below the line of additivity represent an antagonist (sub-additive) or a synergic (super-additive) effect.

Isobologram analysis showed that the combination of imatinib with gemcitabine or pemetrexed resulted in a synergistic cytotoxicity in MMP ($p \leq 0.001$) and REN cells (ranging from $p \leq 0.01$ to $p \leq 0.001$), while the effect was significantly antagonistic in ISTMES2 (ranging from $p \leq 0.05$ to $p \leq 0.001$) (**Figure 9**).

The effectiveness of this combined therapy was further confirmed when cell death was investigated by TUNEL staining (**Table 3**). The apoptotic rates obtained in MMP and REN cells with the combination of imatinib and gemcitabine or pemetrexed, was significantly increased ($p \leq 0.001$) as compared to the theoretical additive effect of the single agents themselves. Interestingly, the concentrations of single agents used in this combined treatment were far lower than those obtainable

at therapeutic dosages, strongly supporting the evaluation of these synergic associations in early phase clinical trials.

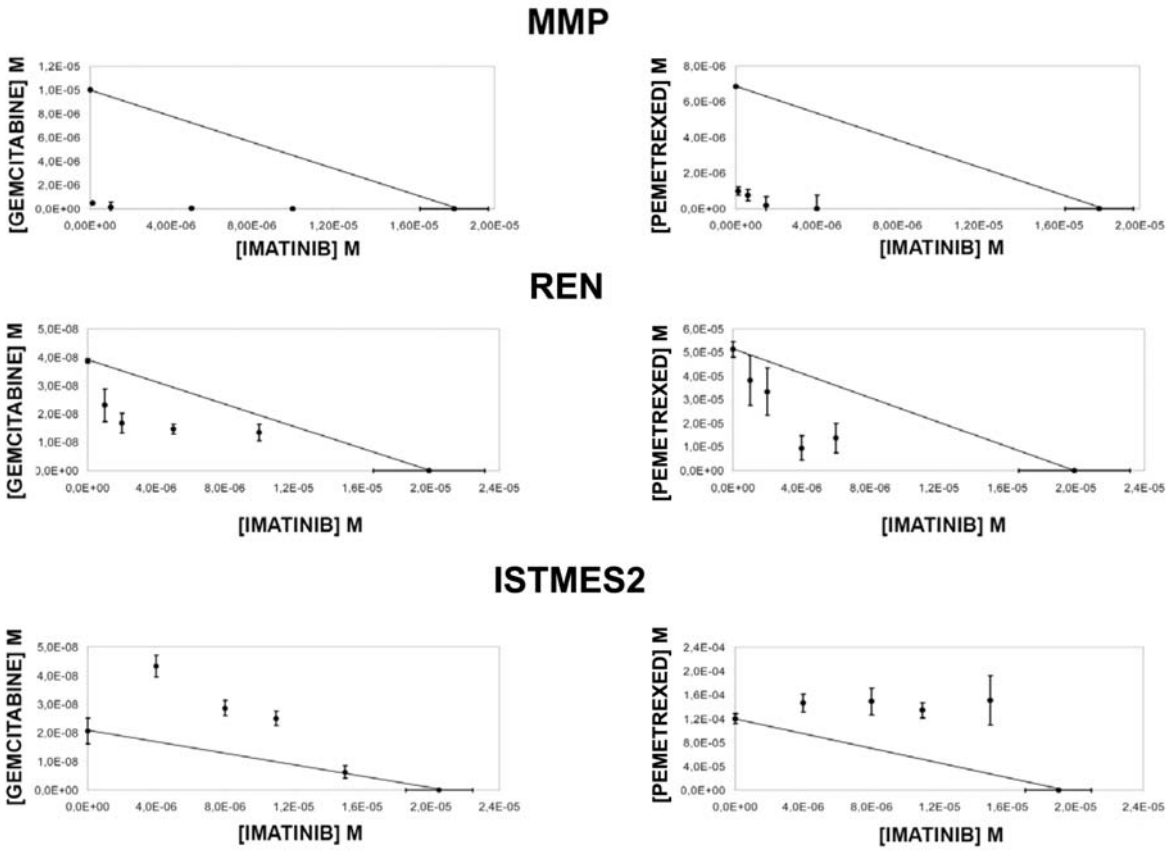


Figure 9: The combination of imatinib with gemcitabine or pemetrexed is synergic in MMP and REN cells. 50% isobologram plot for imatinib in combination with gemcitabine (**left**) or pemetrexed treatment (**right**) in MMP, REN and ISTMES2 cells. Points are LC50 ± S.E., calculated by regression analysis. Both agents, when combined with imatinib, displayed synergic effects in MMP and REN but not in ISTMES2 cells.

Treatment	MMP	REN	ISTMES2
Imatinib	1.1 ± 0.35	1.00 ± 0.23	1.70 ± 0.19
Gemcitabine	1.58 ± 0.42	3.07 ± 0.51	2.80 ± 0.32
Pemetrexed	0.98 ± 0.47	1.04 ± 0.26	1.00 ± 0.26
Imatinib + Gemcitabine	5.34 ± 0.40 (*)	9.72 ± 0.48 (*)	1.02 ± 0.48 (*)
Imatinib + Pemetrexed	8.48 ± 0.40 (*)	4.72 ± 0.26 (*)	0.04 ± 0.26 (*)

Table 3: Imatinib combination with gemcitabine or pemetrexed significantly induces more apoptosis compared to single agents in MMP and REN cells. UNEL analysis of apoptosis induced in mesothelioma cells by single drugs or by drugs combinations at 48 hours. Imatinib, when combined with gemcitabine or pemetrexed, resulted in a significantly higher percentage of apoptotic cells only in MMP and REN cells than results obtained with single agents alone. Data are expressed as the percentage of Biotin-dU positive nuclei for 100 counted cells at a magnification of 100X ± S.E.. Values of each treatment were subtracted of untreated control values. Drug concentrations were as follows: **MMP** Imatinib 3x10-7M, Gemcitabine $5x10^{-7}$M, Pemetrexed $6.5x10^{-6}$M. **REN** Imatinib $1x10^{-6}$M, Gemcitabine $5x10^{-9}$M, Pemetrexed $1x10^{-5}$M. **ISTMES2** Imatinib $4x10^{-6}$M, Gemcitabine $1x10^{-9}$M, Pemetrexed $5x10^{-6}$M.

4.2. Taurolidine in the treatment of mesothelioma

Taurolidine (TN) is a small dimeric molecule (bis(1,1-dioxoperhydro-1,2,4-thiodiazinyl-4)-methane) possessing bactericidal properties against a broad spectrum of bacteria ranging from aerobic to anaerobic species (Browne, Leslie et al. 1977). Tauroldine's mechanism of action as an antibiotic agent is associated with a chemical reaction between the active TN metabolites, taurultam and taurinamide (**Figure 10**), and bacterial wall structures (Gorman, McCafferty et al. 1987). In addition, TN exhibits the ability to neutralize bacterial endotoxins, exotoxins, and lipo-polysaccharides (Monson, Ramsey et al. 1993; Watson, Redmond et al. 1995; Leithauser, Rob et al. 1997).

Taurolidine's current clinical application is as catheter lock solution to reduce the incidence of vascular access-associated bloodstream infections (Jurewitsch and Jeejeebhoy 2005). Other clinical applications include peritoneal lavage for prophylaxis against post-operational bacterial infections and as an antiendotoxic agent in patients with systemic inflammatory response syndrome (Browne 1981; Willatts, Radford et al. 1995).

Taurolidine has also been investigated extensively as an experimental antineoplastic

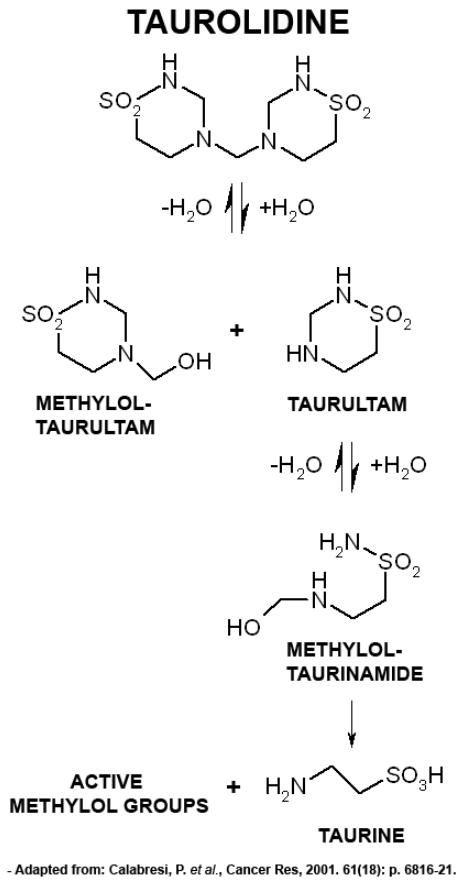


Figure 10: Schematic diagram of the hydrolysis products of Taurolidine. The active methylol groups, Taurultam and Taurinamide mediate the biological effects of Taurolidine.

agent in a few *in vitro* and *in vivo* studies. (Jacobi, Sabat et al. 1997; McCourt, Wang et al. 2000; Calabresi, Goulette et al. 2001).

Over the last years, *in vitro* studies have highlighted the role of TN as pro-apoptotic agent (Jacobi, Menenakos et al. 2005) in brain tumor (Stendel, Stoltenburg-Didinger et al. 2002) and prostate cancer cells.

Moreover, intraperitoneal administration of TN in experimental rats inhibited the growth of injected ovarian and colon cancer cells (McCourt, Wang et al. 2000). The safety and efficacy of intracavitary administration of TN, along with the need of a prolonged i.v. administration for obtaining anti-neoplastic effects (Jacobi, Menenakos et al. 2005), indicate that this drug may be of particular help in the treatment of tumors with prevalent local spreading such as peritoneal and pleural mesothelioma.

TN exerts pro-apoptotic effects with marked selectivity on cancer cells (Calabresi, Goulette et al. 2001), and recent studies (Nici, Monfils et al. 2004) have highlighted a clear induction of apoptosis in three MPM cell lines, and the inhibition of growth and development of MPM in mice, although no specific molecular mechanism has been elucidated. It has been proposed that TN may function via activation of CD95 (Stendel, Scheurer et al. 2003), by involvement of mitochondria (Han, Ribbizi et al. 2002), by the inhibition of protein synthesis (Braumann, Henke et al. 2004) or by the production of reactive oxygen species (ROS) (Rodak, Kubota et al. 2005), which are able to interfere with Akt activity (Gao, Rahmani et al. 2005). As previously reported, the PI3K / Akt / mTOR signalling pathway is of pivotal importance in the maintenance of anti-apoptotic survival signals in MPM cells (Altomare, You et al. 2005), and is responsible for onset and progression of MPM.

Given these proposed functions of TN, we then hypothesized that TN selective induction of apoptosis may reside in an alternative pattern of Akt pathway activation; a pathway utilized only by neoplastic cells and not by their normal non-transformed counterpart.

The cytotoxic effect of TN was first evaluated in a panel of two MPM cells (MMB, MMP), a normal mesothelial cells (HMC) and an immortalized mesothelial

cell line (MET-5A). Human dermal fibroblasts (HDF) and non small cell lung carcinoma (NSCLC) A549 cells were also used as normal and neoplastic controls. A strong cytotoxic effect was observed in all neoplastic cell lines, MMB cells being most sensitive, although non-neoplastic cells, mesothelial or not in origin, displayed a negligible decrease in survival. These cytotoxic properties of TN were also time dependent, reaching a maximal effect within 72 hours (**Figure 11**).

TN-induced reduction of cell viability was also examined in terms of programmed cell death, by evaluating Annexin V staining of cells, the presence of DNA fragmentation with Propidium Iodide (PI) staining and the presence of cleaved forms of caspase 8.

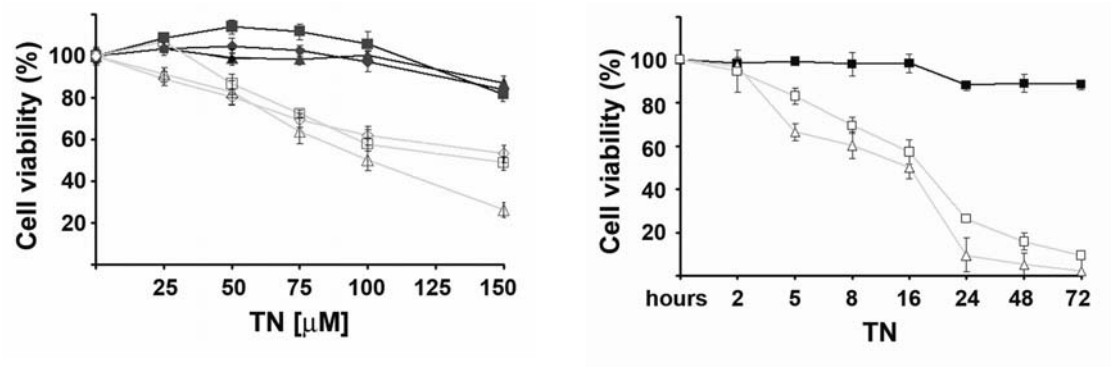


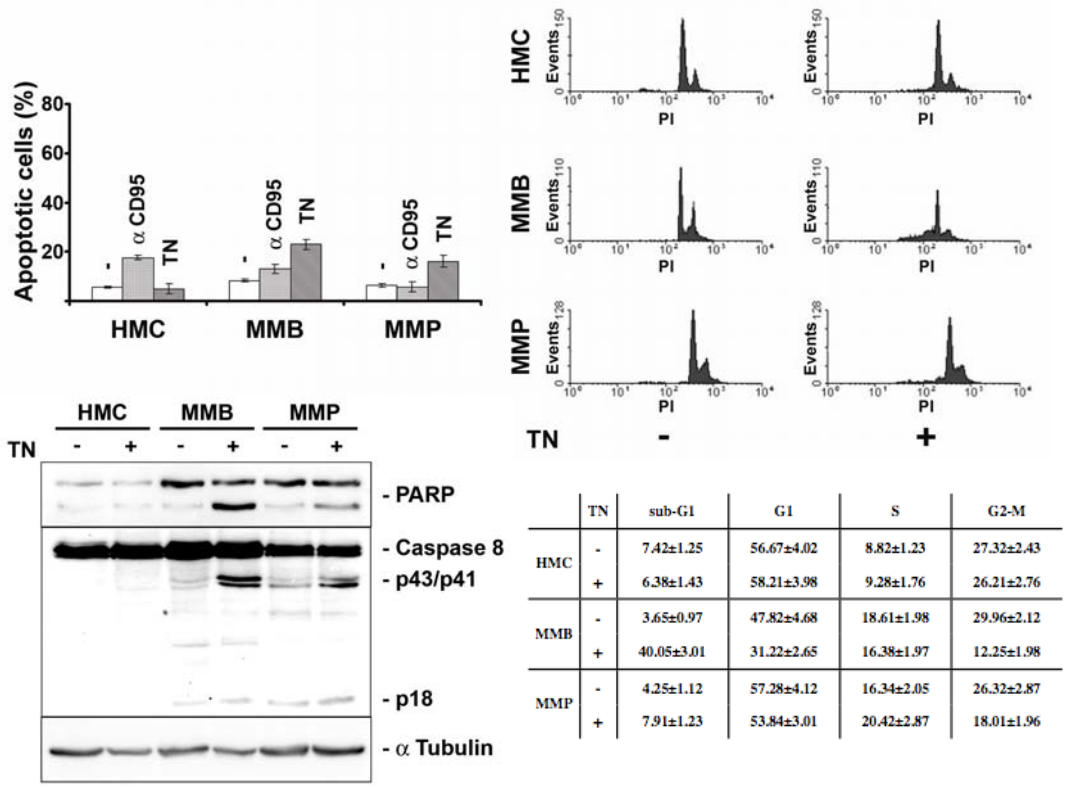
Figure 11: TN is citotoxic only to tumor cells. Non-neoplastic HDF, MET-5A and HMC cells together with mesothelioma MMB, MMP and NSCLC A549 cells, were treated with a range of TN concentrations (25-150 μM) for 16 hours (**left**). Non-transformed cells (filled markers), of different origin, were less sensitive to TN-induced cytotoxicity. Reduction of cell viability was also time-dependent (**right**). HMC, MMB and MMP cells were treated with TN (100 μM) for up to 72 hours. Mesothelioma cells began to display reduced viability after 5 hours of treatment reaching a maximum levels after 48-72 hours. MMB cells were more rapidly affected by TN treatment than MMP cells.

Data obtained from flow cytometric analysis of annexin V staining showed that TN induced a significant amount of apoptosis in MMB and MMP cells, whereas normal cells were unaffected (**Figure 12**).

Interestingly, CD95-activating antibodies, used as positive control, were able to reduce viability of mesothelial cells and, at a lower level, MMB cells but not MMP cells, suggestive that the mere activation of the extrinsic pathway was not sufficient for MPM cells to undergo apoptosis and TN may also affect the mitochondrial

membrane potential, lowering the threshold required for BH3 only molecules to begin the apoptotic program.

Staining with propidium iodide and flow cytometric analysis of the cell cycle of HMC, MMB and MMP evidenced the presence of an hypoploid population in MMB and MMP cells upon TN treatment (**Figure 12**). The presence of a sub-G1 population is indicative of nuclear fragmentation and resembles the data obtained with annexin V, where mesothelial cells displayed no sign of TN-induced apoptosis.



	TN	sub-G1	G1	S	G2-M
HMC	-	7.42±1.25	56.67±4.02	8.82±1.23	27.32±2.43
	+	6.38±1.43	58.21±3.98	9.28±1.76	26.21±2.76
MMB	-	3.65±0.97	47.82±4.68	18.61±1.98	29.96±2.12
	+	40.05±3.01	31.22±2.65	16.38±1.97	12.25±1.98
MMP	-	4.25±1.12	57.28±4.12	16.34±2.05	26.32±2.87
	+	7.91±1.23	53.84±3.01	20.42±2.87	18.01±1.96

Figure 12: TN induces apoptosis in mesothelioma cells. Evaluation of TN-induced apoptosis. FACS analysis of Annexin V binding of HMC, MMB and MMP cells upon treatment with TN (100 μM) for 24 hours (**upper-left**). CD95 activating antibodies (100 ng/ml) were used as positive controls. TN clearly increased the apoptotic cell population in mesothelioma cells but not in mesothelial cells. Cell cycle analysis analysis of the same cell panel after TN (100 μM) treatment for 6 hours (**upper-right**). Only MMB and MMP cells displayed an increased sub-G1 population, representing nuclear fragmentation (**lower-right**). Data are expressed as average percentage of cells in different phases of cell cycle ± S.E.. Immunoblotting with PARP and caspase 8 antibodies (**lower-left**). Cells were treated with TN 150 μM for 6 hours. TN-induced apoptosis is shown by the appearance of cleaved forms of PARP and caspase 8.

Also, to provide a biochemical characterization of the induced apoptosis, the cleavage of PARP and caspase 8 were assessed after TN treatment (**Figure 12**). While MMB and MMP cells clearly showed the cleaved forms of the two enzymes, both proteolytically generated when apoptosis process is activated, HMC did not display any PARP cleavage, and caspase 8 fragments were faintly detectable, further supporting the poor effectiveness of TN treatment on non-transformed cells.

TN has been reported to interfere with protein synthesis and, because mTOR is a known translational regulator downstream of Akt, the activity of these pathways after TN treatment was evaluated (**Figure 13**). The TN-induced inhibition of Akt activity (measured as Ser473 phosphorylation (Alessi and Cohen 1998), was dose-dependent, becoming evident at 50 μM and maximal at 150 μM, and was also time-dependent, beginning at 30 minutes and lasting up to eight hours. To verify the specificity of TN-induced Akt inhibition in MPM cells, the activities of other pathways known to be regulated by phosphorylation such as ERK1/2, JNK and p38 were analyzed by immunoblotting.

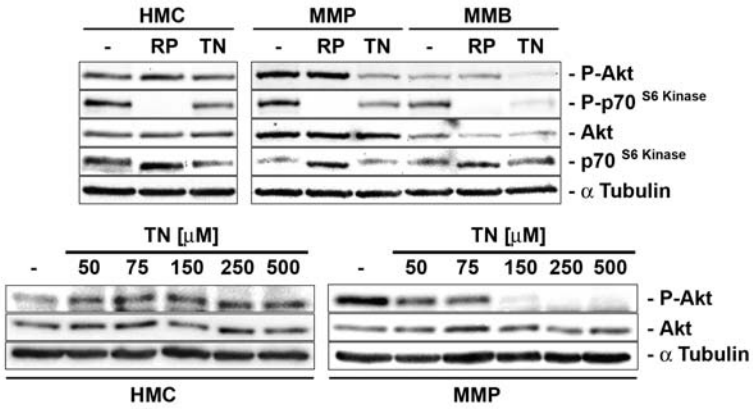


Figure 13: TN treatment reduces Akt activity selectively in mesothelioma cells. Immunoblotting of HMC, MMB and MMP cells after treatment with TN (150 μM) for 30 minutes (**upper**). Rapamycin (200 nM) was used as a control for mTOR inhibition. Akt / mTOR pathway activity was clearly reduced upon TN treatment in mesothelioma cells, while no change in phosphorylation levels was detected in mesothelial cells. Akt activity was lowered by TN in a dose-dependent fashion, starting at 50 μM and progressively increasing, reaching maximal inhibition at 250-500 μM (**lower**).

Furthermore, to better define the molecular mechanism driving Akt dephosphorylation, activities of protein-phosphatases PP1α and PP2A and lipid-phosphatase PTEN, previously reported to regulate Akt phosphorylation (Millward,

Zolnierowicz et al. 1999; Garcia, Cayla et al. 2003; Baker 2007), were evaluated. Since these phosphatases are positively regulated by serine/threonine or tyrosine de-phosphorylation (Chen, Martin et al. 1992; Gericke, Munson et al. 2006), detection of their status was performed by immunoblotting with antibodies directed against residues critical for their activities (**Figure 14**).

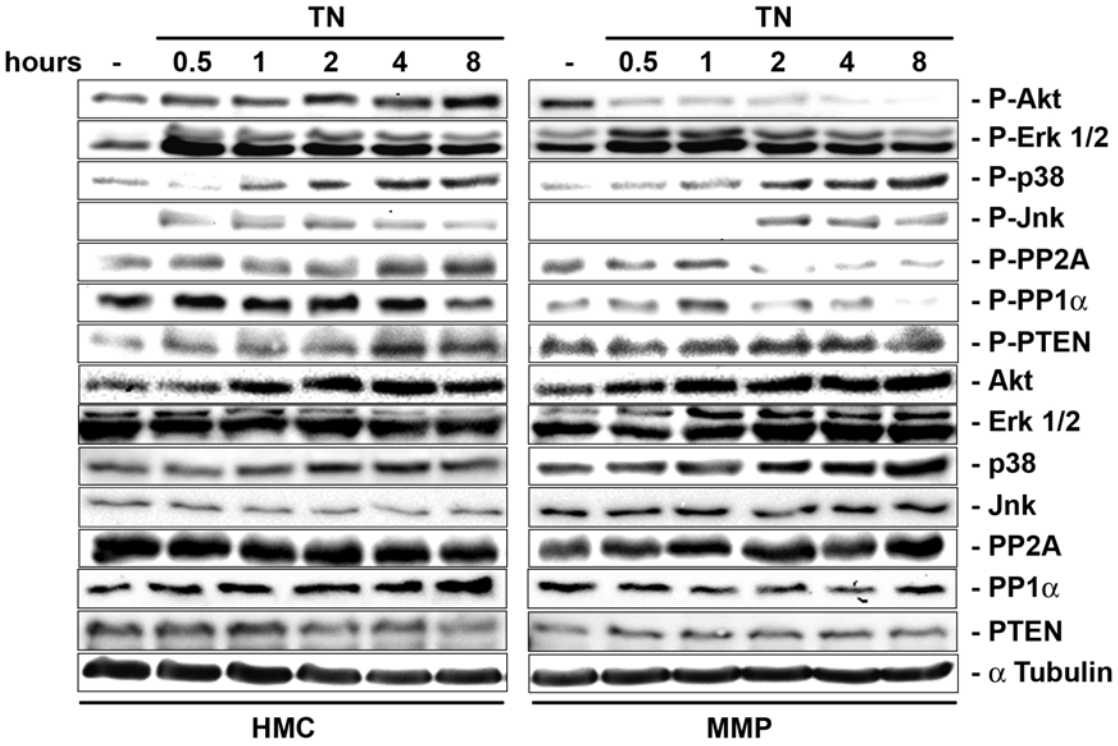


Figure 14: TN treatment activates stress proteins in mesothelial and mesothelioma cells and induces activation of phosphatases activity in mesothelioma cells only. Immunoblotting of HMC and MMP cells treated with TN (150 μM) in a range of 30 minutes to 8 hours, with antibodies directed against the phosphorylated forms of several transduction pathways molecules and phosphatases. TN was able to reduce Akt phosphorylation only in mesothelioma cells, as previously shown, and to activate ERK1/2, JNK and p38 pathways in both cells, suggesting the presence of a TN-induced non-specific stress mechanism. Activities of protein-phospatases PP1α and PP2A were increased, as their dephosphorylation occurred after 2 hours of TN treatment, while the activity of the lipid-phosphatase PTEN activity was not affected.

Analysis of phosphatase activities revealed that PP2A became de-phosphorylated upon TN treatment, although delayed by 2 hours compared to Akt de-phosphorylation pattern. Also, the significant activation after 2 hours of TN treatment may indicate a progressive activation of phosphohydrolase activity that may sustain the reduced phosphorylation of Akt. Conversely, PP1α

phosphorylation levels appeared to be reduced only after 8 hours of treatment, while PTEN activity was not affected by TN.

Altogether, these findings illustrate a specific time- and dose-dependent inhibition of Akt signalling only in mesothelioma cells, while normal mesothelial counterpart did not display any impairment of this pivotal survival pathway.

According to previous reports describing production of reactive oxygen species (ROS) upon TN treatment, ERK1/2, JNK and p38 phosphorylation levels were increased by treatment in both HMC and MMP cells (McCubrey, Lahair et al. 2006).

The increased phosphorylation of MAP kinases (McCubrey, Lahair et al. 2006) and reduced Akt activity are well described intracellular consequences of oxidative stress (Gao, Rahmani et al. 2005) (Zundel and Giaccia 1998) (Zhou, Summers et al. 1998). This prompted us to verify if the mechanism of TN pro-apoptotic activity in mesothelioma cells might rely on the activation of an oxidative pathway and if this might be responsible for the biochemical changes occurring in Akt / mTOR signalling.

To evaluate the hypothesis that TN may induce oxidative or osmotic stress in mesothelioma cells, nitrite production upon treatment with TN was assessed by the Griess assay (**Figure 15**). Nitrite production was observed in a time-dependent fashion only in mesothelioma cells, but not in mesothelial cells, which displayed and maintained a low level of nitrites only after 4 hours of TN treatment. The presence of 8-hydroxy-2' deoxy-guanosine (8-OH-dG) DNA adducts, a consequence of intracellular reactive oxygen species (ROS) production (Wu, Chiou et al. 2004) was also tested (**Figure 16**).

TN increased the presence of DNA adducts in mesothelioma cells as compared to non-transformed mesothelial counterpart and these results further confirmed that neoplastic cells are more sensitive to TN-mediated oxidation.

To verify the hypothesis that oxidative stress mechanisms might be responsible for the TN-induced reduction of Akt phosphorylation, the anti-oxidants agents glutathione mono-ethyl ester (GSH) and L-N-acetylcysteine (L-NAC) were utilized. These compounds, rich in reduced sulphidrilic groups are able to promptly

neutralize the damaging effects of ROS by acting as free-radical scavengers, and have already been used with success in the modulation of inflammatory processes (Santangelo 2003).

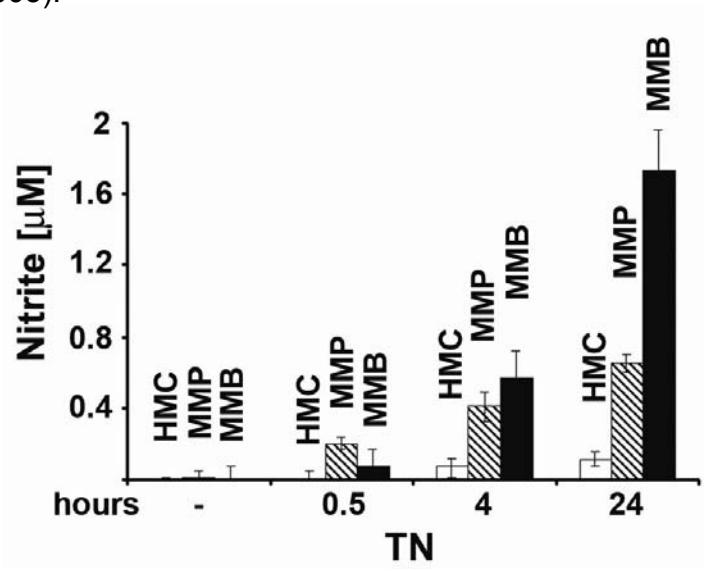


Figure 15: TN treatment causes nitrite production only in mesothelioma cells. Griess assay was performed after treatment of cells with TN (100 μM) for 30 minutes up to 24 hours. Mesothelioma cells progressively increased nitrite production while mesothelial cells displayed a markedly lower oxidative response, increasing and maintaining a very low concentration of nitrites only after 4 hours.

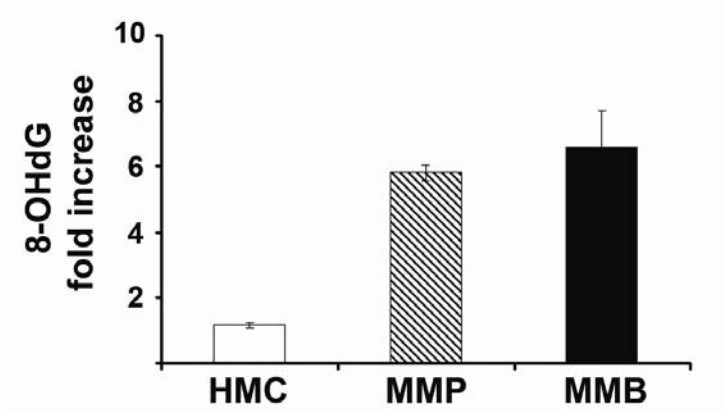


Figure 16: TN-induced oxidative stress causes formation of DNA adducts only in mesothelioma cells. Presence of 8-Oh-dG was evaluated by HPLC after treatment of cells with TN (100 μM) for 16 hours. Mesothelial cells displayed a low amount of DNA adducts formation while significant signs of oxidation occurred in MMB and MMP cells, where 8-OHdG levels increased 5 fold.

MMP cells were pre-treated with GSH or L-NAC and then exposed to TN for 30 minutes. GSH pre-treatment did not affect Akt phosphorylation in the absence

of TN. However, GSH completely prevented TN-induced Akt inhibition, arguing for a role of oxidative stress pathways in TN mechanism of action. Similar results were also obtained using L-NAC, which has already been shown to prevent the apoptosis of neuronal cells (Ferrari, Yan et al. 1995; Yan, Ferrari et al. 1995). Pre-treatment with anti-oxidant agents was also able to dramatically rescue MMP cells viability, as measured by MTT, also when treated with high concentrations of TN for long periods of time (**Figure 17**).

Aiming to determine the molecular mechanism driving the reduction of Akt phosphorylation we analyzed the association of the molecular chaperone heat shock protein HSP-90 to Akt (**Figure 18**). HSP-90 plays an important role in maintaining Akt activity, and oxidative stress or heat are able to induce its dissociation from Akt, reducing its phosphorylation and increasing cell sensitivity to treatment (Pespeni, Hodnett et al. 2007).

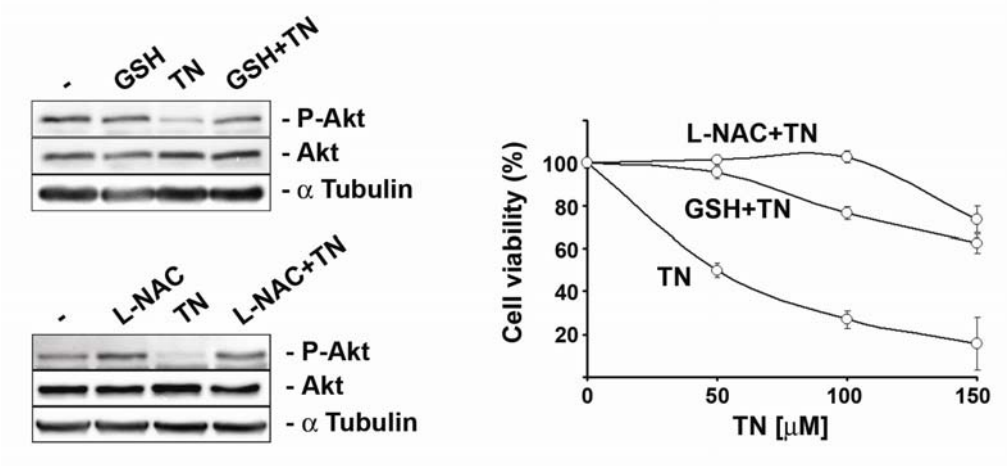


Figure 17: **Anti-oxidant agents prevent TN-induced Akt activity reduction and citotoxicity.** Immunoblotting for Akt phosphorylation in MMP cells upon TN (100μM) treatment for 30 minutes in the presence or absence of anti-oxidant agents (**left**). Pretreatment with GSH or L-NAC (10 mM) did not alter Akt signalling, and completely rescued the TN-induced reduction of Akt phosphorylation. Viability of MMP cells treated with TN (50-150 μM) for 24 hours in the presence or absence of GSH or L-NAC (**right**). Anti-oxidants were able to efficiently inhibit TN-induced cytotoxicity.

No detectable differences in the association of HSP-90 or PP2A to Akt kinase were observed upon TN treatment, suggesting that reduction of Akt phosphorylation is not due to an impairment of Akt binding to its molecular partners. Furthermore, we measured tyrosine kinase receptors (RTK)

phosphorylation, hypothesizing a general effect of TN upstream of Akt activation but we could not detect any variation in the phosphorylation of Met and PDGFRβ, two RTK shown to be important in MPM progression (Rascoe, Cao et al. 2005; Jagadeeswaran, Ma et al. 2006) (**Figure 19**).

We thereby concluded that the pro-apoptotic effects of TN on mesothelioma cells, driven by the inhibition of Akt signalling, is derived from oxidative stress, involves nitrite production and is reversed with efficacy by general anti-oxidant agents.

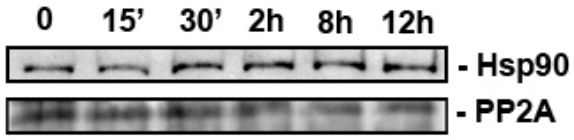


Figure 18: TN does not affect Akt association with HSP-90 or PP2A. Immunoprecipitation with Akt antibodies of MMP cells treated with TN 150 μM for 15 minutes to 12 hours. Precipitated proteins were immunoblotted in non-reducing condition. Membrane was probed with HSP-90 and PP2A antibodies. No detectable differences in the association of Akt to HSP-90 or PP2A upon TN treatment could be detected.

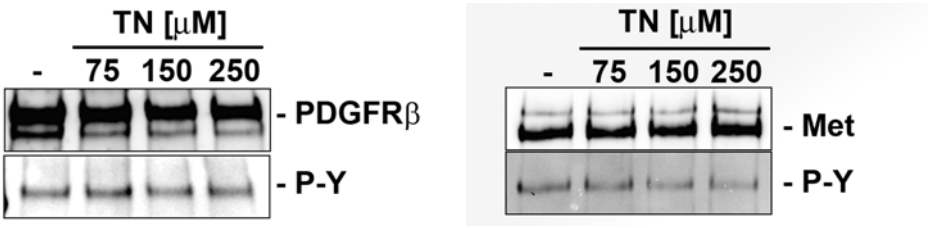


Figure 19: TN does not affect tyrosine phosphorylation of PDGFRβ and Met RTKs. Immunoprecipitation of PDGFRβ and Met of MMP cells treated with TN 75, 150 and 250 μM for 30 minutes. No significant reduction in tyrosine-phosphorylation was detectable upon TN treatment, even at high concentration.

4.3. Acquired resistance of mesothelioma spheroids

Signal transduction pathways and growth factors play a critical role in the development and progression of MPM (Fitzpatrick, Peroni et al. 1995), and the data previously shown further support a strong rationale for Akt and its downstream substrates to be potential therapeutic targets. Besides the pivotal importance of the Akt pathway in many solid tumors, it has also been recently demonstrated that inhibitors of PI3K and mammalian target of rapamycin (mTOR) significantly reduced the apoptotic rate of mesothelioma tumor fragment spheroids as compared to monolayers after TRAIL plus cycloheximide treatment (Kim, Wilson et al. 2005). The authors concluded that tumor fragment spheroids are not only a convenient method of culturing MPM samples *in vitro*, but are also a biologically relevant model, because they maintain the original tumor characteristics (i.e. apoptotic resistance) thereby allowing the study of tumor biology and novel approaches to therapy.

In this perspective we evaluated the importance of the Akt pathway in a three-dimensional cell system formed by an homogenous cell population, namely multi-cellular spheroids. These type of three-dimensional cell structures can be generated through different approaches; our first aim was to develop a reproducible cell culture system that could provide a rapid formation of aggregates, granting control on their size and cellular composition.

The “liquid overlay” system utilizes a non-sticking compound like agar to prevent cell adhesion to plastic. Although this is a very efficient culture system for tumor fragment spheroids, it does not provide the reproducibility necessary to perform molecular assays or allow a high degree of control on the number of cells per spheroids, and takes several days to obtain fully formed spheroids. Because of these shortcomings, we preferred the use of round bottomed 96 multi-well plates

coated with a thin layer of 2-hydroxylethyl methacrylate (polyHEMA), to inhibit cell-matrix adhesion and facilitate cell-cell aggregation.

This system facilitates highly reproducible formation of homogeneous mesothelioma cells aggregates after 24 hours, allowing control of their size by seeding different numbers of cells into wells.

Another spheroid generation technique, the rotatory cell culture system (RCCS), was also evaluated, aiming to increase the overall yield of mesothelioma spheroids. This system is based on a proprietary cell culture chamber mounted on a rotatory apparatus that spins the culture, favoring cell-cell adhesion while allowing gas exchange and maintenance of proper cell culture conditions. Cell aggregates produced by the RCCS method were more compact and smaller in size compared to the spheroids obtained by seeding cells on polyHEMA coated round-bottomed wells (**Figure 20**). However, the RCCS derived spheroids required two weeks of culture for full formation, therefore the age of the aggregates (critical in the development of a necrotic center), and the absence of control on the cell number are clear limitations to the use of this technique for the purpose of this study. We then chose the polyHEMA method as it provides control on every aspect of the spheroid formation and only requires 24 hours of cell culture.

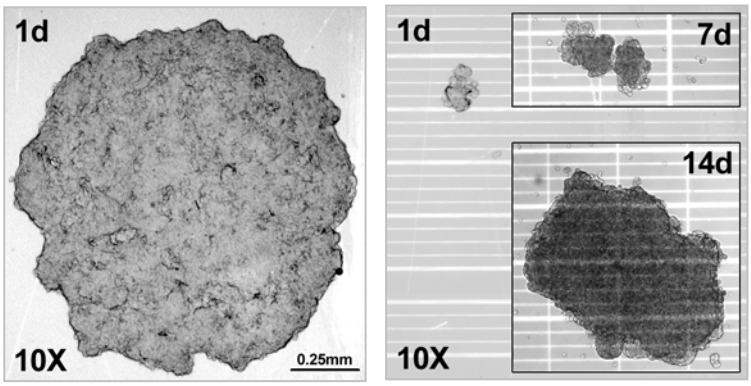


Figure 20: MPM multi-cellular spheroids obtained with polyHEMA coated wells (1 day - left) and with the RCCS method at 1, 7 and 14 days of culture. Depending on the number of cells seeded, the polyHEMA method generated spheroids in the 500-1000 µm range, while the RCCS method only generated spheroid with comparable size after 14 days of culture.

It has been reported that spheroids develop an apo/necrotic core, due to the lack of oxygen and nutrients reaching the inner layers of the aggregates (Mueller-Klieser 1997). The presence of such cells in the study of resistance to apoptosis creates a source of experimental background noise that can hinder the accuracy of many assays. We then assessed the correlation between spheroids size and basal levels of apoptosis, as indicated by the presence of the cleaved form of Poly (ADP-ribose) polymerase (PARP), an enzyme assisting in the repair of single-strand DNA nicks that is degraded by executioner caspases during the apoptotic process. The seeding of 5.000 cells was insufficient to generate a full spheroid, instead a loose mass of cells formed. Furthermore, spheroids originated from 25.000 cells began to exhibit the cleaved form of the enzyme PARP, which increased as the number of seeded cells was augmented. Therefore, spheroids formed with 10.000 cells were chosen for further experiments (**Figure 21**).

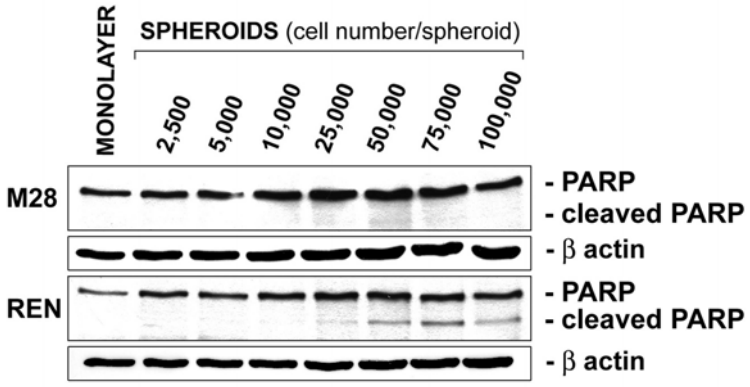


Figure 21: Spheroids formed by more than 10.000 cells begin to show an apoptotic core. Western-blot analysis of Poly (ADP-Ribose) polymerase (PARP), in spheroids formed in 24 hours by different amount of cells (M28 and REN), showed the appearance of a cleaved fragment when seeded at 25.000 cells/spheroid.

MPM cells are resistant to a variety of chemotherapeutics, and several groups have shown that three-dimensional cell structures also acquire resistance to a plethora of agents, a phenomenon termed multi-cellular resistance (Desoize and Jardillier 2000).

It has been recently demonstrated that mitochondria sensitizers such as etoposide can improve the efficiency of apoptosis inducers like TNF-related apoptosis inducing ligand (TRAIL) in mesothelioma (Broaddus, Dansen et al.

2005). This death ligand has shown great promise for the selective induction of apoptosis in tumors (LeBlanc and Ashkenazi 2003), and co-treatment with DNA-damaging agents like etoposide or chemotherapy (doxorubicin, cisplatin, gemcitabine) (Liu, Bodle et al. 2001) is able to increase the poor sensitivity of cells by augmenting levels of TRAIL-activated Bid, that would otherwise be insufficient to start the apoptotic process.

Anisomycin, a potent JNK inducer (Torocsik and Szeberenyi 2000), is able to mimic the DNA-damaging effects of chemotherapy, sensitizing the mitochondria without having intrinsic toxicity and in our system provided a rapid and reliable method of induction of apoptosis in mesothelioma cells (Vivo, Liu et al. 2003).

The MPM cell lines M28 and REN, as monolayers and spheroids, were treated with TRAIL (2.5 ng/ml) plus a non-toxic anisomycin concentration (25 ng/ml) (T+A) for 6 hours (**Figure 22**).

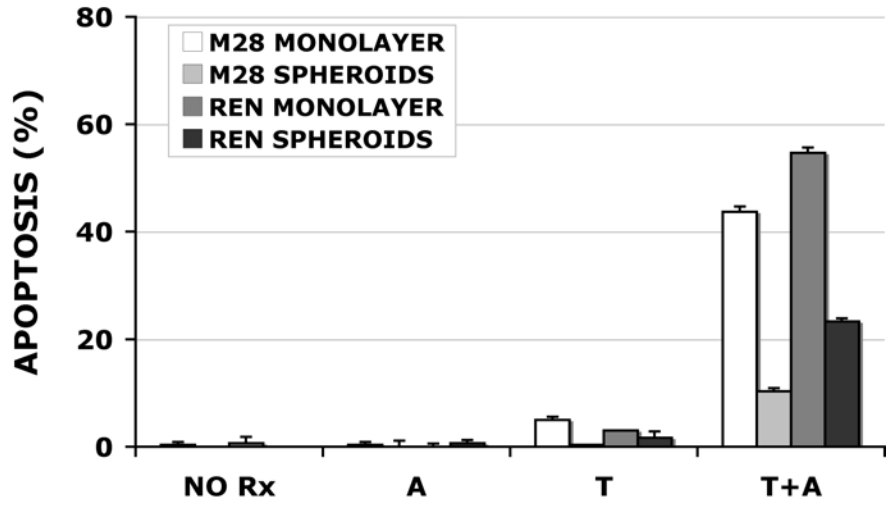


Figure 22: MPM spheroids are resistant to TRAIL plus anisomycin treatment. Hoechst staining of M28 and REN monolayers and spheroids, after treatment with anisomycin (25 ng/ml), TRAIL (2.5 ng/ml) and both for 6 hours. Cells with distinctive signs of nuclear chromatin condensation and blebbing were considered apoptotic. Spheroids displayed a marked resistance when compared to respective monolayer.

Spheroids displayed a marked resistance to the synergic TRAIL plus anisomycin combination, highlighting how the transition to the third dimension can greatly influence cells behavior and more closely reflect tumor responsiveness to treatment. We hypothesized that one major limitation to the efficacy of TRAIL

treatment could result from the limited diffusion of the drug / treatment within the spheroids, reducing the ability of the ligand to effectively trigger death receptor activation. To test this hypothesis, spheroids were treated with TRAIL (2.5 ng/ml), embedded in paraffin blocks, and then immunohistochemistry was performed to detect the presence of TRAIL (**Figure 23**).

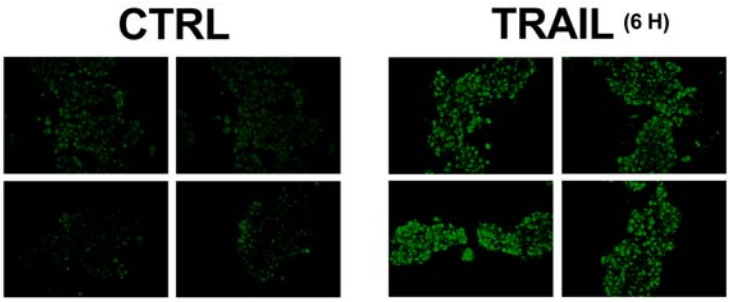


Figure 23: TRAIL diffuses uniformly within spheroids. M28 spheroids were treated with TRAIL (2.5 ng/ml) for 6 hours, then stained IHC with byotinilated TRAIL antibody was performed. TRAIL diffusion within the spheroids was not impaired as demonstrated by the uniform staining upon treatment.

To elucidate the progressive onset of resistance to treatment, apoptosis rates of monolayers, sparse cells plated on polyHEMA-coated flat wells, and spheroids 6 and 24 hours old, were then evaluated upon T+A (**Figure 24**).

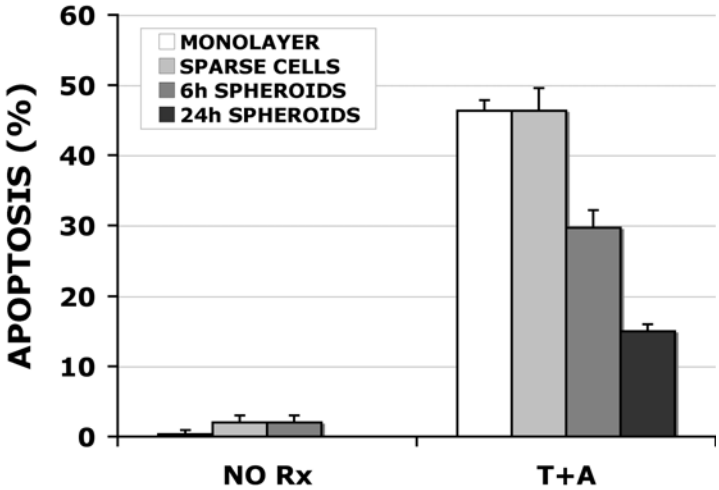


Figure 24: Resistance to T+A appears early, before full spheroids formation. Hoechst staining of M28 cells plated as monolayers, sparse on polyHEMA-coated flat wells, and as spheroids allowed to form for 6 and 24 hours, treated with TRAIL (2.5 ng/ml) and anisomycin (25 ng/ml) for 6 hours. While monolayers and sparse cells exhibited a comparable amount of apoptotic cells, 6 hours spheroids, although not fully formed, began to display resistance to treatment.

Analysis of apoptotic hallmarks (i.e. nuclear condensation and fragmentation) with Hoechst staining, found that monolayers and sparse cells displayed the same level of apoptosis while 6 hours spheroids started to acquire a certain degree of resistance that increased when spheroids were allowed full formation (24 hours). We concluded that spheroids acquired resistance to apoptosis as early as 6 hours after seeding cells into wells, even if not yet fully formed, highlighting the gradual loss of sensitivity to treatment.

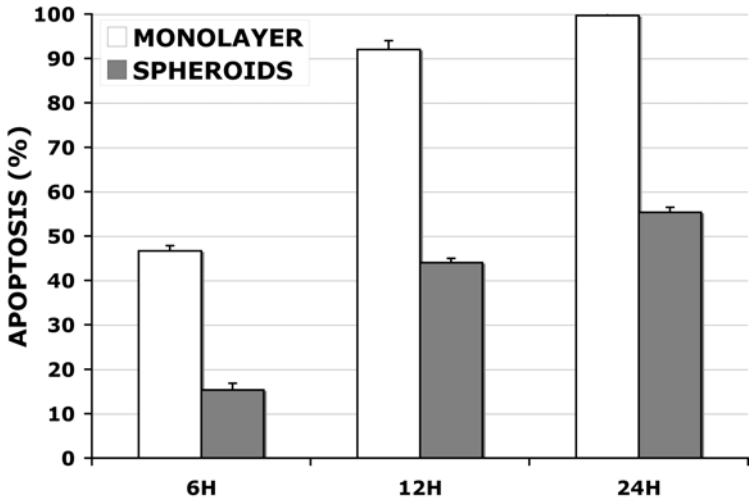


Figure 25: Spheroids display resistance to T+A prolonged treatment. Time course analysis of the apoptosis rate (Hoechst staining) induced by T+A treatment in M28 monolayers and spheroids. Cells with distinctive signs of nuclear chromatin condensation and blebbing were considered apoptotic. Spheroids displayed a remarkable resistance to treatment even at 12 and 24 hours of treatment, while monolayers appeared to be completely apoptotic.

Even at longer time points, spheroids maintained their resistant phenotype (12 and 24 hours) (**Figure 25**), whereas normal monolayers appeared completely apoptotic. Together, these results demonstrate that resistance to treatment is an acquired characteristic of MPM cells when grown in a three-dimensional structure, and not a mere delay of the apoptotic stimulus due to a reduced diffusion of TRAIL.

The resistance of spheroids compared to their monolayers control was also confirmed using various synergic combinations of TRAIL with recently reported effective proteasome inhibitor MG-132 (Sohn, Totzke et al. 2006) and the histone deacetylase inhibitor trichostatin A (Sonnemann, Gange et al. 2005) (**Figure 26**).

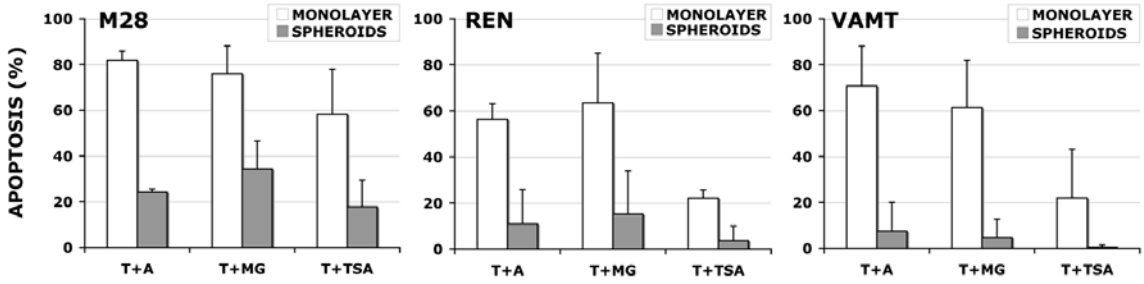


Figure 26: MPM spheroids are resistant to multiple treatments. Flow-cytometric analysis of annexin V / propidium iodide staining of M28, REN and VAMT monolayers and spheroids treated with TRAIL (2.5 ng/ml) in combination with anisomycin (25 ng/ml), MG-132 (2.5 μM) or trichostatin A (250 nM) for 24 hours. Cells positive for annexin V staining were considered apoptotic. Spheroids consistently showed resistance to the different treatments, in all the cells tested.

Further confirming the widespread phenomenon of MPM spheroids acquiring resistance to various treatments, we found that when monolayer were treated with different agents such as the association of MG-132 with the histone deacetylase inhibitor sodium butyrate or trichostatin A, a consistent level of apoptosis was induced. Interestingly, when spheroids were treated with the same combinations, the amount of apoptotic cells was significantly decreased, highlighting the three-dimensional structure as a key factor governing multi-cellular resistance in mesothelioma cells (**Figure 27**).

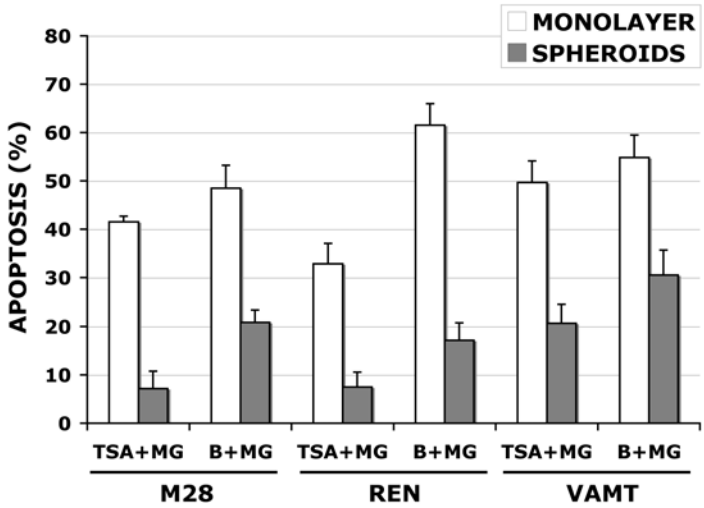


Figure 27: Spheroids displayed resistance also to non-TRAIL combinations. Apoptotic rates (Hoechst staining) M28, REN and VAMT monolayers and spheroids following treatment with non-TRAIL combinations of agents. Cells with distinctive signs of nuclear chromatin condensation and blebbing were considered apoptotic. MG-132 (2.5 μM) synergized with sodium butyrate (10 mM) and trichostatin A (250 nM) in inducing mesothelioma cell lines apoptosis. Consistently with the results obtained with TRAIL plus anisomycin, spheroid were more resistant to treatments, compared to respective monolayers.

Since PI3K / Akt /mTOR pathway has been credited as one of the most important intracellular signalling pathways involved in the protection from apoptosis and consistently found to have a critical role in the survival of many tumors, inhibitors of PI3K (LY294002) and mTOR (rapamycin), or both (PI-103) (Fan, Knight et al. 2006), were used in combination with TRAIL plus anisomycin treatment to evaluate the role of this signalling axis in spheroids acquired resistance (**Figure 28**).

PI3K / Akt / mTOR inhibitors increased the efficiency of treatment and interestingly, affected spheroids more than monolayers, where only a 10% increment in cell death was induced. These results suggested that spheroids seem to rely on the Akt pathway activity more than monolayers and also that mTOR plays a major role in the displayed resistance since specific inhibition of its activity almost resembles the result obtained with a less specific inhibitor like LY294002 (Knight, Gonzalez et al. 2006).

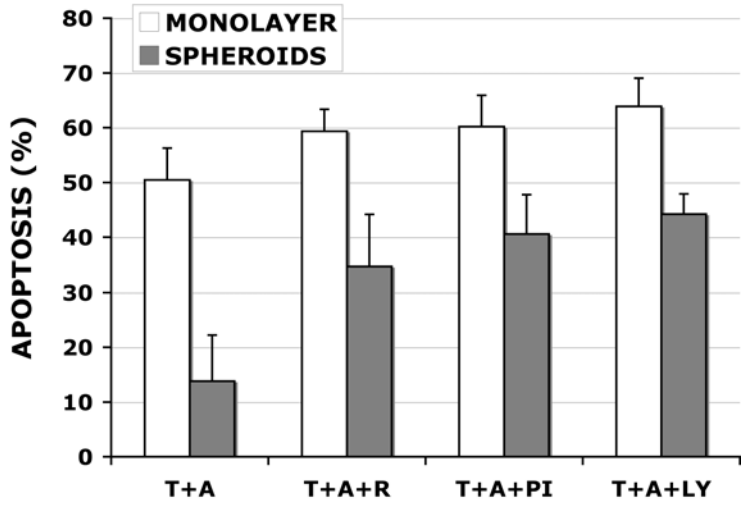


Figure 28: PI3K/mTOR inhibitors sensitize spheroids to treatment. M28 monolayers and spheroids were treated with TRAIL (2.5 ng/ml) plus anisomycin (25 ng/ml) with PI3K / Akt / mTOR pathway inhibitors rapamycin (5 nM), PI-103 (1 μM) and LY294002 (5 μM) for 6 hours. Cells with distinctive signs of nuclear chromatin condensation and blebbing were considered apoptotic. Inhibitors affected resistance to apoptosis more efficiently in spheroids than in monolayers.

Baseline activity of Akt and mTOR pathways in monolayers and spheroids was then evaluated with antibodies targeted to the phosphorylated forms of Akt (phospho-Ser473, required for full Akt activity (Alessi and Cohen 1998)) and phospho-p70S6K (a downstream target of mTOR) (**Figure 29**).

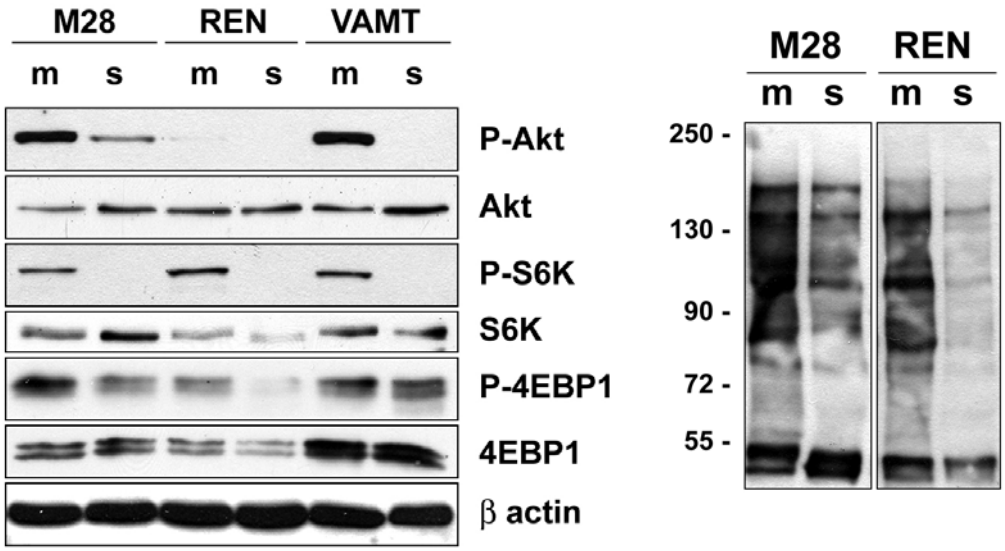


Figure 29: Spheroids displayed reduced Akt and p70S6K activities. Western blot analysis of Akt / mTOR pathway activity of M28, REN and VAMT monolayers and spheroids. (**left**) Phosphorylation of Akt Ser473 and p70S6K Thr389 was markedly reduced in all cells tested when forming spheroids, and Akt activity was noticeably reduced in M28 and REN spheroids (**right**) as evidenced by a decreased phosphorylation levels of Akt substrates (antibody against phosphorylated Akt-kinase consensus sequences).

Intriguingly, spheroids showed a marked reduction of Akt and p70S6K phosphorylation compared to monolayers, while total protein levels tended to increase. Also, the pattern of Akt phosphorylated substrates, detected with an antibody specifically recognizing the phosphorylated form of consensus sequences targeted by Akt kinase (RXRXXS/T), was notably decreased (**Figure 29**).

Immunohistochemical analysis of the phosphorylation levels of Akt and p70S6K revealed that the reduced activity of these proteins is featured by the entire spheroid cell population and is not a characteristic of the inner layers, where supposedly it should be expected (**Figure 30**).

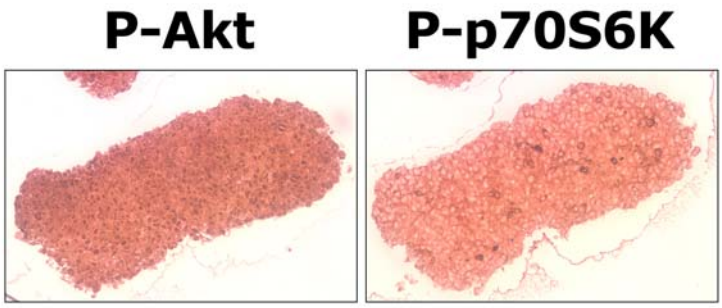


Figure 30: Spheroids display uniform Akt and p70S6K phosphorylation. Immunohistochemistry of M28 spheroids (paraffin sections) with antibodies against Akt phospho-Ser473 and p70S6K phospho-Thr389. The analysis of spheroids cross-sections evidenced the uniform pattern of Akt and p70S6K phosphorylation among the whole cells population.

Several hypothesis may explain why the inhibition of a signalling pathway already turned down could be more effective in modulating response to chemotherapeutics and this paradox can be overcome by considering intracellular signals within a specific environment. For example, it is well known that canonical pathways can have different behaviors in different cells (i.e. NF-κB as pro- or anti-apoptotic factor (Kucharczak, Simmons et al. 2003)) and unexpectedly, overexpression of Akt1 has been shown to display tumor suppressing activity and to reduce the invasivity of breast cancer cells by targeting TSC2 (Wyszomierski and Yu 2005; Liu, Radisky et al. 2006). Furthermore, studies on three-dimensional aggregates of breast cancer cells, highlighted how these structures acquired resistance to apoptosis without relying on the PI3K / Akt and ERK pathways (Zahir, Lakins et al. 2003).

We focused our studies on the mTOR pathway since rapamycin was able to rescue most of the resistance to apoptosis displayed by spheroids. The downstream substrates of mTOR are mainly, p70S6Kinase, responsible for translational control of 5'-TOP mRNAs, and 4EBP1, a scaffolding protein for the translation initiation factor eiF4E, responsible for the translation of capped mRNAs (i.e. cyclin D1, c-myc and VEGF). mTOR function is also regulated by other proteins with which it is complexed: regulatory associated protein of mTOR (raptor) and rapamycin insensitive companion of mTOR (rictor) (Averous and Proud 2006).

Currently, little is known about the fine regulation of mTOR activity and recent data has identified the mTOR/rictor complex as the kinase responsible for the phosphorylation of serine 473 of Akt (Sarbassov, Guertin et al. 2005), shedding new light on the regulation of this signalling pathway. However, to complicate matters, it is known that the modulation of eiF4E activity may result in uncontrolled cross-talk mechanisms downstream of mTOR that may alter interpretation of results (Khaleghpour, Pyronnet et al. 1999).

To further address the involvement of mTOR in the acquired resistance of spheroids, we inhibited its function more specifically, by means of small RNA duplexes aimed to interfere with p70S6K translation (p70S6K-*kd*). Interestingly, the reduction of p70S6K protein translation did not exert any basal cytotoxicity, but increased the apoptosis rate of spheroids when treated with TRAIL plus anisomycin in all tested cells, to a level comparable to the control spheroids when rapamycin was added to treatment. Monolayers did not seem to be affected by the p70S6K knock-down as much as spheroids and furthermore, adding rapamycin to treatment in p70S6K-*kd* cells did not increase the apoptosis rate significantly either in monolayer or spheroids, suggesting that most of the resistance demonstrated by spheroids resides in p70S6K signalling (**Figure 31**).

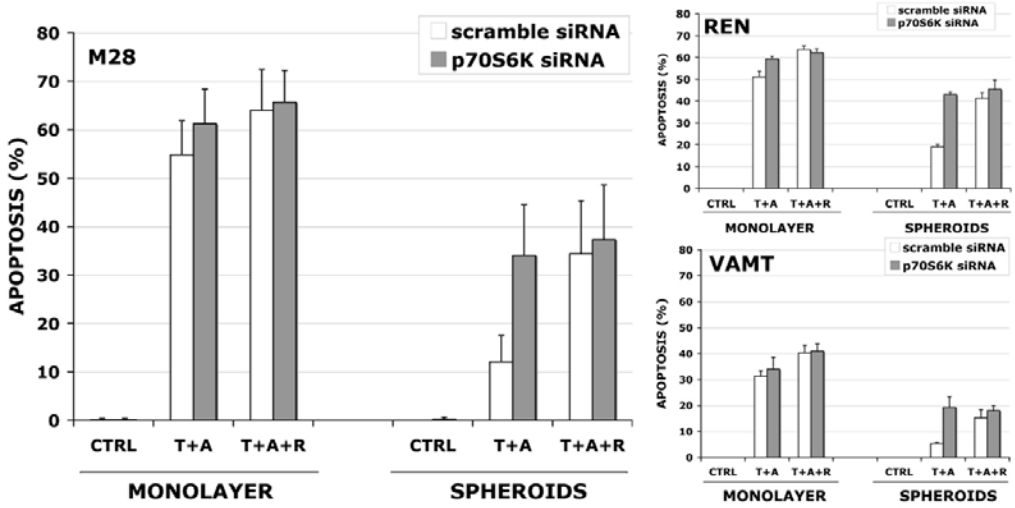


Figure 31: p70S6K siRNA restored spheroids sensitivity to treatment. Apoptosis rates (Hoechst counts) of M28, REN and VAMT cells transfected with p70S6K siRNA duplexes (p70S6K-*kd*) or a scrambled control sequence, grown as monolayers or spheroids and treated with TRAIL (2.5 ng/ml) plus anisomycin (25 ng/ml) (T+A) in presence or absence of rapamycin (5 nM) for 6 hours. Spheroids formed by p70S6K-*kd* cells, when treated with T+A, displayed the same apoptotic rate as control cells treated with T+A in the presence of rapamycin. Conversely, monolayers were not significantly affected by p70S6K siRNA or by rapamycin.

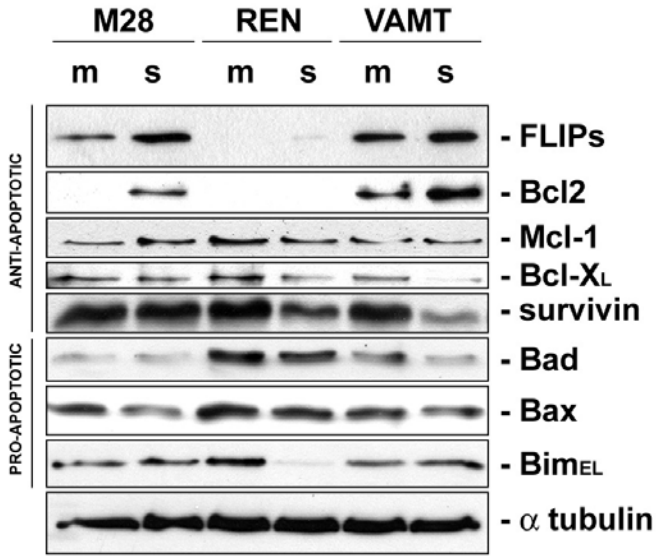


Figure 32: Panel of pro-/anti-apoptotic proteins of M28, REN and VAMT monolayers and spheroids. Spheroids formed by all tested cell lines displayed increased levels of FLIPs and Bcl2 proteins whereas no significant changes were found in other modulators of apoptosis.

Rapamycin may control multiple aspects of resistance to apoptosis (Asnaghi, Bruno et al. 2004), and the translational control of pro- and anti-apoptotic molecules levels could represent an important mechanism of acquired resistance (Gomez-Benito, Balsas et al. 2007).

Expression levels of a panel of pro- and anti-apoptotic Bcl2 family proteins was then analyzed in monolayers and spheroids of all MPM cells tested, to determine if the transition to a three-dimensional structure might result in an impairment of the modulators of the apoptotic-machinery (**Figure 32**).

Interestingly, all spheroids displayed an increase in Flice Inhibitory Protein Short isoform (FLIPs) and Bcl2 levels, whereas other Bcl2 family members analyzed did not show any variation that may correlate to the acquired resistance of spheroids to treatment, although they are known to be important for MPM (Fennell and Rudd 2004).

Both FLIPs and Bcl2 mediate anti-apoptotic effects, by preventing caspase 8 cleavage induced by death receptor ligands or by blocking Bax-mediated loss of mitochondrial membrane potential respectively (Gupta 2001). Recently it has been demonstrated that the p70S6K arm of mTOR signalling positively regulates the

cellular abundance of FLIPs and that pharmacological or molecular inhibition of p70S6K is able to restore glioblastoma multiform cells to TRAIL sensitivity (Panner, James et al. 2005). These results prompted us to verify if mTOR / p70S6K signalling pathway could control FLIPs or Bcl2 protein levels in spheroids and possibly provide a mechanism for the induced chemosensitization of spheroids upon mTOR inhibition.

FLIPs and Bcl2 protein levels of M28 monolayer and spheroids, transfected with p70S6K or scramble siRNA duplexes, were then analyzed by western blot, in the presence or absence of rapamycin (**Figure 33**).

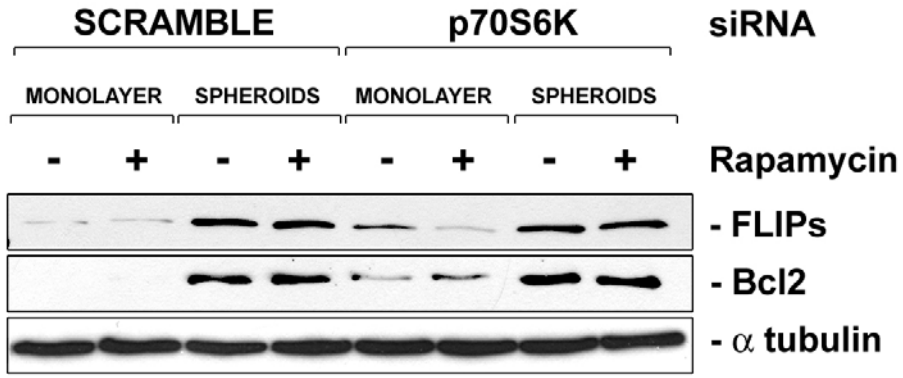


Figure 33: mTOR inhibition did not interfere with FLIPs and Bcl2 protein levels. Immunoblot of monolayers and spheroids formed from scramble or p70S6K siRNA transfected M28 cells, treated or not with rapamycin 5 nM for 4 hours. Neither pharmacological nor molecular inhibition of mTOR signalling altered FLIPs and Bcl2 protein levels.

As expected, the increased expression levels of both FLIPs and Bcl2 of spheroids were confirmed, whereas they were not modified by rapamycin treatment or by the reduction of p70S6K levels by siRNA. Intriguingly, p70S6K-*kd* cells displayed a slight increase of both FLIPs and Bcl2 protein levels.

To further verify the molecular mechanism driving rapamycin mediated chemosensitization, we addressed the question of whether mTOR inhibition could interfere with TRAIL signalling and death-inducing signalling complex (DISC) formation or by lowering the mitochondrial threshold for apoptosis induction. Caspase 8 cleavage after T+A was then analyzed in M28 monolayers and spheroids, in the presence or absence of the mTOR inhibitor rapamycin, or the dual PI3K/mTOR inhibitor PI-103 (**Figure 34**).

While monolayers did not display any increase in caspase 8 cleaved fragments upon treatment, spheroids clearly demonstrated sensitivity to the inhibitors-mediated chemosensitization, as shown by the augmented caspase 8 cleavage. Apoptosis is a dynamic process, where the balance between pro- and anti-apoptotic proteins and the enzymatic activation of caspases, determines cell fate (Green 2005). Particularly, mesothelioma cells have been shown to require the involvement of the intrinsic apoptotic pathway, via cleavage of the BH3-only protein Bid (tBid), to efficiently start the apoptotic program (Broaddus, Dansen et al. 2005).

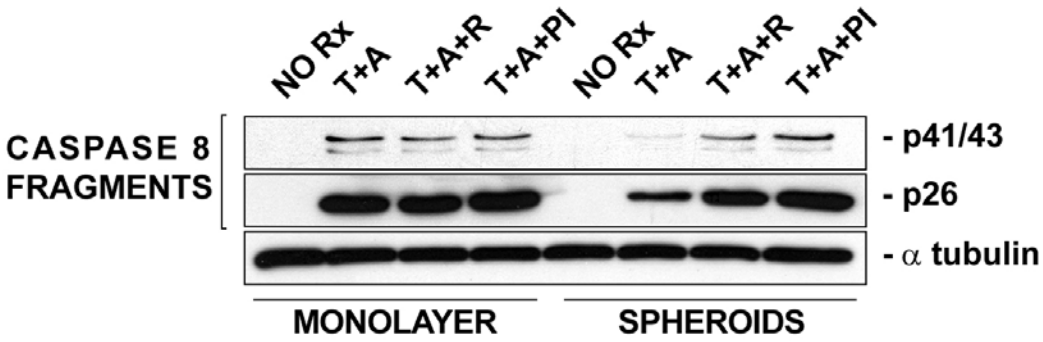


Figure 34: PI3K/mTOR inhibitors induced an increase in caspase 8 cleavage selectively in spheroids. Immunoblot of M28 monolayers and spheroids treated with TRAIL (2.5 ng/ml) plus anisomycin (25 ng/ml) in the presence or absence of rapamycin (5 nM) or PI-103 (1 μM) for 6 hours. Only spheroids displayed increased caspase cleavage when inhibitors were added to treatment.

Increased cleavage of caspase 8 may result from positive amplifying feedbacks by caspase 9, 3 and 7, activated by formation of a functional apoptosome following activation of the mitochondrial apoptosis pathway. To evaluate if rapamycin facilitated either the extrinsic or the intrinsic apoptosis program, cells were then transfected with Bid siRNA duplexes (Bid-*kd*), as to remove an essential cross-talk between the two pathways. Without tBid, mesothelioma cells are unable to involve mitochondrial pathway upon caspase 8 activation, resulting in a marked protection from apoptosis. In these condition we could evaluate if mTOR inhibition interfered with DISC formation and caspase 8 cleavage or with BH3 molecules and the mitochondria-controlled apoptosis pathways.

As expected, the knock-down of Bid completely abolished apoptosis of M28 monolayers and spheroids after T+A treatment. mTOR inhibition was able to rescue sensitivity to treatment in control spheroids while Bid siRNA prevented the rapamycin-mediated chemosensitization of spheroids (**Figure 35**).

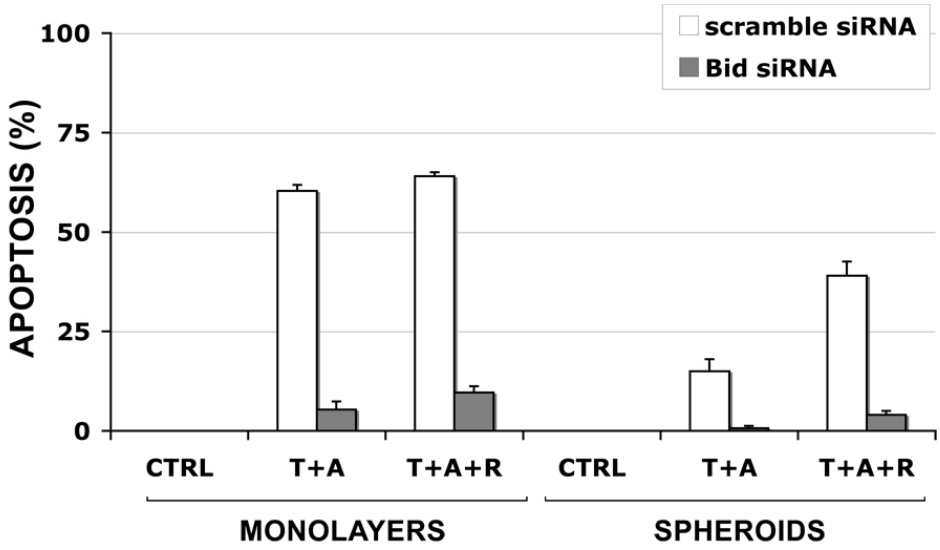


Figure 35: Bid siRNA completely abolished monolayer and spheroids apoptosis upon T+A treatment. Monolayers and spheroids formed from cells transfected with Bid or scramble siRNA duplexes, were treated with TRAIL (2.5 ng/ml) plus anisomycin (25 ng/ml) in the presence or absence of rapamycin (5 nM) for 6 hours. Hoechst staining was then performed on disaggregated cells. Cells with clear signs of nuclear fragmentation and blebbing were considered apoptotic. Bid-*kd* cells displayed a significant reduction of apoptosis.

Since caspases cleavage is an amplifying process mediated by homo- or hetero-typic proteolytical activations, to better evaluate caspase 8 cleavage differences we analyzed cells at an earlier time point of treatment (5 hours), where apoptosis signs started to be seen.

Analysis of caspase 8 cleavage upon T+A treatment of control or Bid-*kd* spheroids, demonstrated that caspase 8 fragments abundance and the cleavage of caspase 3 increased when rapamycin was added to treatment in control spheroids while Bid siRNA prevented any proteolytical cleavage of caspase 8 and 3 cleavage, strongly supporting the hypothesis that rapamycin is controlling mitochondrial apoptosis pathway in spheroids (**Figure 36**).

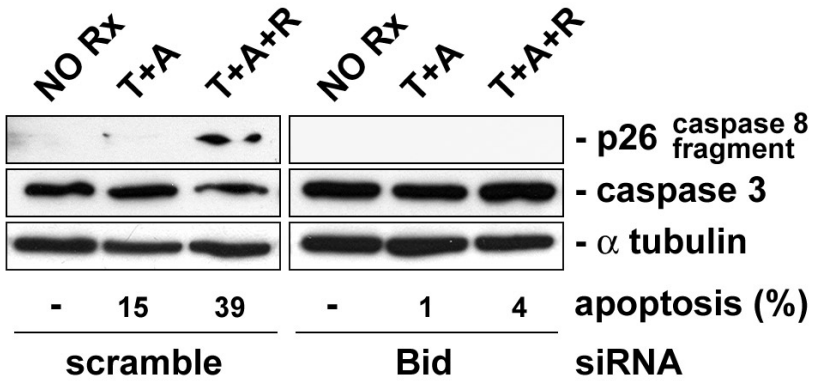


Figure 36: Bid knockdown prevents rapamycin-induced caspase 8 cleavage in spheroids. Spheroids formed from cells transfected with Bid or scramble siRNA duplexes, were treated with TRAIL (2.5 ng/ml) plus anisomycin (25 ng/ml) in the presence or absence of rapamycin (5 nM) for 5 hours. Rapamycin increased caspase 8 and 3 cleavage of control spheroids while Bid siRNA blocked this activity.

We concluded that MPM multi-cellular spheroids display resistance to apoptosis when exposed to different treatments, a property acquired during spheroids formation and maintained after long periods of treatment. PI3K / mTOR inhibitors such as PI-103 and rapamycin were able to rescue chemo-sensitivity of MPM three-dimensional structures while monolayers were not significantly affected. Although spheroids displayed lower Akt / mTOR activities, mTOR / p70S6K inhibition was able to rescue spheroids chemo-sensitivity by affecting the mitochondrial apoptosis pathway.

5. Discussion

Human malignant pleural mesothelioma (MPM), a lethal thoracic cancer which arises from the parietal pleural mesothelium, is characterized by a profound resistance to standard anti-neoplastic therapies and, at present, patients survival rate remains poor, while no standard of therapy is available. The incidence of mesothelioma has been constantly increasing and, while the disease may have already peaked in the USA, other countries such as the United Kingdom and Australia (which regulated asbestos use later than the USA) may expect a high incidence for decades to come. Already, the death rate is high and asbestos-related diseases cause more deaths each year than skin cancer.

Recent advances in chemotherapy studies have highlighted the potential benefits of a multi-targeted approach to overcome the pronounced drug-resistance displayed by tumors (Collins and Workman 2006; Smalley, Haass et al. 2006) and specifically by mesotheliomas (Vogelzang, Porta et al. 2005).

Preclinical studies on several human solid tumors, have demonstrated the efficacy of imatinib mesylate (Gleevec®) as a cytotoxic agent (Krystal, Honsawek et al. 2000; Wang, Healy et al. 2000; Druker, Sawyers et al. 2001; Gonzalez, Andreu et al. 2004). In chronic myeloid leukemia and gastro-intestinal stromal tumors, the carcinogenic role of the fusion protein BCR-ABL and activating mutations of c-Kit (Heinrich, Corless et al. 2003), respectively, are predictive of a clinical response to imatinib mesylate. However, similarly to other molecules, imatinib showed little or no benefit in the therapy of MPM when utilized as single agent (Mathy, Baas et al. 2005; Porta, Mutti et al. 2007), whereas its combination with other chemotherapeutic agents has been shown to be effective in mice (Pietras, Stumm et al. 2003; Yokoi, Sasaki et al. 2005).

One promising therapeutic target for the treatment of MPM is the receptor for platelet derived growth factor B (PDGFRβ). Overexpression of PDGFRβ, characterized by an interrupted tyrosine-kinase domain in the intracellular region (split kinase domain), has been demonstrated in MPM cell lines, xenografts, as well as in MPM cancer patient specimens. Autocrine or paracrine stimulation may activate PDGFRβ *in vivo* and the stromal microenvironment has been proposed as a fundamental source of activating ligands for PDGF receptors in human tumors (Sawyers 2004). Furthermore, one of the most common genetic abnormalities observed in MPM involves chromosome 22q13, which encodes the β-chain of PDGF.

The expression pattern of PDGFRβ found in the tested MPM cells was in accordance with previous findings, reporting a percentage of 30-45% of positive specimens. Interestingly it has been reported that the blockade of PDGF receptors results in MPM growth inhibition (Vogelzang, Porta et al. 2005), offering a strong rationale for testing imatinib in combination with well described agents currently clinically used in combination such as cisplatin, gemcitabine and pemetrexed (Steele and Klabatsa 2005).

Tyrosine phosphorylation of PDGFRβ in MMP and REN cells was inhibited by imatinib treatment, resulting in the inhibition of Akt signalling, a crucial pathway contributing to the MPM malignant phenotype (Cacciotti, Barbone et al. 2005; Ramos-Nino, Vianale et al. 2005; Rascoe, Cao et al. 2005). Specific inhibition of Akt signalling obtained by interfering with PDGFRβ tyrosine kinase activity exerted a relevant increase in cell chemosensitivity.

Results clearly indicate that PDGFRβ expression in MPM is mandatory for the sensitivity to imatinib treatment and for the synergy observed between imatinib and gemcitabine or pemetrexed. When the receptors sensitive to imatinib PDGFRβ, c-Kit and c-Fms (Pardanani and Tefferi 2004; Guo, Marcotte et al. 2006), were all expressed in the same cell type, such as MMP cells, characterized by a strong autonomous Akt activation, the obtained synergistic effect of imatinib was higher than in REN cells, where the Akt pathway was weakly activated.,

Interestingly, ISTMES2 cells, which express very low levels of PDGFRβ, failed to show any benefit from the combined use of imatinib together with either gemcitabine or pemetrexed, indicating the important role played by receptor activation in the observed synergy of imatinib combinatorial therapies.

This observed *in vitro* synergy is most likely the result of the imatinib-dependent PDGFRβ inhibition, subsequent Akt inactivation and the sensitization of MPM cells to lower chemotherapeutic concentrations. This mechanism is of particular clinical relevance because it reveals that very low doses of chemotherapeutic agents may be sufficient to exert beneficial therapeutic effects.

Other biological effects exerted by imatinib may play a role *in vivo* such as the reduction of intratumoral interstitial fluid pressure and increased drug uptake (Pietras, Stumm et al. 2003) as well as interference with VEGF expression and the associated angiogenesis (Beppu, Jaboine et al. 2004). Imatinib has also been shown to synergize with TRAIL, inducing apoptosis in

Altogether these results further donfirm the importance of a multi-targeted approach in the therapy of MPM and the specific involvement of Akt signalling in the resistance of MPM cell lines to treatment. If the mechanism of this resistance can be uncovered, the benefits resulting from the therapeutic synergism obtained with inhibition of a survival pathway and a classic chemotherapeutic agent may be fully realized.

We found that imatinib-induced Akt inhibition was the key mechanism leading to chemosensitization in MPM cell lines. Given the crucial role played by Akt signalling in tumors, we verified whether Taurolidine (TN), which displays selective anticancer properties and has been recognized a possible role in MPM therapy, might interfere with Akt activity in MPM cells..

Clinically available therapeutic options for the treatment of MPM can modify the progression of the neoplastic process but unfortunately still give unsatisfactory results. Recent data identified a TN-induced interference with protein translation (Braumann, Henke et al. 2004); we hypothesized that this translational block could be a consequence of effects on the Akt signalling pathway, as it controls most of

the translational machinery activity via mTOR and its downstream effectors 4E-BP1 and p70S6K (Mamane, Petroulakis et al. 2006).

Our research clearly demonstrates that TN inhibits Akt / mTOR activity specifically in MPM cells, accounting for its selective action on neoplastic cells, as described for other tumor types (Stendel, Stoltenburg-Didinger et al. 2002) while activity of other survival pathways such as ERK 1/2 are not affected.

The kinetics of Akt inhibition induced by TN, which is consistent with the time course of cytotoxicity, and the fact that higher Akt activity correlates with lower TN sensitivity provide further support for this serine/threonine kinase to be a key mediator of MPM cell survival. Both normal and neoplastic cells displayed a progressive increase of p38 and JNK activities upon TN treatment, features of a cell stress response. Because MPM cells have been shown to be sensitive to oxidative stress, this has been proposed as a potential therapeutic strategy (Stapelberg, Gellert et al. 2005). Similar to previous findings showing TN-induced apoptosis via oxygen intermediates in glioma cells (Rodak, Kubota et al. 2005), a MPM specific and time dependent nitrite production upon TN treatment was observed. The kinetics of TN-induced nitrite production in both MMB and MMP cells, congruent with Akt inhibition and cytotoxicity patterns, led us to conclude that TN induces oxidative stress, which drives biochemical effects such as Akt activity inhibition and apoptotic machinery activation. These conclusions are strongly supported by the significant increase of 8OH-dG DNA adducts production upon TN treatment, in MPM cells compared to normal mesothelial cells. The mechanism of action of TN was also assessed by restoration of cell viability when general anti-oxidants such as GSH or L-NAC are added to treatment. Both agents were able to rescue TN-induced Akt activity inhibition, highlighting the close association between cytotoxicity and a reduced phosphorylation and activity of Akt.

Dephosphorylation of Akt by phosphatases is an important regulatory mechanism for cell survival and proliferation moreover, mutations in the lipid phosphatase PTEN have been detected in many human cancers (Garcia, Cayla et al. 2003; Thompson and Thompson 2004). Analysis of phosphatases activity upon TN treatment revealed that PP2A activity was selectively increased by TN in MPM

cells, starting after two hours of exposure to drug, whereas the activity of PP1α was affected at a later time point and PTEN activity was not affected. PP2A has been studied as a modulator of kinase activities (Millward, Zolnierowicz et al. 1999), as a key regulator of human tumors (Janssens and Goris 2001) and as a specific Akt inhibitor (Ruvolo 2001).

The discrepancy between Akt inhibition and PP2A activation may be explained by the hypothesis that a very low PP2A activity, below the immunoblotting detection threshold, may be sufficient to inhibit Akt activity in an early stage or that PP2A and PP1α increased activities may sustain Akt inhibition, provoked by another unknown phosphatase, for a prolonged period of time.

Recently, two novel phosphatases have been demonstrated to regulate Akt activity and apoptosis of glioblastoma cells, PH domain leucine-rich protein phosphatase 1 and 2 (PHLPP1 and PHLPP2), although little is known on their activation mechanism and regulation (Gao, Furnari et al. 2005; Brognard, Sierecki et al. 2007; Mendoza and Blenis 2007).

Akt inhibition may also derive from nitration of the p85 subunit of PI3K upon induction of oxidative stress (el-Remessy, Bartoli et al. 2005) although other reports suggested a stimulating effect of peroxynitrite on Akt activity. Interestingly, no p85 regulatory subunit nitration upon TN treatment was detected in MPM cells, a result that prompted us to find another mechanism by which Akt is inhibited.

The molecular chaperone heat-shock protein HSP-90 plays an important role in maintaining Akt kinase activity by preventing PP2A-mediated dephosphorylation (Sato, Fujita et al. 2000). We then verified if TN could interfere with the activity of HSP-90 but no difference in HSP-90 or PP2A association with Akt kinase was observed, suggesting than another yet unidentified mechanism is driving TN-induced effects on Akt.

More generally, a reduction in tyrosine phosphorylation of membrane tyrosine kinase receptors (RTK) could be the cause of the reduced Akt activity. We then measured the phosphorylation levels of expressed RTKs in MPM cells such as Met and PDGFRβ but we were not able to detect any relevant reduction in their phosphorylation levels upon TN treatment even at high TN concentrations.

The pro-apoptotic effects exerted by TN on MPM cells are hence mediated by oxidative stress in time- and dose-dependent manner, accompanied by the impairment of Akt / mTOR signalling. Other groups have already demonstrated the anti-tumor effects of locally injected TN in mice with intra-peritoneal mesothelioma (Nici, Monfils et al. 2004), thereby this study provides further mechanistic evidence supporting a convincing rationale for using TN as a novel local treatment for the treatment of MPM.

Both presented studies highlighted Akt inhibition as a crucial step towards chemosensitization and efficient induction of the apoptotic process in MPM cells.

Increased resistance to apoptosis of mesothelioma tissue grown in vitro as tumor fragment spheroids (TFS) has already been analyzed (Kim, Wilson et al. 2005). The authors were able to address the importance of Akt / mTOR pathway in the acquired resistance to treatment of these three-dimensional structures, modulating their apoptotic response by means of specific inhibitors of these pathways.

In the last decade multi-cellular spheroids (MCS) have been a valuable tool in the study of solid tumors as they represent a three-dimensional system of intermediate cellular complexity between monolayers, commonly used in molecular biology studies, and structures found *in vivo* (organs and tumors).

We aimed to assess the molecular mechanism at the basis of acquired resistance of MPM cells, when grown three-dimensionally, by using an homogeneous cell culture model that would allow a reduction in the cell type heterogeneity and the complexity displayed by TFS.

MCS generated by M28, REN and VAMT cells, demonstrated a high level of apoptotic resistance to a variety of treatments that were effective in killing the cells in monolayers. The treatment with TRAIL plus anisomycin, has been used as one of the most potent approach for inducing apoptosis in mesothelioma cells in monolayers, and one that induces apoptosis in only 6-8 hours. This apoptotic approach depends on the interaction of TRAIL-induced tBid together with anisomycin-induced Bim at the mitochondria, resulting in a synergistic apoptosis. Importantly, we were able to show that MCS develop resistance to treatment as

soon as six hours after cells have been plated for spheroid formation, suggesting that cells reduce their sensitivity to treatment as they start to engage inter-cellular connections. Fully formed spheroids, after 24 hours of culture, maintained a reduced apoptotic rate even when treatment was prolonged to 24 hours, a time point where monolayers appear to be completely apoptotic.

Although the response of three-dimensional cellular aggregates to chemotherapy may be influenced by diffusion of agents within the layered structure of cells, in our system, TRAIL was able to diffuse uniformly within the spheroids (Fracasso and Colombatti 2000). Cells, when grown into spheroids, developed resistance also to other treatments such as the combination of the proteasome inhibitor MG-132 and the histone deacetylase inhibitors sodium butyrate or trichostatin A, further confirming the multi-drug resistance acquired by these structures.

Inhibitors of PI3K / Akt / mTOR signalling pathway were able to restore a significant degree of MCS sensitivity to TRAIL plus anisomycin treatment while modestly affecting the apoptotic rate of monolayers. The increase of MCS apoptotic rate, obtained with the inhibition of PI3K with LY294002 or with PI-103 (CAY-10009209), a novel dual inhibitor of PI3K and mTOR, was similar to the results achieved by the specific inhibition of mTOR with low doses of rapamycin. These results prompted us to focus on mTOR signalling and its downstream effectors 4E-BP1 and p70S6K.

Surprisingly, spheroids displayed a reduced activation of the Akt/mTOR pathway, a property of all cells forming the spheroids. Previous findings on breast cancer cells aggregates have demonstrated the same pattern of reduced Akt activation upon three-dimensional structure formation (Zahir, Lakins et al. 2003) and overexpression of Akt1 has also been shown to reduce breast cancer cells invasivity (Wyszomierski and Yu 2005), suggesting an intriguing role of this pathway in a new cellular three-dimensional environment.

Recently, the complex of mTOR and rictor (rapamycin insensitive companion of mTOR) has been proposed to be the kinase responsible for Akt phosphorylation on serine 473, a finding that modified the current beliefs on

molecules positioning within this pathway (Sarbassov, Guertin et al. 2005). mTOR has classically been proposed to be positioned downstream of Akt signalling either as a direct substrate or via phosphorylation of tuberous sclerosis complex 2 (TSC2) (Potter, Pedraza et al. 2002), a GTPase-activating protein (GAP) toward Rheb, a Ras family GTPase, which stimulates phosphorylation of mTOR and activation of its downstream effectors 4E-BP1 and p70S6K. More recently, it has been also proposed that p70S6K inhibition can be mediated by TSC2 independently of mTOR activation, via inhibition of the PI3K pathway only (Jaeschke, Hartkamp et al. 2002). Hence, to clearly define the molecular pathway blocked by rapamycin in our system, we aimed to interfere with mTOR specific downstream signalling pathways, transfecting cells with siRNA duplexes targeting p70S6K.

Interference with eIF4E signalling has been demonstrated to be problematic because of unknown cross-talks as overexpression of eIF4E scaffolding protein 4E-BP1 resulted in p70S6K aberrant phosphorylation (Panner, James et al. 2005) and rapamycin itself has been demonstrated to phosphorylate eIF4E, although the significance of this phosphorylation is not completely known (Scheper and Proud 2002).

Also, rapamycin, when complexed to its intracellular receptor FK506-binding protein 12KDa (FKBP12), inhibits the formation of the complex mTOR/ regulatory associated protein of mTOR (raptor) switching the binding of mTOR to rictor and it has already been proposed that raptor mediates most of the functions of mTOR (Hara, Maruki et al. 2002).

Spheroids formed by cells transfected with p70S6K siRNA duplexes (p70S6K-*kd*) and treated with TRAIL plus anisomycin showed an increased apoptotic rate compared to control spheroids, transfected with a scrambled non-targeting siRNA duplex. Interestingly p70S6K-*kd* spheroids treated with TRAIL plus anisomycin showed a percentage of apoptotic cells comparable to control spheroids when rapamycin was added to treatment. Furthermore, when p70S6K-*kd* spheroids were treated together with rapamycin, they did not show any

additional apoptosis, strongly suggesting that p70S6K is the molecule responsible for the amount of apoptotic resistance that is recovered by mTOR inhibition.

The limited effectiveness of treatments in MPM has been considered the result of an important functional defect in the apoptosis pathways, that may account for the profound chemoresistance of this tumor (Fennell and Rudd 2004). Although previous studies demonstrated that mTOR signalling controls FLIPs translation (Panner, James et al. 2005), on the contrary we found that $FLIP_S$ and Bcl2 protein levels were elevated in spheroids, independently of reduced mTOR pathway activity. In addition, their expression levels were not modulated by rapamycin or p70S6K-*kd*. We hypothesized that the increased protein levels of FLIPs and Bcl2 could be considered responsible for the residual spheroids resistance to treatment, which was not rescued by rapamycin. Also, compared to rapamycin, PI-103 and LY294002 were able to induce a more pronounced recovery of chemo-sensitivity in spheroids, because of the additional upstream inhibition of PI3K signalling, which in turn initiates multiple pro-apoptotic signals (Franke, Hornik et al. 2003).

Death receptors and mitochondrial apoptosis pathways can be effectively separated by Bid siRNA in mesothelioma cells (Broaddus, Dansen et al. 2005). Under these experimental conditions, the increased caspase 8 cleavage shown by spheroids upon addition of rapamycin to treatment, a molecular hallmark of chemosensitization, was blocked by Bid siRNA strongly supporting the involvement of mitochondrial apoptosis pathways in the rapamycin-mediated chemo-sensitization.

A mitochondrial involvement in the resistance demonstrated by multi-cellular spheroids against a variety of treatments is conceivable and can be explained by considering spheroids as a link of intermediate cellular complexity between artificial monolayers and tumors found in patients. It has already been proposed that tumors may be sensitive to metabolic stress (Jin, DiPaola et al. 2007) because of an extreme instability in their metabolic rates. Therefore, PI3K / mTOR inhibition, may selectively affect spheroids because of their metabolic vulnerability while

monolayers can easily adapt to the induced blockade of growth factor activated pathways.

We conclude that mesothelioma cell lines, when grown in three-dimensional aggregates, acquire resistance to apoptosis which can be modulated by PI3K / Akt / mTOR inhibitors. RNA interference studies allowed us to identify p70S6K as the kinase responsible for the acquired resistance mediated by mTOR signalling. Altogether these results highlight the importance of the study of MPM multi-cellular spheroids and their acquired resistance to treatments, to increase the knowledge of the molecular mechanisms driving the profound unresponsiveness to treatment of this malignancy.

6. Bibliography

(1947). "Case records of the Massachussetts General Hospital (case 33111)." N. Engl. J. Med. **236**: 407-412.

(2006). "Abstracts of the 8th International Conference of the International Mesothelioma Interest Group. October 19-22, 2006. Chicago, Illinois, USA." Lung Cancer **54 Suppl 1**: S1-60.

Abutaily, A. S., J. E. Collins, et al. (2003). "Cadherins, catenins and APC in pleural malignant mesothelioma." J Pathol **201**(3): 355-62.

Alessi, D. R. and P. Cohen (1998). "Mechanism of activation and function of protein kinase B." Curr Opin Genet Dev **8**(1): 55-62.

Alexiadis, V., L. Halmer, et al. (1997). "Influence of core histone acetylation on SV40 minichromosome replication in vitro." Chromosoma **105**(6): 324-31.

Altomare, D. A., H. You, et al. (2005). "Human and mouse mesotheliomas exhibit elevated AKT/PKB activity, which can be targeted pharmacologically to inhibit tumor cell growth." Oncogene **24**(40): 6080-9.

Andjelkovic, M., D. R. Alessi, et al. (1997). "Role of translocation in the activation and function of protein kinase B." J Biol Chem **272**(50): 31515-24.

Andrews, P. M. and K. R. Porter (1973). "The ultrastructural morphology and possible functional significance of mesothelial microvilli." Anat Rec **177**(3): 409-26.

Arora, A. and E. M. Scholar (2005). "Role of tyrosine kinase inhibitors in cancer therapy." J Pharmacol Exp Ther **315**(3): 971-9.

Asnaghi, L., P. Bruno, et al. (2004). "mTOR: a protein kinase switching between life and death." Pharmacol Res **50**(6): 545-9.

Ault, J. G., R. W. Cole, et al. (1995). "Behavior of crocidolite asbestos during mitosis in living vertebrate lung epithelial cells." Cancer Res **55**(4): 792-8.

Avantaggiati, M. L., M. Carbone, et al. (1996). "The SV40 large T antigen and adenovirus E1a oncoproteins interact with distinct isoforms of the transcriptional co-activator, p300." Embo J **15**(9): 2236-48.

Averous, J. and C. G. Proud (2006). "When translation meets transformation: the mTOR story." Oncogene **25**(48): 6423-35.

Bajorin, D., D. Kelsen, et al. (1987). "Phase II trial of mitomycin in malignant mesothelioma." Cancer Treat Rep **71**(9): 857-8.

Baker, S. J. (2007). "PTEN enters the nuclear age." Cell **128**(1): 25-8.

Baldini, E. H. (2004). "External beam radiation therapy for the treatment of pleural mesothelioma." Thorac Surg Clin **14**(4): 543-8.

Baldwin, A. S. (2001). "Control of oncogenesis and cancer therapy resistance by the transcription factor NF-kappaB." J Clin Invest **107**(3): 241-6.

Baldwin, A. S., Jr. (1996). "The NF-kappa B and I kappa B proteins: new discoveries and insights." Annu Rev Immunol **14**: 649-83.

Baranowska-Kortylewicz, J., M. Abe, et al. (2005). "Effect of platelet-derived growth factor receptor-beta inhibition with STI571 on radioimmunotherapy." Cancer Res **65**(17): 7824-31.

Baris, I., L. Simonato, et al. (1987). "Epidemiological and environmental evidence of the health effects of exposure to erionite fibres: a four-year study in the Cappadocian region of Turkey." Int J Cancer **39**(1): 10-7.

Baris, Y. I. (1991). "Fibrous zeolite (erionite)-related diseases in Turkey." Am J Ind Med **19**(3): 374-8.

Baris, Y. I., R. Saracci, et al. (1981). "Malignant mesothelioma and radiological chest abnormalities in two villages in Central Turkey. An epidemiological and environmental investigation." Lancet **1**(8227): 984-7.

Bates, R. C., N. S. Edwards, et al. (2000). "Spheroids and cell survival." Crit Rev Oncol Hematol **36**(2-3): 61-74.

Batra, S., Y. Shi, et al. (2006). "Wnt inhibitory factor-1, a Wnt antagonist, is silenced by promoter hypermethylation in malignant pleural mesothelioma." Biochem Biophys Res Commun **342**(4): 1228-32.

Beppu, K., J. Jaboine, et al. (2004). "Effect of imatinib mesylate on neuroblastoma tumorigenesis and vascular endothelial growth factor expression." J Natl Cancer Inst **96**(1): 46-55.

Bertino, P., A. Marconi, et al. (2007). "Erionite and asbestos differently cause transformation of human mesothelial cells." Int J Cancer.

Blume-Jensen, P. and T. Hunter (2001). "Oncogenic kinase signalling." Nature **411**(6835): 355-65.

Blumen, S. R., K. Cheng, et al. (2007). "Unique uptake of Acid-prepared mesoporous spheres by lung epithelial and mesothelioma cells." Am J Respir Cell Mol Biol **36**(3): 333-42.

Borczuk, A. C., G. C. Cappellini, et al. (2007). "Molecular profiling of malignant peritoneal mesothelioma identifies the ubiquitin-proteasome pathway as a therapeutic target in poor prognosis tumors." Oncogene **26**(4): 610-7.

Braumann, C., W. Henke, et al. (2004). "The tumor-suppressive reagent taurolidine is an inhibitor of protein biosynthesis." Int J Cancer **112**(2): 225-30.

Broaddus V.C., Y. L., Scavo L.M., Ernst J.D. & Boylan A.M. (1996). "Asbestos induces apoptosis of human and rabbit pleural mesothelial cells via reactive oxygen species." J. Clin. Invest. **98**: 2050-2059.

Broaddus, V. C., T. B. Dansen, et al. (2005). "Bid mediates apoptotic synergy between tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and DNA damage." J Biol Chem **280**(13): 12486-93.

Brognard, J., E. Sierecki, et al. (2007). "PHLPP and a second isoform, PHLPP2, differentially attenuate the amplitude of Akt signaling by regulating distinct Akt isoforms." Mol Cell **25**(6): 917-31.

Browne, M. K. (1981). "The treatment of peritonitis by an antiseptic - taurolin." Pharmatherapeutica **2**(8): 517-22.

Browne, M. K., G. B. Leslie, et al. (1977). "The in vitro and in vivo activity of taurolin against anaerobic pathogenic organisms." Surg Gynecol Obstet **145**(6): 842-6.

Buchdunger, E., C. L. Cioffi, et al. (2000). "Abl protein-tyrosine kinase inhibitor STI571 inhibits in vitro signal transduction mediated by c-kit and platelet-derived growth factor receptors." J Pharmacol Exp Ther **295**(1): 139-45.

Bueno, R., K. Appasani, et al. (2001). "The alpha folate receptor is highly activated in malignant pleural mesothelioma." J Thorac Cardiovasc Surg **121**(2): 225-33.

Burri, C. (1990). "[Treatment of local infection with taurolin]." Aktuelle Probl Chir Orthop **34**: 78-84.

Byrne, M. J. and A. K. Nowak (2004). "Modified RECIST criteria for assessment of response in malignant pleural mesothelioma." Ann Oncol **15**(2): 257-60.

Cacciotti, P., D. Barbone, et al. (2005). "SV40-dependent AKT activity drives mesothelial cell transformation after asbestos exposure." Cancer Res **65**(12): 5256-62.

Cacciotti, P., R. Libener, et al. (2001). "SV40 replication in human mesothelial cells induces HGF/Met receptor activation: a model for viral-related carcinogenesis of human malignant mesothelioma." Proc Natl Acad Sci U S A **98**(21): 12032-7.

Cacciotti, P., L. Strizzi, et al. (2002). "The presence of simian-virus 40 sequences in mesothelioma and mesothelial cells is associated with high levels of vascular endothelial growth factor." Am J Respir Cell Mol Biol **26**(2): 189-93.

Calabresi, P., F. A. Goulette, et al. (2001). "Taurolidine: cytotoxic and mechanistic evaluation of a novel antineoplastic agent." Cancer Res **61**(18): 6816-21.

Cao, X., C. Rodarte, et al. (2007). "Bcl2/bcl-x(L) Inhibitor Engenders Apoptosis and Increases Chemosensitivity in Mesothelioma." Cancer Biol Ther **6**(2).

Cao, X. X., I. Mohuiddin, et al. (2001). "Histone deacetylase inhibitor downregulation of bcl-xl gene expression leads to apoptotic cell death in mesothelioma." Am J Respir Cell Mol Biol **25**(5): 562-8.

Carbone, M., H. I. Pass, et al. (1994). "Simian virus 40-like DNA sequences in human pleural mesothelioma." Oncogene **9**(6): 1781-90.

Carbone, M., P. Rizzo, et al. (1997). "Simian virus-40 large-T antigen binds p53 in human mesotheliomas." Nat Med **3**(8): 908-12.

Carbone, M., P. Rizzo, et al. (1997). "Simian virus 40, poliovaccines and human tumors: a review of recent developments." Oncogene **15**(16): 1877-88.

Castagneto, B., S. Zai, et al. (2001). "Palliative and therapeutic activity of IL-2 immunotherapy in unresectable malignant pleural mesothelioma with pleural effusion: Results of a phase II study on 31 consecutive patients." Lung Cancer **31**(2-3): 303-10.

Cepeda, V., M. A. Fuertes, et al. (2007). "Biochemical mechanisms of cisplatin cytotoxicity." Anticancer Agents Med Chem **7**(1): 3-18.

Chawla-Sarkar, M., S. I. Bae, et al. (2004). "Downregulation of Bcl-2, FLIP or IAPs (XIAP and survivin) by siRNAs sensitizes resistant melanoma cells to Apo2L/TRAIL-induced apoptosis." Cell Death Differ **11**(8): 915-23.

Chen, J., B. L. Martin, et al. (1992). "Regulation of protein serine-threonine phosphatase type-2A by tyrosine phosphorylation." Science **257**(5074): 1261-4.

Cheng, J. Q., W. C. Lee, et al. (1999). "Frequent mutations of NF2 and allelic loss from chromosome band 22q12 in malignant mesothelioma: evidence for a two-hit mechanism of NF2 inactivation." Genes Chromosomes Cancer **24**(3): 238-42.

Chilosi, M., F. Facchettti, et al. (1997). "bcl-2 expression in pleural and extrapleural solitary fibrous tumours." J Pathol **181**(4): 362-7.

Cho, S., Y. Tian, et al. (2001). "Binding of p300/CBP co-activators by polyoma large T antigen." J Biol Chem **276**(36): 33533-9.

Ciardiello, F., R. Caputo, et al. (2000). "Antitumor effect and potentiation of cytotoxic drugs activity in human cancer cells by ZD-1839 (Iressa), an epidermal growth factor receptor-selective tyrosine kinase inhibitor." Clin Cancer Res **6**(5): 2053-63.

Cole, G. W., Jr., A. M. Alleva, et al. (2006). "Suppression of pro-metastasis phenotypes expression in malignant pleural mesothelioma by the PI3K inhibitor LY294002 or the MEK inhibitor UO126." Anticancer Res **26**(2A): 809-21.

Collins, I. and P. Workman (2006). "New approaches to molecular cancer therapeutics." Nat Chem Biol **2**(12): 689-700.

Cooper, C. S., M. Park, et al. (1984). "Molecular cloning of a new transforming gene from a chemically transformed human cell line." Nature **311**(5981): 29-33.

Cortese, J. F., A. L. Gowda, et al. (2006). "Common EGFR mutations conferring sensitivity to gefitinib in lung adenocarcinoma are not prevalent in human malignant mesothelioma." Int J Cancer **118**(2): 521-2.

Cutrone, R., J. Lednicky, et al. (2005). "Some oral poliovirus vaccines were contaminated with infectious SV40 after 1961." Cancer Res **65**(22): 10273-9.

Da Costa, M. L., H. P. Redmond, et al. (2001). "Taurolidine improves survival by abrogating the accelerated development and proliferation of solid tumors and development of organ metastases from circulating tumor cells released following surgery." J Surg Res **101**(2): 111-9.

Dai, J. and A. Churg (2001). "Relationship of fiber surface iron and active oxygen species to expression of procollagen, PDGF-A, and TGF-beta(1) in tracheal explants exposed to amosite asbestos." Am J Respir Cell Mol Biol **24**(4): 427-35.

Dai, Y., C. W. Bedrossian, et al. (2005). "The expression pattern of beta-catenin in mesothelial proliferative lesions and its diagnostic utilities." Diagn Cytopathol **33**(5): 320-4.

Dancey, J. E. (2006). "Therapeutic targets: MTOR and related pathways." Cancer Biol Ther **5**(9): 1065-73.

Davidson, J. A., A. W. Musk, et al. (1998). "Intralesional cytokine therapy in cancer: a pilot study of GM-CSF infusion in mesothelioma." J Immunother **21**(5): 389-98.

De Luca, A., A. Baldi, et al. (1997). "The retinoblastoma gene family pRb/p105, p107, pRb2/p130 and simian virus-40 large T-antigen in human mesotheliomas." Nat Med **3**(8): 913-6.

de Melo, M., M. W. Gerbase, et al. (2006). "Phosphorylated extracellular signal-regulated kinases are significantly increased in malignant mesothelioma." J Histochem Cytochem **54**(8): 855-61.

Degiovanni, D., B. Pesce, et al. (2004). "Asbestos in Italy." Int J Occup Environ Health **10**(2): 193-7.

Desoize, B. and J. Jardillier (2000). "Multicellular resistance: a paradigm for clinical resistance?" Crit Rev Oncol Hematol **36**(2-3): 193-207.

Destro, A., G. L. Ceresoli, et al. (2006). "EGFR overexpression in malignant pleural mesothelioma. An immunohistochemical and molecular study with clinico-pathological correlations." Lung Cancer **51**(2): 207-15.

Deveraux, Q. L. and J. C. Reed (1999). "IAP family proteins--suppressors of apoptosis." Genes Dev **13**(3): 239-52.

Deveraux, Q. L., N. Roy, et al. (1998). "IAPs block apoptotic events induced by caspase-8 and cytochrome c by direct inhibition of distinct caspases." Embo J **17**(8): 2215-23.

Dewar, A. L., A. C. Zannettino, et al. (2005). "Inhibition of c-fms by imatinib: expanding the spectrum of treatment." Cell Cycle **4**(7): 851-3.

Dianzani, I., L. Gibello, et al. (2006). "Polymorphisms in DNA repair genes as risk factors for asbestos-related malignant mesothelioma in a general population study." Mutat Res **599**(1-2): 124-34.

Dogan, A. U., Y. I. Baris, et al. (2006). "Genetic predisposition to fiber carcinogenesis causes a mesothelioma epidemic in Turkey." Cancer Res **66**(10): 5063-8.

Druker, B. J., C. L. Sawyers, et al. (2001). "Activity of a specific inhibitor of the BCR-ABL tyrosine kinase in the blast crisis of chronic myeloid leukemia and acute lymphoblastic leukemia with the Philadelphia chromosome." N Engl J Med **344**(14): 1038-42.

Edwards, J. G., D. E. Swinson, et al. (2006). "EGFR expression: associations with outcome and clinicopathological variables in malignant pleural mesothelioma." Lung Cancer **54**(3): 399-407.

el-Remessy, A. B., M. Bartoli, et al. (2005). "Oxidative stress inactivates VEGF survival signaling in retinal endothelial cells via PI 3-kinase tyrosine nitration." J Cell Sci **118**(Pt 1): 243-52.

Emri, S., T. Kocagoz, et al. (2000). "Simian virus 40 is not a cofactor in the pathogenesis of environmentally induced malignant pleural mesothelioma in Turkey." Anticancer Res **20**(2A): 891-4.

Erlichman, C. and D. Vidgen (1984). "Cytotoxicity of adriamycin in MGH-U1 cells grown as monolayer cultures, spheroids, and xenografts in immune-deprived mice." Cancer Res **44**(11): 5369-75.

Falleni, M., C. Pellegrini, et al. (2005). "Quantitative evaluation of the apoptosis regulating genes Survivin, Bcl-2 and Bax in inflammatory and malignant pleural lesions." Lung Cancer **48**(2): 211-6.

Fan, Q. W., Z. A. Knight, et al. (2006). "A dual PI3 kinase/mTOR inhibitor reveals emergent efficacy in glioma." Cancer Cell **9**(5): 341-9.

Feng, F. Y., S. Varambally, et al. (2006). "Role of epidermal growth factor receptor degradation in gemcitabine-mediated cytotoxicity." Oncogene.

Fennell, D. A. and R. M. Rudd (2004). "Defective core-apoptosis signalling in diffuse malignant pleural mesothelioma: opportunities for effective drug development." Lancet Oncol **5**(6): 354-62.

Ferrari, G., C. Y. Yan, et al. (1995). "N-acetylcysteine (D- and L-stereoisomers) prevents apoptotic death of neuronal cells." J Neurosci **15**(4): 2857-66.

Fitzpatrick, D. R., D. J. Peroni, et al. (1995). "The role of growth factors and cytokines in the tumorigenesis and immunobiology of malignant mesothelioma." Am J Respir Cell Mol Biol **12**(5): 455-60.

Fizazi, K., R. Caliandro, et al. (2000). "Combination raltitrexed (Tomudex(R))-oxaliplatin: a step forward in the struggle against mesothelioma? The Institut Gustave Roussy experience with chemotherapy and chemo-immunotherapy in mesothelioma." Eur J Cancer **36**(12): 1514-21.

Foddis, R., A. De Rienzo, et al. (2002). "SV40 infection induces telomerase activity in human mesothelial cells." Oncogene **21**(9): 1434-42.

Fong, T. A., L. K. Shawver, et al. (1999). "SU5416 is a potent and selective inhibitor of the vascular endothelial growth factor receptor (Flk-1/KDR) that inhibits tyrosine kinase catalysis, tumor vascularization, and growth of multiple tumor types." Cancer Res **59**(1): 99-106.

Fracasso, G. and M. Colombatti (2000). "Effect of therapeutic macromolecules in spheroids." Crit Rev Oncol Hematol **36**(2-3): 159-78.

Franke, T. F., C. P. Hornik, et al. (2003). "PI3K/Akt and apoptosis: size matters." Oncogene **22**(56): 8983-98.

Furge, K. A., Y. W. Zhang, et al. (2000). "Met receptor tyrosine kinase: enhanced signaling through adapter proteins." Oncogene **19**(49): 5582-9.

Galffy, G., K. A. Mohammed, et al. (1999). "Interleukin 8: an autocrine growth factor for malignant mesothelioma." Cancer Res **59**(2): 367-71.

Gao, N., M. Rahmani, et al. (2005). "2-Methoxyestradiol-induced apoptosis in human leukemia cells proceeds through a reactive oxygen species and Akt-dependent process." Oncogene **24**(23): 3797-809.

Gao, T., F. Furnari, et al. (2005). "PHLPP: a phosphatase that directly dephosphorylates Akt, promotes apoptosis, and suppresses tumor growth." Mol Cell **18**(1): 13-24.

Garcia, A., X. Cayla, et al. (2003). "Serine/threonine protein phosphatases PP1 and PP2A are key players in apoptosis." Biochimie **85**(8): 721-6.

George, D. (2001). "Platelet-derived growth factor receptors: a therapeutic target in solid tumors." Semin Oncol **28**(5 Suppl 17): 27-33.

Gericke, A., M. Munson, et al. (2006). "Regulation of the PTEN phosphatase." Gene **374**: 1-9.

Gerwin, B. I., J. F. Lechner, et al. (1987). "Comparison of production of transforming growth factor-beta and platelet-derived growth factor by normal human mesothelial cells and mesothelioma cell lines." Cancer Res **47**(23): 6180-4.

Ghosh, S., M. J. May, et al. (1998). "NF-kappa B and Rel proteins: evolutionarily conserved mediators of immune responses." Annu Rev Immunol **16**: 225-60.

Goldberg, J. L., C. L. Zanella, et al. (1997). "Novel cell imaging techniques show induction of apoptosis and proliferation in mesothelial cells by asbestos." Am J Respir Cell Mol Biol **17**(3): 265-71.

Gomez-Benito, M., P. Balsas, et al. (2007). "Mechanism of apoptosis induced by IFN-alpha in human myeloma cells: Role of Jak1 and Bim and potentiation by rapamycin." Cell Signal **19**(4): 844-54.

Gonzalez, I., E. J. Andreu, et al. (2004). "Imatinib inhibits proliferation of Ewing tumor cells mediated by the stem cell factor/KIT receptor pathway, and sensitizes cells to vincristine and doxorubicin-induced apoptosis." Clin Cancer Res **10**(2): 751-61.

Gordon, G., M. Mani, et al. (2007). "Inhibitor of apoptosis proteins are regulated by tumour necrosis factor-alpha in malignant pleural mesothelioma." J Pathol **211**(4): 439-446.

Gordon, G. J., K. Appasani, et al. (2002). "Inhibitor of apoptosis protein-1 promotes tumor cell survival in mesothelioma." Carcinogenesis **23**(6): 1017-24.

Gorman, S. P., D. F. McCafferty, et al. (1987). "Reduced adherence of micro-organisms to human mucosal epithelial cells following treatment with Taurolin, a novel antimicrobial agent." J Appl Bacteriol **62**(4): 315-20.

Gottlieb, K. A. and L. P. Villarreal (2001). "Natural biology of polyomavirus middle T antigen." Microbiol Mol Biol Rev **65**(2): 288-318 ; second and third pages, table of contents.

Govindan, R., R. A. Kratzke, et al. (2005). "Gefitinib in patients with malignant mesothelioma: a phase II study by the Cancer and Leukemia Group B." Clin Cancer Res **11**(6): 2300-4.

Green, D. R. (2005). "Apoptotic pathways: ten minutes to dead." Cell **121**(5): 671-4.

Gross, A., J. M. McDonnell, et al. (1999). "BCL-2 family members and the mitochondria in apoptosis." Genes Dev **13**(15): 1899-911.

Gross, A., X. M. Yin, et al. (1999). "Caspase cleaved BID targets mitochondria and is required for cytochrome c release, while BCL-XL prevents this release but not tumor necrosis factor-R1/Fas death." J Biol Chem **274**(2): 1156-63.

Guo, J., P. A. Marcotte, et al. (2006). "Inhibition of phosphorylation of the colony-stimulating factor-1 receptor (c-Fms) tyrosine kinase in transfected cells by ABT-869 and other tyrosine kinase inhibitors." Mol Cancer Ther **5**(4): 1007-13.

Gupta, S. (2001). "Molecular steps of death receptor and mitochondrial pathways of apoptosis." Life Sci **69**(25-26): 2957-64.

Hamilton, G. (1998). "Multicellular spheroids as an in vitro tumor model." Cancer Lett **131**(1): 29-34.

Han, Z., I. Ribbizi, et al. (2002). "The antibacterial drug taurolidine induces apoptosis by a mitochondrial cytochrome c-dependent mechanism." Anticancer Res **22**(4): 1959-64.

Hanahan, D. and J. Folkman (1996). "Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis." Cell **86**(3): 353-64.

Hanauske, A. R., V. Chen, et al. (2001). "Pemetrexed disodium: a novel antifolate clinically active against multiple solid tumors." Oncologist **6**(4): 363-73.

Hara, E., R. Smith, et al. (1996). "Regulation of p16CDKN2 expression and its implications for cell immortalization and senescence." Mol Cell Biol **16**(3): 859-67.

Hara, K., Y. Maruki, et al. (2002). "Raptor, a binding partner of target of rapamycin (TOR), mediates TOR action." Cell **110**(2): 177-89.

Harada, H., J. S. Andersen, et al. (2001). "p70S6 kinase signals cell survival as well as growth, inactivating the pro-apoptotic molecule BAD." Proc Natl Acad Sci U S A **98**(17): 9666-70.

Harvey, P., A. Warn, et al. (1996). "Immunoreactivity for hepatocyte growth factor/scatter factor and its receptor, met, in human lung carcinomas and malignant mesotheliomas." J Pathol **180**(4): 389-94.

Hay, N. (2005). "The Akt-mTOR tango and its relevance to cancer." Cancer Cell **8**(3): 179-83.

He, B., L. You, et al. (2004). "A monoclonal antibody against Wnt-1 induces apoptosis in human cancer cells." Neoplasia **6**(1): 7-14.

Heinrich, M. C., C. L. Corless, et al. (2003). "Kinase mutations and imatinib response in patients with metastatic gastrointestinal stromal tumor." J Clin Oncol **21**(23): 4342-9.

Herrick, S. E. and S. E. Mutsaers (2004). "Mesothelial progenitor cells and their potential in tissue engineering." Int J Biochem Cell Biol **36**(4): 621-42.

Hessel, P. A., J. F. Gamble, et al. (2005). "Asbestos, asbestosis, and lung cancer: a critical assessment of the epidemiological evidence." Thorax **60**(5): 433-6.

Hirao, T., R. Bueno, et al. (2002). "Alterations of the p16(INK4) locus in human malignant mesothelial tumors." Carcinogenesis **23**(7): 1127-30.

Holtfreter, J. (1944). "A study of the mechanism of gastrulation." J. Exp. Zool **95**: 171-212.

Hopkins-Donaldson, S., R. Cathomas, et al. (2003). "Induction of apoptosis and chemosensitization of mesothelioma cells by Bcl-2 and Bcl-xL antisense treatment." Int J Cancer **106**(2): 160-6.

Huang, S. and P. J. Houghton (2003). "Targeting mTOR signaling for cancer therapy." Curr Opin Pharmacol **3**(4): 371-7.

Inch, W. R., J. A. McCredie, et al. (1970). "Growth of nodular carcinomas in rodents compared with multi-cell spheroids in tissue culture." Growth **34**(3): 271-82.

Iordanov, M. S., O. P. Ryabinina, et al. (2000). "Molecular determinants of apoptosis induced by the cytotoxic ribonuclease onconase: evidence for cytotoxic mechanisms different from inhibition of protein synthesis." Cancer Res **60**(7): 1983-94.

Ishikawa, Y. and M. Kitamura (1999). "Dual potential of extracellular signal-regulated kinase for the control of cell survival." Biochem Biophys Res Commun **264**(3): 696-701.

Jacobi, C. A., C. Menenakos, et al. (2005). "Taurolidine--a new drug with anti-tumor and anti-angiogenic effects." Anticancer Drugs **16**(9): 917-21.

Jacobi, C. A., R. Sabat, et al. (1997). "[Peritoneal instillation of taurolidine and heparin for preventing intraperitoneal tumor growth and trocar metastases in

laparoscopic operations in the rat model]." Langenbecks Arch Chir **382**(4 Suppl 1): S31-6.

Jaeschke, A., J. Hartkamp, et al. (2002). "Tuberous sclerosis complex tumor suppressor-mediated S6 kinase inhibition by phosphatidylinositide-3-OH kinase is mTOR independent." J Cell Biol **159**(2): 217-24.

Jagadeeswaran, R., P. C. Ma, et al. (2006). "Functional analysis of c-Met/hepatocyte growth factor pathway in malignant pleural mesothelioma." Cancer Res **66**(1): 352-61.

Janne, P. A., M. L. Taffaro, et al. (2002). "Inhibition of epidermal growth factor receptor signaling in malignant pleural mesothelioma." Cancer Res **62**(18): 5242-7.

Janssens, V. and J. Goris (2001). "Protein phosphatase 2A: a highly regulated family of serine/threonine phosphatases implicated in cell growth and signalling." Biochem J **353**(Pt 3): 417-39.

Jeffers, M., M. Fiscella, et al. (1998). "The mutationally activated Met receptor mediates motility and metastasis." Proc Natl Acad Sci U S A **95**(24): 14417-22.

Jeffers, M., S. Koochekpour, et al. (1998). "Signaling requirements for oncogenic forms of the Met tyrosine kinase receptor." Oncogene **17**(21): 2691-700.

Jeffers, M., L. Schmidt, et al. (1997). "Activating mutations for the met tyrosine kinase receptor in human cancer." Proc Natl Acad Sci U S A **94**(21): 11445-50.

Jimenez, L. A., C. Zanella, et al. (1997). "Role of extracellular signal-regulated protein kinases in apoptosis by asbestos and H2O2." Am J Physiol **273**(5 Pt 1): L1029-35.

Jin, S., R. S. DiPaola, et al. (2007). "Metabolic catastrophe as a means to cancer cell death." J Cell Sci **120**(Pt 3): 379-83.

Jurewitsch, B. and K. N. Jeejeebhoy (2005). "Taurolidine lock: the key to prevention of recurrent catheter-related bloodstream infections." Clin Nutr **24**(3): 462-5.

Kamp, D. W., P. Graceffa, et al. (1992). "The role of free radicals in asbestos-induced diseases." Free Radic Biol Med **12**(4): 293-315.

Kamp, D. W., V. A. Israbian, et al. (1995). "Asbestos causes DNA strand breaks in cultured pulmonary epithelial cells: role of iron-catalyzed free radicals." Am J Physiol **268**(3 Pt 1): L471-80.

Kamp, D. W. and S. A. Weitzman (1999). "The molecular basis of asbestos induced lung injury." Thorax **54**(7): 638-52.

Karin, M. and A. Lin (2002). "NF-kappaB at the crossroads of life and death." Nat Immunol **3**(3): 221-7.

Khaleghpour, K., S. Pyronnet, et al. (1999). "Translational homeostasis: eukaryotic translation initiation factor 4E control of 4E-binding protein 1 and p70 S6 kinase activities." Mol Cell Biol **19**(6): 4302-10.

Kikani, C. K., L. Q. Dong, et al. (2005). ""New"-clear functions of PDK1: beyond a master kinase in the cytosol?" J Cell Biochem **96**(6): 1157-62.

Kim, K. U., S. M. Wilson, et al. (2005). "A Novel In Vitro Model of Human Mesothelioma for Studying Tumor Biology and Apoptotic Resistance." Am J Respir Cell Mol Biol.

Kim, R., M. Emi, et al. (2005). "Chemosensitization by STI571 targeting the platelet-derived growth factor/platelet-derived growth factor receptor-signaling pathway in the tumor progression and angiogenesis of gastric carcinoma." Cancer **103**(9): 1800-9.

Kindler, H. L. (2000). "Malignant pleural mesothelioma." Curr Treat Options Oncol **1**(4): 313-26.

Kindler, H. L. (2001). "A double-blind placebo-controlled randomized phase II trial of gemcitabine and cisplatin with or without the VEGF inhibitor bevacizumab in patients with malignant mesothelioma." Curr Clin Trials Thorac Oncol **4**(2).

Kindler, H. L. and J. P. van Meerbeeck (2002). "The role of gemcitabine in the treatment of malignant mesothelioma." Semin Oncol **29**(1): 70-6.

Kitamura, F., S. Araki, et al. (2002). "Assessment of the mutations of p53 suppressor gene and Ha- and Ki-ras oncogenes in malignant mesothelioma

in relation to asbestos exposure: a study of 12 American patients." Ind Health **40**(2): 175-81.

Klein, G., A. Powers, et al. (2002). "Association of SV40 with human tumors." Oncogene **21**(8): 1141-9.

Klominek, J., B. Baskin, et al. (1998). "Hepatocyte growth factor/scatter factor stimulates chemotaxis and growth of malignant mesothelioma cells through c-met receptor." Int J Cancer **76**(2): 240-9.

Knight, Z. A., B. Gonzalez, et al. (2006). "A pharmacological map of the PI3-K family defines a role for p110alpha in insulin signaling." Cell **125**(4): 733-47.

Konig, J., E. Tolnay, et al. (2000). "Co-expression of vascular endothelial growth factor and its receptor flt-1 in malignant pleural mesothelioma." Respiration **67**(1): 36-40.

Kosaka, T., Y. Yatabe, et al. (2006). "Analysis of epidermal growth factor receptor gene mutation in patients with non-small cell lung cancer and acquired resistance to gefitinib." Clin Cancer Res **12**(19): 5764-9.

Krystal, G. W., S. Honsawek, et al. (2000). "The selective tyrosine kinase inhibitor STI571 inhibits small cell lung cancer growth." Clin Cancer Res **6**(8): 3319-26.

Kucharczak, J., M. J. Simmons, et al. (2003). "To be, or not to be: NF-kappaB is the answer--role of Rel/NF-kappaB in the regulation of apoptosis." Oncogene **22**(56): 8961-82.

Langerak, A. W., P. A. De Laat, et al. (1996). "Expression of platelet-derived growth factor (PDGF) and PDGF receptors in human malignant mesothelioma in vitro and in vivo." J Pathol **178**(2): 151-60.

LeBlanc, H. N. and A. Ashkenazi (2003). "Apo2L/TRAIL and its death and decoy receptors." Cell Death Differ **10**(1): 66-75.

Lee, J. H., S. U. Han, et al. (2000). "A novel germ line juxtamembrane Met mutation in human gastric cancer." Oncogene **19**(43): 4947-53.

Lee, W. C. and J. R. Testa (1999). "Somatic genetic alterations in human malignant mesothelioma (review)." Int J Oncol **14**(1): 181-8.

Leigh, J. and T. Driscoll (2003). "Malignant mesothelioma in Australia, 1945-2002." Int J Occup Environ Health **9**(3): 206-17.

Leithauser, M. L., P. M. Rob, et al. (1997). "Pentoxifylline, cyclosporine A and taurolidine inhibit endotoxin-stimulated tumor necrosis factor-alpha production in rat mesangial cell cultures." Exp Nephrol **5**(1): 100-4.

Leland, P. A. and R. T. Raines (2001). "Cancer chemotherapy--ribonucleases to the rescue." Chem Biol **8**(5): 405-13.

Lerner, H. J., D. A. Schoenfeld, et al. (1983). "Malignant mesothelioma. The Eastern Cooperative Oncology Group (ECOG) experience." Cancer **52**(11): 1981-5.

Leung, D. W., G. Cachianes, et al. (1989). "Vascular endothelial growth factor is a secreted angiogenic mitogen." Science **246**(4935): 1306-9.

Levresse, V., A. Renier, et al. (2000). "DNA breakage in asbestos-treated normal and transformed (TSV40) rat pleural mesothelial cells." Mutagenesis **15**(3): 239-44.

Light, W. G. and E. T. Wei (1977). "Surface charge and asbestos toxicity." Nature **265**(5594): 537-9.

Lim, Y., S. H. Kim, et al. (1997). "Involvement of protein kinase C, phospholipase C, and protein tyrosine kinase pathways in oxygen radical generation by asbestos-stimulated alveolar macrophage." Environ Health Perspect **105 Suppl 5**: 1325-7.

Lin, A. and M. Karin (2003). "NF-kappaB in cancer: a marked target." Semin Cancer Biol **13**(2): 107-14.

Lin, J. Y. and J. A. DeCaprio (2003). "SV40 large T antigen promotes dephosphorylation of p130." J Biol Chem **278**(47): 46482-7.

Linder, C., S. Linder, et al. (1998). "Independent expression of serum vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) in patients with carcinoma and sarcoma." Anticancer Res **18**(3B): 2063-8.

Liu, H., D. C. Radisky, et al. (2006). "Mechanism of Akt1 inhibition of breast cancer cell invasion reveals a protumorigenic role for TSC2." Proc Natl Acad Sci U S A **103**(11): 4134-9.

Liu, W., E. Bodle, et al. (2001). "Tumor necrosis factor-related apoptosis-inducing ligand and chemotherapy cooperate to induce apoptosis in mesothelioma cell lines." Am J Respir Cell Mol Biol **25**(1): 111-8.

Liu, W., Ernst, J.D. & Broaddus, V. (2000). "Phagocytosis of Crocidolite asbestos induces oxidative stress, DNA damage, and apoptosis in mesothelial cells." Am. J. Respir. Cell. Mol. Biol. **23**: 371-378.

Liu, Z. and J. Klominek (2003). "Regulation of matrix metalloprotease activity in malignant mesothelioma cell lines by growth factors." Thorax **58**(3): 198-203.

Longati, P., P. M. Comoglio, et al. (2001). "Receptor tyrosine kinases as therapeutic targets: the model of the MET oncogene." Curr Drug Targets **2**(1): 41-55.

Lopez-Rios, F., P. B. Illei, et al. (2004). "Evidence against a role for SV40 infection in human mesotheliomas and high risk of false-positive PCR results owing to presence of SV40 sequences in common laboratory plasmids." Lancet **364**(9440): 1157-66.

Lu, C., R. Perez-Soler, et al. (2005). "Phase II study of a liposome-entrapped cisplatin analog (L-NDDP) administered intrapleurally and pathologic response rates in patients with malignant pleural mesothelioma." J Clin Oncol **23**(15): 3495-501.

Lumb, P. D. and S. K. Suvarna (2004). "Metastasis in pleural mesothelioma. Immunohistochemical markers for disseminated disease." Histopathology **44**(4): 345-52.

Lynch, T. J., D. W. Bell, et al. (2004). "Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib." N Engl J Med **350**(21): 2129-39.

Mamane, Y., E. Petroulakis, et al. (2006). "mTOR, translation initiation and cancer." Oncogene **25**(48): 6416-22.

Mansbridge, J. N., W. A. Ausserer, et al. (1994). "Adaptation of EGF receptor signal transduction to three-dimensional culture conditions: changes in

surface receptor expression and protein tyrosine phosphorylation." J Cell Physiol **161**(2): 374-82.

Masood, R., A. Kundra, et al. (2003). "Malignant mesothelioma growth inhibition by agents that target the VEGF and VEGF-C autocrine loops." Int J Cancer **104**(5): 603-10.

Mathy, A., P. Baas, et al. (2005). "Limited efficacy of imatinib mesylate in malignant mesothelioma: a phase II trial." Lung Cancer **50**(1): 83-6.

Mayall, F. G., G. Jacobson, et al. (1999). "Mutations of p53 gene and SV40 sequences in asbestos associated and non-asbestos-associated mesotheliomas." J Clin Pathol **52**(4): 291-3.

Mazieres, J., L. You, et al. (2005). "Wnt2 as a new therapeutic target in malignant pleural mesothelioma." Int J Cancer **117**(2): 326-32.

McCourt, M., J. H. Wang, et al. (2000). "Taurolidine inhibits tumor cell growth in vitro and in vivo." Ann Surg Oncol **7**(9): 685-91.

McCubrey, J. A., M. M. Lahair, et al. (2006). "Reactive oxygen species-induced activation of the MAP kinase signaling pathways." Antioxid Redox Signal **8**(9-10): 1775-89.

Mendoza, M. C. and J. Blenis (2007). "PHLPPing it off: phosphatases get in the Akt." Mol Cell **25**(6): 798-800.

Mikulski, S. M., J. J. Costanzi, et al. (2002). "Phase II trial of a single weekly intravenous dose of ranpirnase in patients with unresectable malignant mesothelioma." J Clin Oncol **20**(1): 274-81.

Millward, T. A., S. Zolnierowicz, et al. (1999). "Regulation of protein kinase cascades by protein phosphatase 2A." Trends Biochem Sci **24**(5): 186-91.

Mohiuddin, I., X. Cao, et al. (2002). "Phosphatase and tensin analog gene overexpression engenders cellular death in human malignant mesothelioma cells via inhibition of AKT phosphorylation." Ann Surg Oncol **9**(3): 310-6.

Monson, J. R., P. S. Ramsey, et al. (1993). "Taurolidine inhibits tumour necrosis factor (TNF) toxicity--new evidence of TNF and endotoxin synergy." Eur J Surg Oncol **19**(3): 226-31.

Morinaga, K., T. Kishimoto, et al. (2001). "Asbestos-related lung cancer and mesothelioma in Japan." Ind Health **39**(2): 65-74.

Moscona, A. (1952). "Cell suspensions from organ rudiments of chick embryos." Exp. Cell Res. **3**: 535-539.

Moscona, A. (1957). "The development in vitro of chimeric aggregates of dissociated embryonic chick and mouse cells." Proc. Natl. Acad. Sci. USA **43**: 184-194.

Moscona, A. (1957). "Formation of lentoids by dissociated retinal cells of the chick embryo." Science **125**: 598-599.

Moscona, A. (1961). "Rotation-mediated histogenetic aggregation of dissociated cells." Exp. Cell Res. **22**: 455-475.

Mosmann, T. (1983). "Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays." J Immunol Methods **65**(1-2): 55-63.

Mossman, B. T., J. Bignon, et al. (1990). "Asbestos: scientific developments and implications for public policy." Science **247**(4940): 294-301.

Mossman, B. T. and A. Churg (1998). "Mechanisms in the pathogenesis of asbestosis and silicosis." Am J Respir Crit Care Med **157**(5 Pt 1): 1666-80.

Mossman, B. T., Kamp, D.W. & Weitzman, S.A. (1996). "Mechanisms of carcinogenesis and clinical features of asbestos-associated cancers." Cancer. Invest. **14**: 466-480.

Mueller-Klieser, W. (1997). "Three-dimensional cell cultures: from molecular mechanisms to clinical applications." Am J Physiol **273**(4 Pt 1): C1109-23.

Mukohara, T., G. Civiello, et al. (2005). "Inhibition of the met receptor in mesothelioma." Clin Cancer Res **11**(22): 8122-30.

Mukohara, T., G. Civiello, et al. (2005). "Therapeutic targeting of multiple signaling pathways in malignant pleural mesothelioma." Oncology **68**(4-6): 500-10.

Murthy, S. S. and J. R. Testa (1999). "Asbestos, chromosomal deletions, and tumor suppressor gene alterations in human malignant mesothelioma." J Cell Physiol **180**(2): 150-7.

Musk, A. W. (1995). "More cases of miliary mesothelioma." Chest **108**(2): 587.

Mutsaers, S. E. (2004). "The mesothelial cell." Int J Biochem Cell Biol **36**(1): 9-16.

Mutsaers, S. E., J. E. Bishop, et al. (1997). "Mechanisms of tissue repair: from wound healing to fibrosis." Int J Biochem Cell Biol **29**(1): 5-17.

Narasimhan, S. R., L. Yang, et al. (1998). "Resistance of pleural mesothelioma cell lines to apoptosis: relation to expression of Bcl-2 and Bax." Am J Physiol **275**(1 Pt 1): L165-71.

Nelson, D. J., B. W. Robinson, et al. (2005). "Gene therapy of mesothelioma." Expert Opin Biol Ther **5**(8): 1039-49.

Neragi-Miandoab, S. (2006). "Multimodality approach in management of malignant pleural mesothelioma." Eur J Cardiothorac Surg **29**(1): 14-9.

Nici, L., B. Monfils, et al. (2004). "The effects of taurolidine, a novel antineoplastic agent, on human malignant mesothelioma." Clin Cancer Res **10**(22): 7655-61.

Niklinski, J., W. Niklinska, et al. (2004). "The epidemiology of asbestos-related diseases." Lung Cancer **45 Suppl 1**: S7-S15.

Nolan, R. P., M. Ross, et al. (2005). "Risk assessment for asbestos-related cancer from the 9/11 attack on the World Trade Center." J Occup Environ Med **47**(8): 817-25.

Nowak, A. K., M. J. Byrne, et al. (2002). "A multicentre phase II study of cisplatin and gemcitabine for malignant mesothelioma." Br J Cancer **87**(5): 491-6.

Nowak, A. K., R. A. Lake, et al. (2002). "New approaches for mesothelioma: biologics, vaccines, gene therapy, and other novel agents." Semin Oncol **29**(1): 82-96.

O'Kane, S. L., R. J. Pound, et al. (2006). "Expression of bcl-2 family members in malignant pleural mesothelioma." Acta Oncol **45**(4): 449-53.

Ohta, Y., V. Shridhar, et al. (1999). "VEGF and VEGF type C play an important role in angiogenesis and lymphangiogenesis in human malignant mesothelioma tumours." Br J Cancer **81**(1): 54-61.

Oloumi, A., S. H. MacPhail, et al. (2000). "Changes in subcellular distribution of topoisomerase IIalpha correlate with etoposide resistance in multicell spheroids and xenograft tumors." Cancer Res **60**(20): 5747-53.

Osaki, M., M. Oshimura, et al. (2004). "PI3K-Akt pathway: its functions and alterations in human cancer." Apoptosis **9**(6): 667-76.

Otterson, G. A., J. E. Herndon, 2nd, et al. (2004). "Capecitabine in malignant mesothelioma: a phase II trial by the Cancer and Leukemia Group B (39807)." Lung Cancer **44**(2): 251-9.

Pache, J. C., Y. M. Janssen, et al. (1998). "Increased epidermal growth factor-receptor protein in a human mesothelial cell line in response to long asbestos fibers." Am J Pathol **152**(2): 333-40.

Panner, A., C. D. James, et al. (2005). "mTOR controls FLIPS translation and TRAIL sensitivity in glioblastoma multiforme cells." Mol Cell Biol **25**(20): 8809-23.

Papp, T., H. Schipper, et al. (2001). "Mutational analysis of N-ras, p53, p16INK4a, p14ARF and CDK4 genes in primary human malignant mesotheliomas." Int J Oncol **18**(2): 425-33.

Pardanani, A. and A. Tefferi (2004). "Imatinib targets other than bcr/abl and their clinical relevance in myeloid disorders." Blood **104**(7): 1931-9.

Pass, H. I. (2002). "Photodynamic therapy in thoracic surgery." Ann Thorac Surg **73**(6): 2012-3.

Pass, H. I. and J. S. Donington (1995). "Use of photodynamic therapy for the management of pleural malignancies." Semin Surg Oncol **11**(5): 360-7.

Pass, H. I., E. J. Stevens, et al. (1995). "Characteristics of nine newly derived mesothelioma cell lines." Ann Thorac Surg **59**(4): 835-44.

Pawson, T. and P. Nash (2000). "Protein-protein interactions define specificity in signal transduction." Genes Dev **14**(9): 1027-47.

Pelin, K., A. Hirvonen, et al. (1994). "Expression of cell adhesion molecules and connexins in gap junctional intercellular communication deficient human mesothelioma tumour cell lines and communication competent primary mesothelial cells." Carcinogenesis **15**(11): 2673-5.

Perderiset, M., J. P. Marsh, et al. (1991). "Activation of protein kinase C by crocidolite asbestos in hamster tracheal epithelial cells." Carcinogenesis **12**(8): 1499-502.

Pespeni, M. H., M. Hodnett, et al. (2007). "Sensitization of Mesothelioma Cells to Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand-Induced Apoptosis by Heat Stress via the Inhibition of the 3-Phosphoinositide-Dependent Kinase 1/Akt Pathway." Cancer Res **67**(6): 2865-71.

Pietras, K., M. Stumm, et al. (2003). "STI571 enhances the therapeutic index of epothilone B by a tumor-selective increase of drug uptake." Clin Cancer Res **9**(10 Pt 1): 3779-87.

Planting, A. S., J. H. Schellens, et al. (1994). "Weekly high-dose cisplatin in malignant pleural mesothelioma." Ann Oncol **5**(4): 373-4.

Platanias, L. C. (2003). "Map kinase signaling pathways and hematologic malignancies." Blood **101**(12): 4667-79.

Poole, A., R. C. Brown, et al. (1983). "In vitro genotoxic activities of fibrous erionite." Br J Cancer **47**(5): 697-705.

Porcu, P., A. Ferber, et al. (1992). "The growth-stimulatory effect of simian virus 40 T antigen requires the interaction of insulinlike growth factor 1 with its receptor." Mol Cell Biol **12**(11): 5069-77.

Porta, C., L. Mutti, et al. (2007). "Negative results of an Italian Group for Mesothelioma (G.I.Me.) pilot study of single-agent imatinib mesylate in malignant pleural mesothelioma." Cancer Chemother Pharmacol **59**(1): 149-50.

Pott, F., U. Ziem, et al. (1987). "Carcinogenicity studies on fibres, metal compounds, and some other dusts in rats." Exp Pathol **32**(3): 129-52.

Potter, C. J., L. G. Pedraza, et al. (2002). "Akt regulates growth by directly phosphorylating Tsc2." Nat Cell Biol **4**(9): 658-65.

Poulikakos, P. I., G. H. Xiao, et al. (2006). "Re-expression of the tumor suppressor NF2/merlin inhibits invasiveness in mesothelioma cells and negatively regulates FAK." Oncogene **25**(44): 5960-8.

Powell, A., J. Creaney, et al. (2006). "Recombinant GM-CSF plus autologous tumor cells as a vaccine for patients with mesothelioma." Lung Cancer **52**(2): 189-97.

Raghavan, D., P. Gianoutsos, et al. (1990). "Phase II trial of carboplatin in the management of malignant mesothelioma." J Clin Oncol **8**(1): 151-4.

Ramos-Nino, M. E., C. R. Timblin, et al. (2002). "Mesothelial cell transformation requires increased AP-1 binding activity and ERK-dependent Fra-1 expression." Cancer Res **62**(21): 6065-9.

Ramos-Nino, M. E., G. Vianale, et al. (2005). "Human mesothelioma cells exhibit tumor cell-specific differences in phosphatidylinositol 3-kinase/AKT activity that predict the efficacy of Onconase." Mol Cancer Ther **4**(5): 835-42.

Rascoe, P. A., X. Cao, et al. (2005). "Receptor tyrosine kinase and phosphoinositide-3 kinase signaling in malignant mesothelioma." J Thorac Cardiovasc Surg **130**(2): 393-400.

Ribizzi, I., J. W. Darnowski, et al. (2002). "Taurolidine: preclinical evaluation of a novel, highly selective, agent for bone marrow purging." Bone Marrow Transplant **29**(4): 313-9.

Rinderknecht, E. and R. E. Humbel (1976). "Amino-terminal sequences of two polypeptides from human serum with nonsuppressible insulin-like and cell-growth-promoting activities: evidence for structural homology with insulin B chain." Proc Natl Acad Sci U S A **73**(12): 4379-81.

Robinson, B. W., J. Creaney, et al. (2003). "Mesothelin-family proteins and diagnosis of mesothelioma." Lancet **362**(9396): 1612-6.

Robinson, C., M. Callow, et al. (2000). "Serologic responses in patients with malignant mesothelioma: evidence for both public and private specificities." Am J Respir Cell Mol Biol **22**(5): 550-6.

Robledo, R. and B. Mossman (1999). "Cellular and molecular mechanisms of asbestos-induced fibrosis." J Cell Physiol **180**(2): 158-66.

Rodak, R., H. Kubota, et al. (2005). "Induction of reactive oxygen intermediates-dependent programmed cell death in human malignant ex vivo glioma cells and inhibition of the vascular endothelial growth factor production by taurolidine." J Neurosurg **102**(6): 1055-68.

Rosenberg, B. (1985). "Fundamental studies with cisplatin." Cancer **55**(10): 2303-l6.

Rundell, K. and R. Parakati (2001). "The role of the SV40 ST antigen in cell growth promotion and transformation." Semin Cancer Biol **11**(1): 5-13.

Ruvolo, P. P. (2001). "Ceramide regulates cellular homeostasis via diverse stress signaling pathways." Leukemia **15**(8): 1153-60.

Saenz-Robles, M. T., C. S. Sullivan, et al. (2001). "Transforming functions of Simian Virus 40." Oncogene **20**(54): 7899-907.

Santangelo, F. (2003). "Intracellular thiol concentration modulating inflammatory response: influence on the regulation of cell functions through cysteine prodrug approach." Curr Med Chem **10**(23): 2599-610.

Sarbassov, D. D., D. A. Guertin, et al. (2005). "Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex." Science **307**(5712): 1098-101.

Sato, S., N. Fujita, et al. (2000). "Modulation of Akt kinase activity by binding to Hsp90." Proc Natl Acad Sci U S A **97**(20): 10832-7.

Sattler, M., Y. B. Pride, et al. (2003). "A novel small molecule met inhibitor induces apoptosis in cells transformed by the oncogenic TPR-MET tyrosine kinase." Cancer Res **63**(17): 5462-9.

Sawyers, C. (2004). "Targeted cancer therapy." Nature **432**(7015): 294-7.

Scheper, G. C. and C. G. Proud (2002). "Does phosphorylation of the cap-binding protein eIF4E play a role in translation initiation?" Eur J Biochem **269**(22): 5350-9.

Schnadig, I. D. and C. D. Blanke (2006). "Gastrointestinal stromal tumors: imatinib and beyond." Curr Treat Options Oncol **7**(6): 427-37.

Sebastien, P., X. Janson, et al. (1980). "Asbestos retention in human respiratory tissues: comparative measurements in lung parenchyma and in parietal pleura." IARC Sci Publ(30): 237-46.

Segers, K., M. Ramael, et al. (1994). "Immunoreactivity for bcl-2 protein in malignant mesothelioma and non-neoplastic mesothelium." Virchows Arch **424**(6): 631-4.

Selcuk, Z. T., L. Coplu, et al. (1992). "Malignant pleural mesothelioma due to environmental mineral fiber exposure in Turkey. Analysis of 135 cases." Chest **102**(3): 790-6.

Shah, K. V. (2007). "SV40 and human cancer: a review of recent data." Int J Cancer **120**(2): 215-23.

Shrayer, D. P., H. Lukoff, et al. (2003). "The effect of Taurolidine on adherent and floating subpopulations of melanoma cells." Anticancer Drugs **14**(4): 295-303.

Silverman, N. and T. Maniatis (2001). "NF-kappaB signaling pathways in mammalian and insect innate immunity." Genes Dev **15**(18): 2321-42.

Simpson, L. and R. Parsons (2001). "PTEN: life as a tumor suppressor." Exp Cell Res **264**(1): 29-41.

Smalley, K. S., N. K. Haass, et al. (2006). "Multiple signaling pathways must be targeted to overcome drug resistance in cell lines derived from melanoma metastases." Mol Cancer Ther **5**(5): 1136-44.

Sohn, D., G. Totzke, et al. (2006). "The proteasome is required for rapid initiation of death receptor-induced apoptosis." Mol Cell Biol **26**(5): 1967-78.

Soini, Y., V. Kinnula, et al. (1999). "Apoptosis and expression of apoptosis regulating proteins bcl-2, mcl-1, bcl-X, and bax in malignant mesothelioma." Clin Cancer Res **5**(11): 3508-15.

Sonnemann, J., J. Gange, et al. (2005). "Histone deacetylase inhibitors interact synergistically with tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) to induce apoptosis in carcinoma cell lines." Invest New Drugs **23**(2): 99-109.

Sontag, E., J. M. Sontag, et al. (1997). "Protein phosphatase 2A is a critical regulator of protein kinase C zeta signaling targeted by SV40 small t to promote cell growth and NF-kappaB activation." Embo J **16**(18): 5662-71.

Sorensen, P. G., F. Bach, et al. (1985). "Randomized trial of doxorubicin versus cyclophosphamide in diffuse malignant pleural mesothelioma." Cancer Treat Rep **69**(12): 1431-2.

St Croix, B. and R. S. Kerbel (1997). "Cell adhesion and drug resistance in cancer." Curr Opin Oncol **9**(6): 549-56.

Stapelberg, M., N. Gellert, et al. (2005). "Alpha-tocopheryl succinate inhibits malignant mesothelioma by disrupting the fibroblast growth factor autocrine

loop: mechanism and the role of oxidative stress." J Biol Chem **280**(27): 25369-76.

Steeghs, N., J. W. Nortier, et al. (2007). "Small molecule tyrosine kinase inhibitors in the treatment of solid tumors: an update of recent developments." Ann Surg Oncol **14**(2): 942-53.

Steele, J. P. and A. Klabatsa (2005). "Chemotherapy options and new advances in malignant pleural mesothelioma." Ann Oncol **16**(3): 345-51.

Stella, M. C. and P. M. Comoglio (1999). "HGF: a multifunctional growth factor controlling cell scattering." Int J Biochem Cell Biol **31**(12): 1357-62.

Stendel, R., L. Scheurer, et al. (2003). "Enhancement of Fas-ligand-mediated programmed cell death by taurolidine." Anticancer Res **23**(3B): 2309-14.

Stendel, R., G. Stoltenburg-Didinger, et al. (2002). "The effect of taurolidine on brain tumor cells." Anticancer Res **22**(2A): 809-14.

Strizzi, L., A. Catalano, et al. (2001). "Vascular endothelial growth factor is an autocrine growth factor in human malignant mesothelioma." J Pathol **193**(4): 468-75.

Sutherland, R. M. (1988). "Cell and environment interactions in tumor microregions: the multicell spheroid model." Science **240**(4849): 177-84.

Sutherland, R. M., W. R. Inch, et al. (1971). "Phytohemagglutinin (PHA)-induced transformation of lymphocytes from patients with cancer." Cancer **27**(3): 574-8.

Sutherland, R. M., J. A. McCredie, et al. (1971). "Growth of multicell spheroids in tissue culture as a model of nodular carcinomas." J Natl Cancer Inst **46**(1): 113-20.

Swain, W. A., K. J. O'Byrne, et al. (2004). "Activation of p38 MAP kinase by asbestos in rat mesothelial cells is mediated by oxidative stress." Am J Physiol Lung Cell Mol Physiol **286**(4): L859-65.

Sweet, B. H. and M. R. Hilleman (1960). "The vacuolating virus, S.V. 40." Proc Soc Exp Biol Med **105**: 420-7.

Syrokou, A., G. N. Tzanakakis, et al. (1999). "Proteoglycans in human malignant mesothelioma. Stimulation of their synthesis induced by epidermal, insulin

and platelet-derived growth factors involves receptors with tyrosine kinase activity." Biochimie **81**(7): 733-44.

Tallarida, R. J. (1992). "Statistical analysis of drug combinations for synergism." Pain **49**(1): 93-7.

Tallarida, R. J. (2001). "Drug synergism: its detection and applications." J Pharmacol Exp Ther **298**(3): 865-72.

Testa, J. R. and A. Giordano (2001). "SV40 and cell cycle perturbations in malignant mesothelioma." Cancer Biol. **11**: 31-38.

Therasse, P., S. G. Arbuck, et al. (2000). "New guidelines to evaluate the response to treatment in solid tumors. European Organization for Research and Treatment of Cancer, National Cancer Institute of the United States, National Cancer Institute of Canada." J Natl Cancer Inst **92**(3): 205-16.

Thompson, J. E. and C. B. Thompson (2004). "Putting the rap on Akt." J Clin Oncol **22**(20): 4217-26.

Tomek, S. and C. Manegold (2004). "Chemotherapy for malignant pleural mesothelioma: past results and recent developments." Lung Cancer **45 Suppl 1**: S103-19.

Torocsik, B. and J. Szeberenyi (2000). "Anisomycin uses multiple mechanisms to stimulate mitogen-activated protein kinases and gene expression and to inhibit neuronal differentiation in PC12 phaeochromocytoma cells." Eur J Neurosci **12**(2): 527-32.

Toyokuni, S. and J. L. Sagripanti (1996). "Association between 8-hydroxy-2'-deoxyguanosine formation and DNA strand breaks mediated by copper and iron." Free Radic Biol Med **20**(6): 859-64.

Treasure, T. and A. Sedrakyan (2004). "Pleural mesothelioma: little evidence, still time to do trials." Lancet **364**(9440): 1183-5.

Uematsu, K., S. Kanazawa, et al. (2003). "Wnt pathway activation in mesothelioma: evidence of Dishevelled overexpression and transcriptional activity of beta-catenin." Cancer Res **63**(15): 4547-51.

van der Voort, R., T. E. Taher, et al. (2000). "The hepatocyte growth factor/Met pathway in development, tumorigenesis, and B-cell differentiation." Adv Cancer Res **79**: 39-90.

van Meerbeeck, J. P., P. Baas, et al. (2002). "A phase II EORTC study of temozolomide in patients with malignant pleural mesothelioma." Eur J Cancer **38**(6): 779-83.

van Meerbeeck, J. P., R. Gaafar, et al. (2005). "Randomized phase III study of cisplatin with or without raltitrexed in patients with malignant pleural mesothelioma: an intergroup study of the European Organisation for Research and Treatment of Cancer Lung Cancer Group and the National Cancer Institute of Canada." J Clin Oncol **23**(28): 6881-9.

Versnel, M. A., L. Claesson-Welsh, et al. (1991). "Human malignant mesothelioma cell lines express PDGF beta-receptors whereas cultured normal mesothelial cells express predominantly PDGF alpha-receptors." Oncogene **6**(11): 2005-11.

Vintman, L., S. Nielsen, et al. (2005). "Mitogen-activated protein kinase expression and activation does not differentiate benign from malignant mesothelial cells." Cancer **103**(11): 2427-33.

Vivo, C., W. Liu, et al. (2003). "c-Jun N-terminal kinase contributes to apoptotic synergy induced by tumor necrosis factor-related apoptosis-inducing ligand plus DNA damage in chemoresistant, p53 inactive mesothelioma cells." J Biol Chem **278**(28): 25461-7.

Vogelzang, N. J., M. Goutsou, et al. (1990). "Carboplatin in malignant mesothelioma: a phase II study of the Cancer and Leukemia Group B." Cancer Chemother Pharmacol **27**(3): 239-42.

Vogelzang, N. J., C. Porta, et al. (2005). "New agents in the management of advanced mesothelioma." Semin Oncol **32**(3): 336-50.

Vogelzang, N. J., J. J. Rusthoven, et al. (2003). "Phase III study of pemetrexed in combination with cisplatin versus cisplatin alone in patients with malignant pleural mesothelioma." J Clin Oncol **21**(14): 2636-44.

Volmat, V. and J. Pouyssegur (2001). "Spatiotemporal regulation of the p42/p44 MAPK pathway." Biol Cell **93**(1-2): 71-9.

Wagner, J. C., C. A. Sleggs, et al. (1960). "Diffuse pleural mesothelioma and asbestos exposure in the North Western Cape Province." Br J Ind Med **17**: 260-71.

Wang, N. S. e. a. (1987). "The interactions between asbestos fibers and metaphase chromosomes of rat pleural mesothelial cells in culture. A scanning and transmission electron microscopic study." Am. J. Pathol. **126**: 343-349.

Wang, S. Y., B. Chen, et al. (2004). "SU5416 is a potent inhibitor of hepatocyte growth factor receptor (c-Met) and blocks HGF-induced invasiveness of human HepG2 hepatoma cells." J Hepatol **41**(2): 267-73.

Wang, W. L., M. E. Healy, et al. (2000). "Growth inhibition and modulation of kinase pathways of small cell lung cancer cell lines by the novel tyrosine kinase inhibitor STI 571." Oncogene **19**(31): 3521-8.

Warn, R., P. Harvey, et al. (2001). "HGF/SF induces mesothelial cell migration and proliferation by autocrine and paracrine pathways." Exp Cell Res **267**(2): 258-66.

Watson, R. W., H. P. Redmond, et al. (1995). "Taurolidine, an antilipopolysaccharide agent, has immunoregulatory properties that are mediated by the amino acid taurine." J Leukoc Biol **58**(3): 299-306.

Webster, I., J. W. Cochrane, et al. (1982). "Immunotherapy with BCG vaccine in 30 cases of mesothelioma." S Afr Med J **61**(8): 277-8.

Weitzman, S. A. and P. Graceffa (1984). "Asbestos catalyzes hydroxyl and superoxide radical generation from hydrogen peroxide." Arch Biochem Biophys **228**(1): 373-6.

Whitson, B. A., B. A. Jacobson, et al. (2006). "Effects of insulin-like growth factor-1 receptor inhibition in mesothelioma. Thoracic Surgery Directors Association Resident Research Award." Ann Thorac Surg **82**(3): 996-1001; discussion 1001-2.

Willatts, S. M., S. Radford, et al. (1995). "Effect of the antiendotoxic agent, taurolidine, in the treatment of sepsis syndrome: a placebo-controlled, double-blind trial." Crit Care Med **23**(6): 1033-9.

Willis, S. N. and J. M. Adams (2005). "Life in the balance: how BH3-only proteins induce apoptosis." Curr Opin Cell Biol **17**(6): 617-25.

Wojta, J., C. Kaun, et al. (1999). "Hepatocyte growth factor increases expression of vascular endothelial growth factor and plasminogen activator inhibitor-1 in human keratinocytes and the vascular endothelial growth factor receptor flk-1 in human endothelial cells." Lab Invest **79**(4): 427-38.

Wood, J. M., G. Bold, et al. (2000). "PTK787/ZK 222584, a novel and potent inhibitor of vascular endothelial growth factor receptor tyrosine kinases, impairs vascular endothelial growth factor-induced responses and tumor growth after oral administration." Cancer Res **60**(8): 2178-89.

Wu, L. L., C. C. Chiou, et al. (2004). "Urinary 8-OHdG: a marker of oxidative stress to DNA and a risk factor for cancer, atherosclerosis and diabetics." Clin Chim Acta **339**(1-2): 1-9.

Wyszomierski, S. L. and D. Yu (2005). "A knotty turnabout?: Akt1 as a metastasis suppressor." Cancer Cell **8**(6): 437-9.

Xiong, Y., H. Zhang, et al. (1993). "Subunit rearrangement of the cyclin-dependent kinases is associated with cellular transformation." Genes Dev **7**(8): 1572-83.

Yan, C. Y., G. Ferrari, et al. (1995). "N-acetylcysteine-promoted survival of PC12 cells is glutathione-independent but transcription-dependent." J Biol Chem **270**(45): 26827-32.

Yang, H., M. Bocchetta, et al. (2006). "TNF-alpha inhibits asbestos-induced cytotoxicity via a NF-kappaB-dependent pathway, a possible mechanism for asbestos-induced oncogenesis." Proc Natl Acad Sci U S A **103**(27): 10397-402.

Yokoi, K., T. Sasaki, et al. (2005). "Simultaneous inhibition of EGFR, VEGFR, and platelet-derived growth factor receptor signaling combined with gemcitabine

produces therapy of human pancreatic carcinoma and prolongs survival in an orthotopic nude mouse model." Cancer Res **65**(22): 10371-80.

Zahir, N., J. N. Lakins, et al. (2003). "Autocrine laminin-5 ligates alpha6beta4 integrin and activates RAC and NFkappaB to mediate anchorage-independent survival of mammary tumors." J Cell Biol **163**(6): 1397-407.

Zanella, C. L., J. Posada, et al. (1996). "Asbestos causes stimulation of the extracellular signal-regulated kinase 1 mitogen-activated protein kinase cascade after phosphorylation of the epidermal growth factor receptor." Cancer Res **56**(23): 5334-8.

Zhong, J., M. M. Gencay, et al. (2006). "ERK1/2 and p38 MAP kinase control MMP-2, MT1-MMP, and TIMP action and affect cell migration: a comparison between mesothelioma and mesothelial cells." J Cell Physiol **207**(2): 540-52.

Zhong, X. S., J. Z. Zheng, et al. (2004). "SU5416 inhibited VEGF and HIF-1alpha expression through the PI3K/AKT/p70S6K1 signaling pathway." Biochem Biophys Res Commun **324**(2): 471-80.

Zhou, H., S. A. Summers, et al. (1998). "Inhibition of Akt kinase by cell-permeable ceramide and its implications for ceramide-induced apoptosis." J Biol Chem **273**(26): 16568-75.

Zinkel, S., A. Gross, et al. (2006). "BCL2 family in DNA damage and cell cycle control." Cell Death Differ **13**(8): 1351-9.

Zundel, W. and A. Giaccia (1998). "Inhibition of the anti-apoptotic PI(3)K/Akt/Bad pathway by stress." Genes Dev **12**(13): 1941-6.