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REVIEW

Preclinical studies identify novel targeted pharmacological strategies for treatment of human malignant pleural mesothelioma

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The incidence of human malignant pleural mesothelioma (hMPM) is still increasing worldwide. hMPM prognosis is poor even if the median survival time has been slightly improved after the introduction of the up-to-date chemotherapy. Nevertheless, large phase II/III trials support the combination of platinum derivatives and pemetrexed or raltitrexed, as preferred first-line schedule. Better understanding of the molecular machinery of hMPM will lead to the design and synthesis of novel compounds targeted against pathways identified as crucial for hMPM cell proliferation and spreading. Among them, several receptors tyrosine kinase show altered activity in subsets of hMPM. This observation suggests that these kinases might represent novel therapeutic targets in this chemotherapy-resistant disease. Over these foundations, several promising studies are ongoing at preclinical level and novel molecules are currently under evaluation as well. Yet, established tumour cell lines, used for decades to investigate the efficacy of anticancer agents, although still the main source of drug efficacy studies, after long-term cultures tend to biologically diverge from the original tumour, limiting the predictive potential of *in vivo* efficacy. Cancer stem cells (CSCs), a subpopulation of malignant cells capable of self-renewal and multilineage differentiation, are believed to play an essential role in cancer initiation, growth, metastasization and relapse, being responsible of chemo- and radiotherapy refractoriness. According to the current carcinogenesis theory, CSCs represent the tumour-initiating cell (TIC) fraction, the only clonogenic subpopulation able to originate a tumour mass. Consequently, the recently described isolation of TICs from hMPM, the proposed main pharmacological target for novel antitumoural drugs, may contribute to better dissect the biology and multidrug resistance pathways controlling hMPM growth.

Abbreviations

CSC, cancer stem cell; GF, growth factor; hMPM, human malignant pleural mesothelioma; MoAb, monoclonal antibody; NSAID, non-steroidal anti-inflammatory drugs; RR, response rate; RTK, receptor tyrosine kinase; SP, side population; TIC, tumour-initiating cells; TSG, tumour suppressor genes

Introduction

History

Aetiology of human malignant mesothelioma as primary tumour of serosa surfaces, such as pleura and peritoneum, has long been controversial. In 1931, Klemperer and Rabin first described the histological features of benign (localized) and malignant (diffused) mesotheliomas (Klemperer and Rabin, 1931). A single case of human malignant pleural mesothelioma (hMPM) analysed in 1947 (Case records of the Massachussetts General Hospital, 1947) excluded to recognize the

asbestos as causative factor even if the patient was an asbestos worker. The controversy lasted until 1960 when, in a fundamental report by JC Wagner and colleagues, asbestos was established as major etiologic factor in 32 of 33 cases of mesothelioma, largely induced by environmental exposure in South Africa (Wagner *et al.*, 1960). That singular relationship, confirmed worldwide, established the disease as a specific nosologic entity (Selikoff *et al.*, 1965).

Asbestos is a set of six natural silicate mineral fibres categorized into two classes: serpentines (chrysotile, white asbestos) and amphiboles (main types are crocidolite, amosite, blue and brown asbestos, respectively; Roach *et al.*, 2002). All of them have excellent fire-resistant characteristics as well as strong resistance to thermal, electric and chemical damage. For these reasons, for decades, asbestos has been extensively used for automobile brake pads and linings, household products, fire-retardant electric cables, heat insulation in shipyards and train building, to wrap pipes and mixed with cement, in construction industries, for fireproof roofing and flooring, corrugates roof sheets for outbuildings, warehouses and garages.

Another naturally occurring fibrous mineral having physical properties similar to asbestos, is erionite. However, this asbestos-like mineral is not regulated by the US Environmental Protection Agency as an asbestos fibre. Nevertheless, erionite is known as a carcinogen and listed by the International Agency for Research on Cancer as a group I carcinogen (IARC Monographs – Classifications – Group I, 2009). It has been shown that exposure to erionite results in pleural and interstitial fibrotic changes, which are similar to those observed with asbestos. Furthermore, *in vitro* studies demonstrated that erionite, but not asbestos, is sufficient to cause malignant transformation of cultured human mesothelial cells (Bertino *et al.*, 2007a).

Aetiology – Epidemiology – Incidence

Chronic exposure and inhalation of the small asbestos fibres, chrysotile and the most carcinogenic amosite and crocidolite, can cause serious illnesses, including malignant lung cancer and hMPM. Tumours have mostly been observed in people occupationally exposed to asbestos, in their family members and in residents who lived close to asbestos factories and mines. The asbestos microfibres are rigid, sharp and resistant to chemical and biological degradation; they gather into the interstitial tissues, accumulate in the lower part of the lungs and finally reach the pleura. Once, this tumour was rare but its incidence grew and it is still increasing in several countries because of the past widespread use of asbestos; the prediction is for a further increase in the next decades, especially in the countries where the use of asbestos has not yet been totally banned (Peto *et al.*, 1999).

Mortality from hMPM depends on exposure to this early-stage carcinogen; the latency between first contact with the agent and tumour diagnosis is long: between 15 and more than 30 years. Even if about 80% of hMPM can be attributed to asbestos fibre inhalation, exposure to SV40 and radiations are recognized as further potential carcinogenic cofactors (Carbone *et al.*, 2002). In Western Europe, 5000 patients each year die of hMPM; the highest incidence rates have been reported in Belgium and Great Britain (approximately 30 cases/million/year; Bianchi and Bianchi, 2007). The incidence is growing up in most developed countries and in Western Europe is expected to rise in the next 15 years: early projections for the 1995–2029 period foresee a doubling of hMPM each year between 1998 and 2018 before the decline (Peto *et al.*, 1999). A 2004 update of mesothelioma trend in the United States described that approximately 2500 persons per year are diagnosed, 19% of which are women, and more than 70 000 cases are expected to occur in the next 20 years with the peak this year (Price and Ware, 2004). Worldwide, the relatively rare incidence is increasing with a peak expected in 10 years. The latest statistics released by the Great Britain's Health and Safety Executive regarding the country's rate of hMPM incidence reveal that at least 5000 deaths from hMPM a year are expected by 2015, a number surprisingly prevalent than in the United States (3000 deaths) (http://www.maacenter.org). In 1978, a remarkable mesothelioma epidemic due to erionite exposure, causing 50% of all deaths, has been reported in three small villages in Cappadocia. More recent studies have shown erionite to cause mesothelioma mostly in families with a genetical predispostion to this tumour (Carbone *et al.*, 2007). Moreover, searching for genetic predisposing factors, germ line mutations in the gene encoding BRCA1-associated protein-1 (BAP-1) were discovered in two families at high incidence of mesothelioma. The identification of a BAP-1-related cancer syndrome characterized by mesothelioma (and uveal melanoma), could help to identify individuals at high risk who could be treated in chemoprevention protocols (Testa *et al.*, 2011).

Histology

hMPM is typically classified into four histological subtypes: epithelioid, sarcomatoid, biphasic and desmoplastic (Beasley *et al.*, 2005). The epithelioid type is the most common variant, comprising 50–60% of the total hMPM. Sarcomatoid subtype is constituted of spindled cells, often they mimic fibrosarcoma; biphasic (or mixed) presents epithelioid and sarcomatoid features, whereas desmoplastic hMPM represents a quite rare variant of the tumour.

Biology and pathogenesis

The clinical evidences of hMPM are thought to arise as a result of the accrual of several molecular alterations. Asbestos fibres induce the expression of the nuclear proto-oncogenes c-fos and c-jun, which result in cell proliferation and gene transcription, representing the initial alteration induced by the chemical exposure. Furthermore, asbestos promotes secretion of the pro-inflammatory cytokine TNF-α by mesothelial cells and macrophages leading to activation of NF-κB, which plays a role in cell proliferation and anti-apoptosis. High-mobility group box 1 (HMGB 1) release has been identified as a critical initial step in the pathogenesis of asbestos-related hMPM. Asbestos-exposed mesothelial cells translocate HMGB 1 from the nucleus across the cytoplasm, into the extracellular space. The release of HMGB 1 induces macrophages to secrete TNF-α, which protects mesothelial cells from asbestos-induced cell death triggering a chronic inflammatory response that ultimately may favor mesothelial cells transformation (Yang *et al.*, 2010). Mesothelial cells are assumed to undergo neoplastic transformation as a result of the activation of the NF-κB pathway (Yang *et al.*, 2006). Many

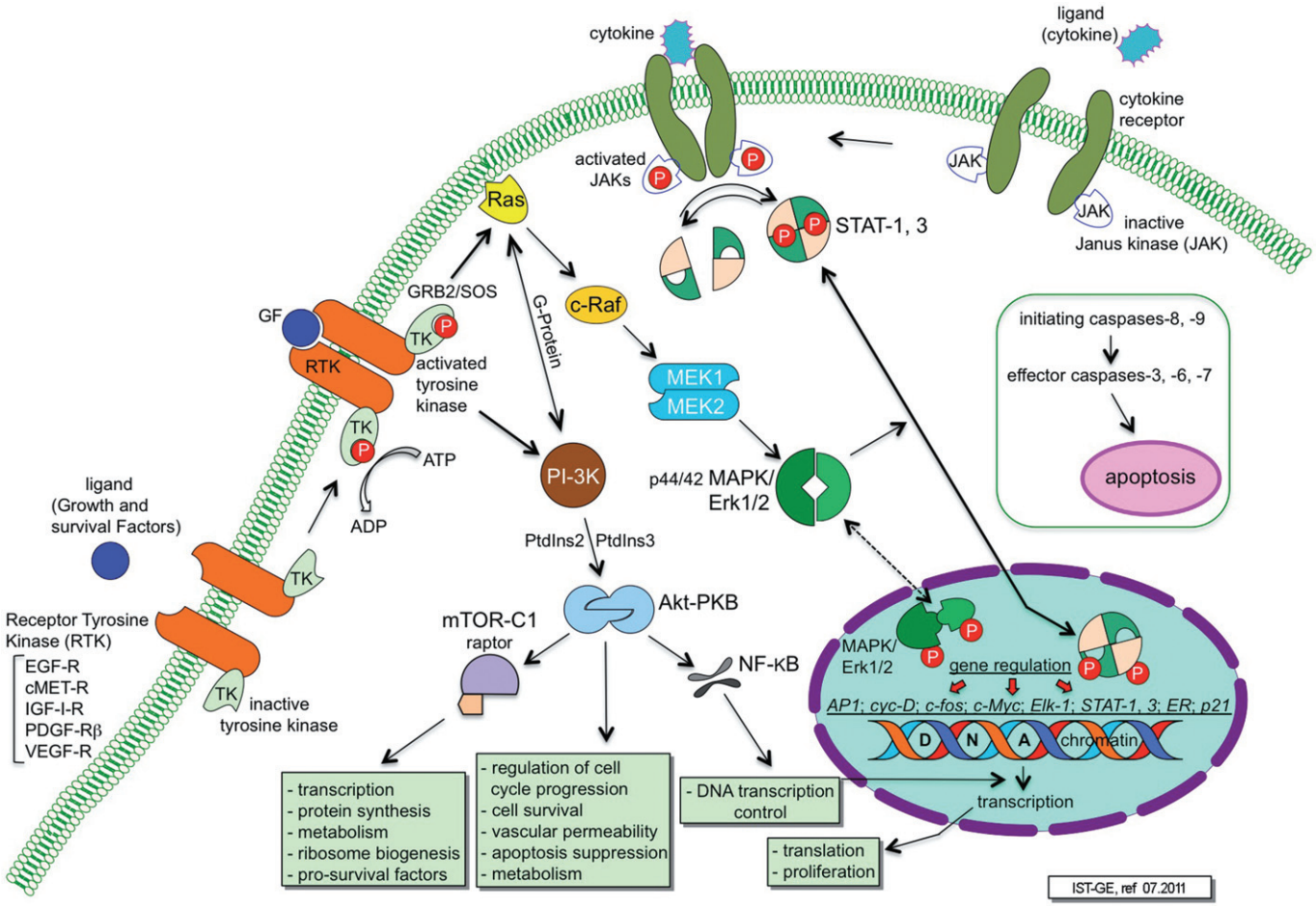


Figure 1

Schematic representation of polypeptide GF-induced activation of TK-linked receptors and cytokines-mediated activation of STAT factors and their affected downstream targets. The activation of their specific metabolic pathways has been shown relevant for gene regulation, DNA transcription, protein synthesis, apoptosis suppression and metabolism in neoplastic cells. In detail, binding of ligands to their transmembrane receptors, such as EGF-R, IGF-I-R, PDGF-R, VEGF-R, activates signalling cascades that, through the Ras, Raf, MEK, ERK, PI-3 K, Akt and mTOR molecules, lead to protein synthesis, cell cycle regulation and metabolism. Non-receptorial JA kinases activation occurs after cytokines-induced dimerization of their cognate receptors followed by trans-phosphorilation of the two subunits on specific tyrosine residues; JAKs than induce dimerization and phosphorylation of latent STATs transcription factors which, migrating from the cytosol and accumulating into the cell nucleus, interact with specific DNA response elements sequences leading to gene transcription and originating biologic proliferative reactions. GRB2/SOS, growth factor receptor-bound protein 2/Son of sevenless (SOS) is a guanine nucleotide exchange factor that activates Ras in response to growth factor stimulation; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase; PtdIns, phosphatidyl-inositol; Ras, small GTPase-protein cycling between an activated (Ras-GTP) and inactivated (Ras-GDP) conformations; Raf, proto-oncogene serine/threonine protein kinase.

alterations, deletions and amplifications, have been found at chromosome level. A particularly high frequency of homodeletion has been detected in the 9p21 region, causing a high frequency of deletion of P16 and P14 genes located on that chromosome: the loss of expression of their proteins causes a breakdown of the cell cycle control mechanisms by inhibiting the phosphorylation of Rb protein and destabilizing p53 proteins, respectively (Musti *et al.*, 2006). Otherwise, mutations in other genes including p53, ras and RB, highly frequent in malignant tumours, are very rare in hMPM.

Promoter methylation and histone deacetylation are epigenetic changes in chromatin structure causing gene silencing, without altering DNA sequence. Methylation of tumour suppressor genes (TSG) has been observed in hMPM strongly suggesting that methylation of the promoter region of TSGs contributes to neoplastic transformation and progression (Batra *et al.*, 2006). Methylation of the insulin-like growth factor binding protein-3 (IGF-BP3) gene, thought to control IGF-I function by suppressing its specific receptor, has been shown to be more frequent in hMPM patients in Japan than in the United States, suggesting racial or regional differences in genes undergoing methylation (Tomii *et al.*, 2007).

Increased hMPM cell proliferation results from the autocrine and paracrine activity of several growth factors (GF), mainly epidermal growth factor (EGF), hepatocyte growth factor (HGF), insulin-like growth factor (IGF)-I and II, platelet-derived growth factor (PDGF), transforming growth factor (TGF)-β, vascular endothelial growth factor (VEGF) and their specific transmembrane receptors (R; Figure 1), all found highly expressed in hMPM. EGF and TGF-α are the main

ligands for EGF receptor (EGFR), a member of the erbB family TK receptors. The ligand-receptor docking and binding leads to receptor dimerization and temporary internalization followed by transphosphorylation of the receptor tail-located TK domains, resulting in activation of signalling pathways involved in cell proliferation, differentiation and survival, such as Raf-MEK-ERK1/2 and phosphoinositide-3kinase (PI-3 K)-Akt (Favoni *et al*., 2010). Overexpression of EGFR has been recognized to play a fundamental role in the pathogenesis and progression of a variety of malignancies including breast and pulmonary carcinomas. The initial involvement of EGFR in hMPM derived from a study by Dazzi *et al*. (1990) who found its expression in 68% tissue specimens, whereas another study reported EGFR immunoreactivity in almost 56% samples and no immunoreactivity in normal pleura expression (Destro *et al*., 2006). It has been demonstrated that asbestos fibres cause aggregation and increased immunoreactivity of EGFR in mesothelial cells and that asbestos-induced EGFR autophosphorylation may lead to the induction of the AP-1 family members, c-fos and c-jun.

VEGF is a potent angiogenesis inducer playing a critical role in tumour progression and whose up-regulation is relevant for mesothelial cell transformation, as well. High levels of VEGF have been detected in serum of mesothelioma patients' versus normal subjects. Recently, it was suggested that SV40 large tumour antigen is involved in VEGF promoter activation, potently increasing VEGF expression level in hMPM cell lines. Besides the stimulation of the neovascularization, VEGF may induce activation of its receptors, VEGFR-1 (Flt-1) and VEGFR-2 (Flk-1/KDR), acting as autocrine GF in hMPM cell (Strizzi *et al*., 2001).

PDGF is the natural ligand of PDGFR, which occurs as α and β homo- or α–β heterodimers. Following the same biochemical scheme occurring for all the receptor TKs (RTKs), PDGF-PDGFR binding switch on the receptor tyrosine residues autophosphorylation and ultimately the downstream transmission of the signal to drive cell growth, morphology changes and apoptosis prevention. Overactivity of PDGF-PDGFR axis has been shown in several proliferation disorders, including ovaric, pancreatic, gastric, pulmonary, prostatic cancers, gliomas and hMPM. Whereas hMPM cell lines show overexpression of PDGFβ receptors, normal mesothelial cells mainly express PDGFα receptors (Langerak *et al*., 1996).

HGF and its receptor, the RTK c-Met, play an important role in hMPM cell motility and invasion into extracellular stroma. HGF was detected in pleural effusion fluids of patients with malignant mesothelioma and in paraffin-embedded tumour tissues, showing higher levels than in control subjects. Similarly, increased co-expression of c-Met was also detected in hMPM, showing a significant co-localization in the same cells. The co-expression of this receptor ligand pair clearly suggests a possible autocrine/paracrine stimulation of hMPM cells (Harvey *et al*., 1998; Mukohara *et al*., 2005a). Moreover, HGF-positive hMPM also showed a significantly higher microvessel density as compared with its negative counterpart (Tolnay *et al*., 1998).

Similarly to other GFs, the 'IGF-I system' plays a central role in cancer cell proliferation and survival (Pasello and Favaretto, 2009). IGF-I can behave in an autocrine or paracrine fashion, stimulating tumour growth; its physiologic receptor (IGF-1R) is a significant regulator of mesothelioma growth through downstream kinases as serine-threonine protein kinase (Akt-PKB; Whitson and Kratzke, 2006). Additional members of the IGF system, including IGF-BP1-6-binding proteins, modulate the pathway. In surveying of hMPM, IGF-BP2, 4 and 5 were found to be present while IGF-BP1, 3 and 6 were absent; the absence of IGF-BP3 together with the presence of deleterious IGF-BP4 would allow for a more aggressive phenotype (Hodzic *et al*., 1997).

The overexpression of IGF-I, IGF-II, their respective receptors and IGF-BP4, together with the underexpression of IGF-BP5 found in a hMPM array analysis (Hoang *et al*., 2004), led to the speculation that IGF-BP5 may act as IGF-IR activation inhibitor and its decreasing allows for over stimulation of the receptor potentially triggering autocrine stimulation. However, the IGF axis, as an important regulator of hMPM growth and tumourigenesis, still needs further elucidation.

Overexpression of the mammalian target of rapamycin (mTOR), a kinase downstream of PI-3 K and Akt, has been identified in mice where the mTOR pathway accounts for the major survival effect of Akt (Kim *et al*., 2005). The high local invasiveness, as well as the distant metastases, which sometimes occur in advanced hMPM, could be related to matrix metalloproteinases, particularly MMP-2 and -9, the first also considered as a negative prognostic factor (Edwards *et al*., 2003). Bcl-2 protein involved in apoptosis is strongly expressed in many malignant tumours whereas is weakly expressed in hMPM; however, expression of a member of Bcl-2 family (Bcl-XL) and the potent anti-apoptotic Bax are frequently found. Also, survivin and inhibitor of apoptosis protein (IAP) expression, considered resistant factors for chemotherapy, have been observed (Kleinberg *et al*., 2007).

Multimodality treatment

In consideration of the elevated rates of therapeutical failures in the treatment of hMPM, many different approaches have been developed, often trying to combine them to maximize the responses.

Radiotherapy. the treatment of the whole pleural surface results technically difficult and jointed with risks of radiation pneumonitis, myelitis and myocarditis (Allen *et al*., 2006). Radiotherapy alone resulted ineffective in prolonging the patient's survival but can be applicated to palliate symptoms even if the duration of response, if any, is very short. Radiotherapy could have a role in hMPM cure if used in combination with other treatments.

Surgery. The complete surgical resection with histologically negative margins, which is theoretically the most effective treatment for hMPM, is difficult to obtain because of the peculiar characteristics of the disease, usually diffused throughout the hemitorax. The removal of the most possible bulk of the tumour results only in a cytoreduction that, generally, left behind microscopic tumour. The most aggressive surgical procedure is the extrapleural pneumonectomy (EPP) consisting in the radical 'en block' removal of the pleura, pericardium, diaphragm along with the whole involved lung and mediastinal lymph nodes. However, this radical procedure is feasible only in 1–5% of cases represented by patients selected by strict criteria: relatively young, in

good general health, with epithelial subtype mesothelioma at I or II stage, and it is associated with significant morbidity and with mortality rate (from 3.4 to >10%) (Sugarbaker, 2006). The effect of EPP versus no-EPP, in a context of trimodal therapy, on survival and quality of life has been recently assessed in a multicenter randomized controlled trial by the mesothelioma and radical surgery feasibility study in 12 UK hospitals. The authors, because of the high morbidity associated with EPP and the no significant differences in quality of life between groups, suggested that radical surgery, as EPP, following a platinum-based chemotherapy inducted in a pre-randomization phase, does not offer benefits (Treasure *et al.*, 2011).

Multidisciplinary approaches. The failure of single modality treatment to improve hMPM patient survival and the general conviction that any of the available techniques can be considered a potentially curative procedure, has led to evaluate their combination. Today, radical debulking surgery (EPP) or pleurectomy/decortication with complementary neoadjuvant treatment and adjuvant chemotherapy, radiotherapy and local or systemic immunotherapy are the cornerstones of multimodality treatments. In any case, based on many evidences coming from the clinical medicine surgery, the prognosis of hMPM remains poor. Other therapeutic options, including intrapleural chemotherapy and gene therapy, which demonstrated some relief, have to be evaluated for their real advantages. Finally, more efficacious systemic therapies, mainly founded on the novel molecular targeted compounds, besides those already under evaluation, must be identified.

Standard and latest therapeutic molecular targets: tested agents and potentially beneficial compounds in mesothelioma therapy

Chemotherapy represents one of the main options for cancer treatment. In the following paragraphs, we review a considerable set of molecules acting on the most common pharmacological targets, expressed or overexpressed, in neoplastic cells, including hMPM; few promising new agents, designed to target very topical biochemical pathways have been tackled, as well (Figure 2).

DNA-targeting drugs: anthracyclines-antibiotics and alkylants

Anthracyclines belong to a class of antitumour-antibiotic, DNA crosslinker drugs used to treat a wide range of tumours, including lymphomas, leukaemia, breast, ovarian and lung cancers. They are among the most effective single or combined treatments, although their utility is limited by cardiotoxicity (Minotti *et al.*, 2004). The main agents today available are daunorubicin and doxorubicin (both existing also as liposomal formulation), epirubicin and mytomicin-C. These drugs act through the inhibition of DNA and RNA synthesis by intercalating between base pairs of the DNA/RNA strand, thus preventing the replication of rapidly growing cancer cells; they also inhibit topoisomerase II enzyme, preventing the relaxing of supercoiled DNA, thus blocking DNA transcription and replication and, finally, creating iron-mediated free oxygen radicals that damage the DNA and cell membranes (Table 1).

The inorganic platinum salt *cis*-diammine-dichloroplatinum (CDDP), cisplatin, is one of the most widely used anticancer molecule. Platinum-based regimens have been the basis for treatment of several solid tumours, including hMPM, and have been extensively analysed as single agent and in combined protocols (Choy *et al.*, 2008). The cytotoxicity of this molecule is due to binding and cross-links the DNA bases (mainly guanine), forming intrastrand bond in DNA. These interferences contribute to inhibition of DNA duplication, RNA transcription, alteration of cell division and activation of apoptosis. Notwithstanding the recent synthesis of several derivatives, such as carboplatin and oxaliplatin, its efficacy in hMPM remains limited (Table 1).

Mitosis-targeting drugs: vinca alkaloids and taxanes

Cytotoxic vinca alkaloid derivatives, vincristine, vinblastine and vinorelbine, interact with tubulin to block microtubule assembly, chromosome segregation and causing metaphase arrest in cell cycle (Jordan and Wilson, 2004). Taxanes, like paclitaxel and docetaxel, plants extracts derivatives, have a different binding site from vinca alkaloids. They bind to the -NH_2 terminal amino acid of the β-tubulin subunit in tubulin oligomers or polymers instead to tubulin dimers. In this way, taxanes shift the dynamic equilibrium between tubulin dimers and microtubules, stabilizing microtubules and preventing depolymerization (Teicher, 2008; Table 1).

Antimetabolites: nucleoside and pyrimidine analogues

Chemically, gemcitabine (2′,2′-difluoro-2′-deoxycytidine) is an 'old' anticancer nucleoside analog, in which the hydrogen atoms on the 2′ carbon of deoxycytidine are replaced by fluorine atoms; the triphosphate analogue of gemcitabine replaces the cytidine of nucleic acids during DNA replication leading to tumour growth arrest. Another target of gemcitabine is the enzyme ribonucleotide reductase (Table 1). Gemcitabine and the vinca-derived vinorelbine, which have shown activity as the first-line setting, have been recently also investigated in association with the objective of evaluating their activity and toxicity in pemetrexed (a new generation antifolate)-pretreated hMPM patients: the combination was only moderately active showing an acceptable toxicity profile (Zucali *et al.*, 2008).

Topoisomerase I/II targeting drugs

Topoisomerase inhibitors are agents designed to interfere with the action of topoisomerase I and II enzymes that control DNA breaking and rejoining of the phosphodiester backbone of strands during the DNA helices separation. Topoisomerase inhibitors block the ligation step of the cell cycle, generating single and double stranded breaks that harm the integrity of the genome and, subsequently, lead to apoptosis. Principal compounds active against topoisomerase I are camptothecin, irinotecan, topotecan (Staker *et al.*, 2002;

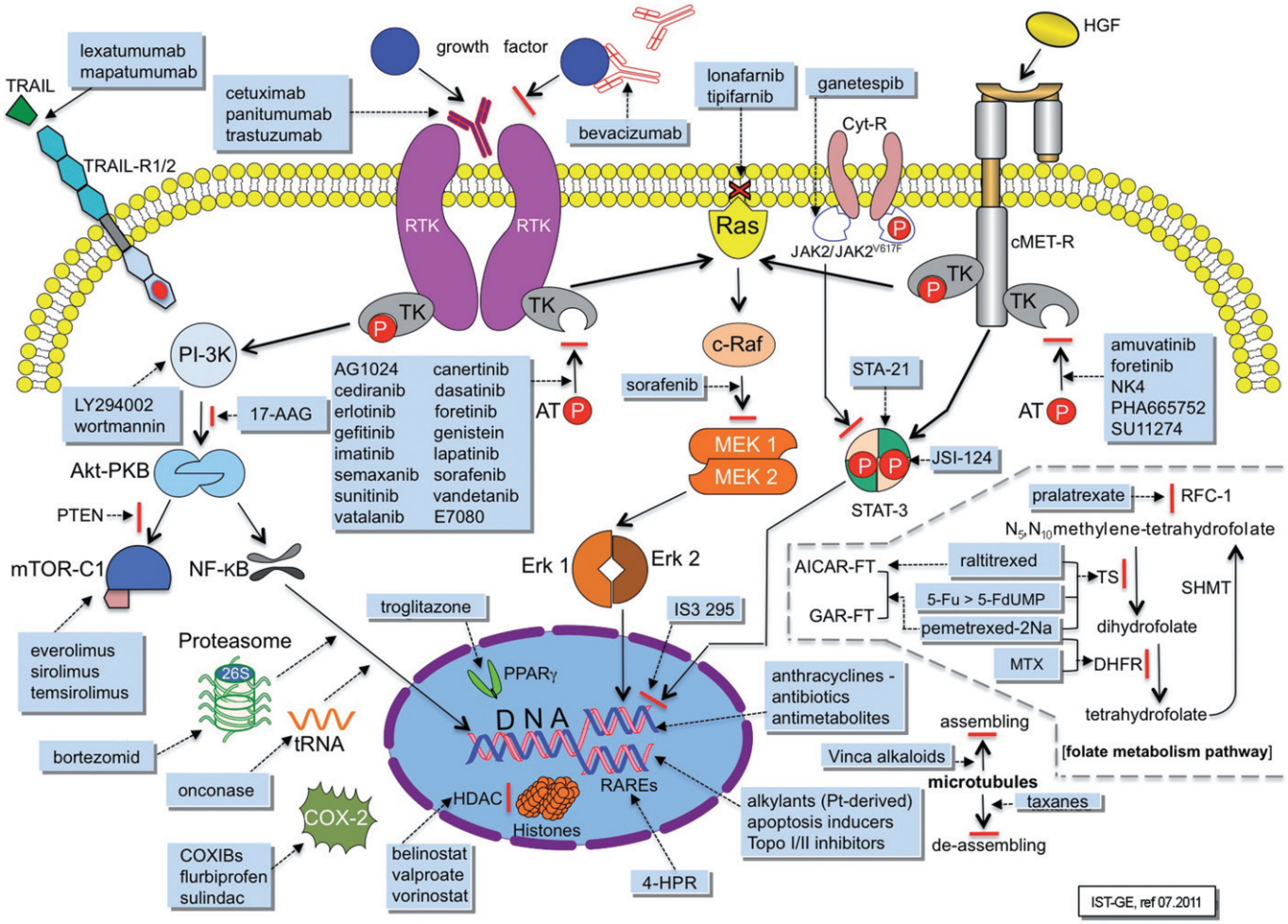


Figure 2

Schematic representation of main biochemical pathways and established targets for pharmacological inhibition of tumour cell growth and their modulation by standard cytotoxic and target-directed drugs already tested in malignant mesothelioma; novel compounds of potential use in mesothelioma are also indicated. Cyt-R, cytokine receptor; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase; cMET-R, proto-oncogene/protein mesenchymal-epithelial transition factor – HGF-receptor; P, phosphorus; PTEN, phosphatase and tensin homolog tumour suppressor gene/protein acting as phosphatase to dephosphorylate phosphatidylinositol (3,4,5)-triphosphate; Raf, proto-oncogene serine/threonine protein kinase; RARE, retinoic acid response elements; Ras, small GTPase-protein cycling between an activated (Ras-GTP) and inactivated (Ras-GDP) conformations; SHMT, serine hydroxy metyl transferase; TS, tymidylate synthase.

Table 1), whereas the most representative of drugs versus topoisomerase II is etoposide (VP16) a podophyllotoxin which, by provoking the DNA unwinding, causes strands to break (Gordaliza *et al.*, 2004).

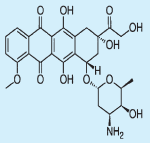
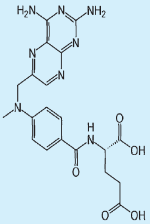
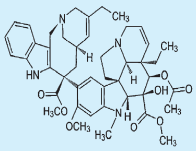
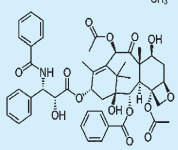
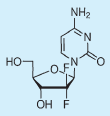
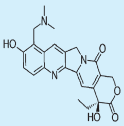
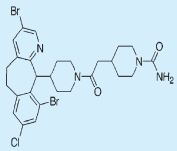
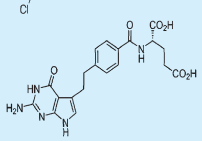
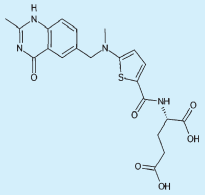
Growth factors and receptor TK-targeting drugs

Upon oncogenic mutations (i.e. gene amplification or over-expression, point mutation, translocation), GFs and their cognate receptors may induce a cell gain of function that ultimately leads to cell transformation. GFs, after binding to their specific transmembrane receptors, promote neoplastic growth, proliferation and invasiveness. EGF, VEGF, HGF and PDGF are autocrine GFs in hMPM; other GFs, such as IGF-I, have been involved in development and progression of the disease (Jagadeeswaran *et al.*, 2006; Whitson and Kratzke, 2006). GFs also provide for neo-angiogenesis that is essential for solid tumour growth and may be considered a critical step in hMPM development. Angiogenesis process is mediated and controlled by FGF and VEGF (Strizzi *et al.*, 2001). Because GFs and their RTKs are often overexpressed in hMPM cell lines, tissues and pleural effusions as well as in non-malignant mesothelial specimens, they represent an appealing target for therapy (Belli *et al.*, 2009). Nevertheless, to date, the blockade of GFs-receptor binding has not been sufficiently exploited with the exception of the recombinant humanized IgG monoclonal antibody (MoAb) bevacizumab that binds VEGF preventing its interaction to its receptors (Rocha-Lima and Caio, 2008; Figure 2).

Among RTKs, EGFR, which belongs to Erb-B receptor family, plays a critical role in cell growth and differentiation; it is overexpressed in many solid tumours including hMPM (Kindler, 2008). The extra-cytoplasmatic portion of EGFR is the molecular target of MoAbs, such as cetuximab,

Table 1

Structures representative of cytotoxic inhibitors tested in human malignant pleural mesothelioma in preclinical studies and clinical trials

Drug name	Target	Chemical class	Molecular formula	Relative mol mass	Structural formula
Doxorubicin (Adryamicin)	DNA strands	Antracyclines	$C_{27}H_{29}NO_{11}$	543.52	
Cisplatin (CDDP)	DNA	Alkylant	$Pt(NH_3)_2Cl_2$	300.05	
Picoplatin [(ammine-dichloro(2-methylpyridine)]	DNA	Alkylant	$C_6H_{10}Cl_2N_2Pt$	376.14	
Methotrexate (MTX)	Folic acid	Antifolate	$C_{20}H_{22}N_8O_5$	454.44	
Vinorelbine	Microtubules	Antimitotic	$C_{45}H_{54}N_4O_8$	778.93	
Paclitaxel (Taxol)	Microtubules	Antimitotic	$C_{47}H_{51}NO_{14}$	853.9	
Gemcitabine	Cytidine	Antimetabolites	$C_9H_{11}F_2N_3O_4$	263.20	
5-Fluorouracil	Thymidylate synth	Antimetabolites	$C_4H_3FN_2O_2$	130.08	
Topotecan (Hycamtin)	Topoisomerase I	Topoiso-inhibitor	$C_{23}H_{23}N_3O_5$	457.9	
Lonafarnib (SCH66336)	Farnesil-transferase	Farnesil-transferase inhibitor	$C_{27}H_{31}N_4O_2ClBr_2$	638.82	
Pemetrexed disodium (LY231514)	Multitargeted folate pathway	Folic acid analogue	$C_{20}H_{19}N_5O_6.\ 2Na$	471.40	
Raltitrexed (ZD1694)	Thymidylate synthasi	Folic acid analogue	$C_{21}H_{22}N_4O_6S$	458.49	

panitumumab and trastuzumab. Cetuximab, a chimeric (mouse/human) MoAb (IgG1), recognizes EGFR (and not other receptors of Her family) with affinity 5- to 10-fold higher than endogenous ligands inhibiting the receptor function (Van Cutsem *et al.*, 2009). Panitumumab is a fully human MoAb (IgG2) specific to EGFR: it works by binding the extracellular domain of the EGFR preventing its activation. Trastuzumab is a humanized MoAb that binds to the domain IV of the extracellular segment of the HER2/neu receptor (Hudis, 2007). Cells treated with trastuzumab undergo arrest during the G1 phase of the cell cycle reducing the proliferation. It has been suggested that trastuzumab induces some of its effects by down-regulating HER2/neu leading to disruption of receptor dimerization and signalling through the downstream PI-3 K cascade. Trastuzumab also suppresses angiogenesis by induction of anti-angiogenic factors and repression of the pro-angiogenic ones.

Upstream steps of signal transduction pathways (Figure 2)

GF binding and activation of RTK is the first fundamental step that triggers the sequences of intracellular signals regulating cell proliferation, survival, progression, metabolism, angiogenesis, metastasis and drug resistance. Generally, RTKs are constituted of an extracellular ligand-binding portion connected to the cytoplasmic domain by a single transmembrane peptide. The cytoplasmic domain contains a conserved TK core and several regulatory sequences that undergo autophosphorylation and phosphorylation by heterologous kinases, after specific ligand binding. GF-RTKs docking first prompts receptor steric changes resulting in homo/eterodimerization, then induces the phosphorylation of specific residues of TKs in the cytoplasmatic tails; the activated receptors recruit and phosphorylate intracellular effectors that initiate a downstream signalling cascade leading to various biological responses (Chung *et al.*, 2010; Lemmon and Schlessinger, 2010; Figure 1).

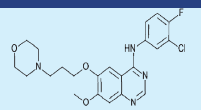
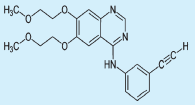
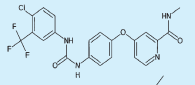
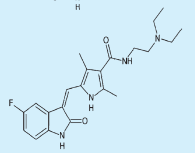
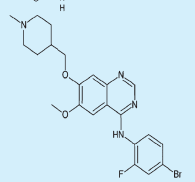
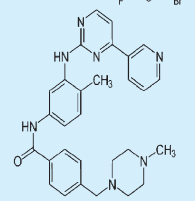
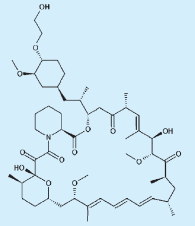
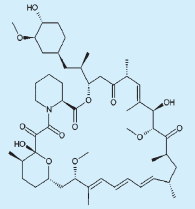
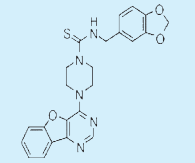
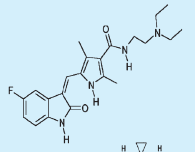
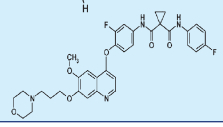
Several agents have been synthesized to target RTKs and block their kinase activity by competing with ATP binding: gefitinib (ZD1839), lapatinib (GW572016) and erlotinib (OSI774), small MW 4-anilino-quinazoline derivatives, are EGFR TK inhibitors; sorafenib (BAY43-9006), sunitinib (SU0011248) and vandetanib (ZD6474) are bis-aryl urea, L-malate salt and 4-anilino-quinazoline, respectively, and have been developed as anti-VEGFRs (Flt1 and Flt4); VEGFRs (Flt1, Flt4, Flt3), PDGFRα–β, c-kit; and anti-VEGFRs, respectively. The blockade of the TK activity induced by PDGF has been obtained by cediranib maleate (AZD2171), a 4-propoxy-quinazoline PDGFR inhibitor, which also acts on VEGFRs; imatinib mesylate (STI571), chemically a 2-fenilamidopirimide derived, active on PDGFRβ, bcr-abl, c-kit and c-fms (Table 2); dasatinib (BMS354825), a pyrimidil aminothiazole carboxamide, a dual inhibitor of bcr-abl and src kinase family; vatalanib (PTK787), an amino-phenazoline derivative that acts as anti-PDGFRβ, anti-c-kit and anti-VEGFRs. Also, semaxanib (SU5416), indolin-2-one derivative, inhibits PDGFR and VEGFR (Flt-1) activity. Other inhibitor of the catalytic activity of EGFR and IGF-IR, is the 'tyrphostin' (tyrosine-phosphorylation inhibitors) AG1024, potentially useful to down-regulating receptor autophosphorylation and phosphorylation of downstream effectors (Levitzki and Mishani, 2006); AG1024 may improve hhMPM cells sensitivity to cisplatin by inhibiting Akt, which seems to be up-regulated in presence of cytotoxic drugs, confirming the hypothesis that an updated managing of hMPM should consider the combination of multiple TK inhibitors associated with cytotoxic drugs (Kai *et al.*, 2009). C-Met, the HGF receptor, is a RTK playing a key role in thoracic tumours (Cipriani *et al.*, 2009). Activation of c-Met is involved in cell growth, survival, invasion, metastasis and angiogenesis conferring poor prognosis. Currently, pharmacological strategies to target HGF/c-Met axis are based on the blockade of the ligand-receptor interaction, the inhibition of TK activation and the interruption of the subsequent biochemical signals. As far as c-Met kinase inhibitors potentially effective on hMPM, here we mention: PHA665752, beyond specific inhibition of c-Met kinase activity it has also been demonstrated to represses both HGF-dependent and constitutive c-Met phosphorylation (Christensen *et al.*, 2003); SU11274 (Wang *et al.*, 2003); NK4 (Suzuki *et al.*, 2010); foretinib (XL880) (Ross *et al.*, 2007) and amuvatinib (MP470-HCl) target c-Met-R kinase, blocking the action of HGF. Interestingly, amuvatinib (which also acts on c-Ret, c-Kit and VEGFR2), evaluated in biochemical assays, was less potent in cells overexpressing c-Met suggesting further still unknown mechanisms of action. Moreover, a synergy with DNA-damaging drugs was reported, implying a role for amuvatinib in combination therapies with platinum and derivatives (Taverna *et al.*, 2010; Table 2).

Recently, heat shock protein (Hsp90) has emerged as being of prime importance to tumour cell growth and survival. Hsp90 is an abundant molecular chaperone protein that mediates the maturation and stability of a variety of proteins, such as Akt, bcr-abl, kit and receptors TK (c-Met, EGFR, PDGFR, VEGFR) that drive the growth proliferation of many types of cancer. Okamoto and colleagues investigated and demonstrated that 17-allylamino-17-demethoxygeldanamycin (17-AAG), a small molecule Hsp90-inhibitor, leads to G1 or G2/M cell cycle arrest, to suppression of cell growth and to apoptosis resulting from decreased levels of AKT and survivin in five human hMPM cell lines. They also demonstrated that this small molecule induces apoptosis in hMPM primary tissue cultures suggesting that inhibition of Hsp90 function is a promising therapeutic target for a highly aggressive and inexorably fatal cancer (Okamoto *et al.*, 2008). 17-AAG is currently used in phase I and II clinical trials.

Ganetespib (formerly STA-9090), another synthetic molecule structurally unrelated to the first-generation Hsp90 inhibitors such as 17-AAG, has shown higher activity in preclinical models (*in vitro*/*in vivo*) of a broad range of cancers including lung, breast, colon, prostate, melanoma, myeloma, lymphoma many of which were resistant to targeted agents. Activated cytokine-Janus kinase (JAK) complexes recruit and phosphorylate effector molecules including signal transducers and activators of transcription (STAT) proteins. STAT proteins mediate a wide range of biological processes, including cell growth, differentiation, apoptosis, inflammation and immune response. Two STATs in particular, STAT-3 and STAT-5, represent the major substrates for JAK2 that govern myelopoeisis and can contribute to cellular transformation. Persistent JAK/STAT activation is oncogenic and characteristic

Table 2

Structures of major novel small TK inhibitors tested in human malignant pleural mesothelioma *in vitro*/*in vivo* studies

Drug name	Target	Chemical class	Molecular formula	Relative mol mass	Structural formula
Gefitinib (ZD1839)	EGF-RPTK	4-anilino-quinazoline	$C_{22}H_{24}ClFN_4O_3$	446.90	
Erlotinib-HCl (OSI774)	EGF-RPTK	quinazoline	$C_{22}H_{23}N_3O_4$	393.44. (429.9 HCl)	
Sorafenib™ (BAY43-9006)	VEGF-R2/3 raf kinase	Bis-aryl urea	$C_{21}H_{16}ClF_3N_4O_3$	464.82	
Sunitinib (SU011248)	VEGF-Rs PDGF-Rα, β, Kit, FLT3	3carboxamide L-malate salt	$C_{22}H_{27}FN_4O_2$	398.47 (532.56 malate salt)	
Vandetanib (ZD6474)	VEGF-RPTK	4-anilino-quinazoline	$C_{22}H_{24}BrFN_4O_2$	475.35	
Imatinib (STI571)	PDGF-Rβ; bcr-abl; c-kit	Imatinib mesilate	$C_{30}H_{35}N_7\ SO_4$	589.72	
Everolimus (RAD001)	mTOR	Rapamicin-derived	$C_{53}H_{83}NO_{14}$	958.22	
Sirolimus (Rapamycin)	mTOR	Macrocyclic poliketide	$C_{51}H_{79}NO_{13}$	914.17	
Amuvatinib (MP470.HCl)	cMET	Piperazinecarbo thioamide	$C_{23}H_{22}ClN_5O_3S$	483.97	
SU11274	cMET	Methyl-piperazine indole sulfonamide	$C_{28}H_{30}ClN_5O_4S$	568.09	
Foretinib (XL880)	cMET		$C_{34}H_{34}F_2N_4O_6$	632.65	

of many human malignancies providing an attractive point of intervention for molecularly targeted therapeutics. It has been shown (Proia *et al.*, 2011) that ganetespib has profound antitumour activity in an array of JAK/STAT-driven cancers and can abrogate aberrant signalling through multiple mechanisms. Ganetespib effectively targets the upstream regulator JAK2, including the constitutively active JAK2^{V617F} mutant, for degradation in a range of hematological and solid tumour types with subsequent prolonged loss of STAT-3 and STAT-5 signalling (Figure 2).

Downstream steps of signal transduction pathways (Figure 2)

The Ras-Raf-ERK, PI-3 K-Akt = > mTOR (and/or = > NF-κB) and STAT-3 pathways.

Ras is a small GTPase, usually tethered inside the cell membrane that functions as early binary 'on/off' player in signal transduction networks. Upon activation by RTKs, Ras is turned 'on' releasing GDP and binding GTP. In this active form, Ras binds and activates the downstream effector Raf that in turn start a cascade of phosphorylation/activation of MEK1/2 and the MAPK ERK1/2. In certain cell types, Ras has been also involved in the activation of the PI-3 K-Akt/PKB cascade. Then Ras is switched 'off' by its intrinsic GTPase activity. Mutations in Ras result in impaired GTPase function causing to remain locked in the GTP-dependent 'on' state; this malfunction leads to increased transcription, translation, cell cycle progression and cell survival. Sorafenib is a novel antitumoural agent showing a dual action on RTKs (VEGFR and PDGFR) and on Raf, resulting a sequential inhibition of the MAPK pathway. LY294002, a morpholine derivative of quercetin, is a potent and reversible inhibitor of PI-3 K, even if it is less potent than wortmannin (a furanosteroid metabolite of the fungi *Penicillium funiculosum*), which acts irreversibly on the same target (Maira *et al.*, 2009).

mTOR and NF-κB are two other downstream targets of Akt activation. Both of them have a variety of roles in cell proliferation, survival, resistance to apoptosis, angiogenesis and invasion. Three non-cytotoxic compounds against mTOR-C1 have been tested *in vitro* with satisfying results and are currently studied in clinical trials: sirolimus (rapamycin) a natural macrocyclic polyketide; temsirolimus (CCI-779), a sirolimus derivative and everolimus (RAD001), a rapamycin-derived macrolide (Table 2).

The nuclear factor-κ light-chain-enhancer of activated B cells (NF-κB) is a dimeric protein complex controlling the transcription of DNA (Gilmore, 2006). Inactive NF-κB is located in the cytosol, bound to its physiological inhibitor IκB. Upon different extracellular stimuli, including activation of the PI-3 K-Akt cascade IκB is phosphorylated and rapidly degraded by proteasome. Free NF-κB is able to translocate into the nucleus where binds to specific response elements on DNA as homo- or heterodimer. Suppression of NF-κB activity, often aberrantly activated in cancer cells, limits their proliferation. A couple of agents that can inhibit the NF-κB activity with different mechanisms are onconase and bortezomib. Onconase is an inhibitor of NF-κB synthesis reducing the amount of tRNA levels in the cell. Bortezomib acts as a selective proteasome inhibitor particularly inhibiting the degradation of I-κB thus preventing the activation of NF-κB (Figure 2). Clinical trials showed that in single or combined administration with chemotherapeutic agents, bortezomib has pro-apoptotic activity in hMPM patients. The combined administration cisplatin-pemetrexed with bortezomid showed synergic activity when the proteasome inhibitor was administered before the cytotoxic agents (Gordon *et al.*, 2008).

The activation of STAT family proteins (seven members have been identified in mammals) is a fundamental event to mediate GF- and cytokine-induced cellular and biological processes included proliferation, differentiation, survival and development (Turkson, 2004). STAT activation by phosphorylation is controlled by RTKs through the activation of cytoplasmic JAKs. Phosphorylation induces dimerization of two STAT monomers that, from cytoplasm, accumulate into the nucleus where mediate gene transcription by binding to specific DNA response elements (Darnell Jr, 1997; Figure 2). In contrast to normal cells, many solid and haematological tumours contain constitutive STAT3 activity. Aberrant STAT3 promotes tumour progression, invasion and metastasis (Haura *et al.*, 2005). Numerous studies validated STAT3 as cancer drug target and several strategies have been explored. A variety of agents with different mode of inhibition of STAT functions have been developed (Peibin and Turkson, 2009): (i) direct targeting STAT3 protein by dimerization inhibitors (STA-21; Siddiquee *et al.*, 2007); (ii) DNA-binding domain inhibitors (IS3 295; Turkson *et al.*, 2005); (iii) indirect targeting of the upstream components of STAT3 pathway, TK phosphorylation inhibitors (JSI-124 and derivatives; Blaskovich *et al.*, 2003; Figure 2). Although not yet tested in hMPM due to their specific mechanism of action, the efficacy of this class of drugs in this tumour is very likely: STAT3 is overexpressed in hMPM (Achcar Rde *et al.*, 2007) and the JAK/STAT system was shown to be involved in hMPM cell lines proliferation (Buard *et al.*, 1998).

Drugs targeting other intracellular pathways and molecules

COX enzymes convert arachidonic acid into prostaglandins. COX-1 is constitutively expressed, whereas COX-2 is induced by inflammatory stimuli, cytokines, GFs and tumour promoters. The mechanisms of COX-2-mediated tumour development involve multiple mitogenic signalling pathways and molecules that mediate resistance to apoptosis, cell migration, invasion, angiogenesis and peroxidation of procarcinogens to carcinogens. Synthetic COX-2-selective non-steroidal anti-inflammatory drugs (NSAID), like celecoxib, show antitumour activity even if it does not correlate with COX-2 inhibition suggesting alternative mechanisms involved (Chuang *et al.*, 2008; Figure 2).

Histones acetyltransferase and deacetylase (HDAC) regulate the equilibrium between the acetylated or deacetylated configurations of histone proteins in the core of the nucleosomes. Deacetylated histones are linked to DNA, which results transcriptionally inactive. HDAC inhibitors, able to activate the transcription of genes involved in apoptosis induction, cell proliferation and angiogenesis suppression, are then promising anti-cancer agents. Two molecules are in course of development; vorinostat (suberoylanilide hydroxamic acid) studied as a new therapeutic option for many solid malignancies, and belinostat (PXD101) currently in late-stage clinical development for the treatment of hematological malignancies and solid tumours (Figure 2). In 2007, prelimi-

nary results were released from the phase II clinical trial of intravenous belinostat in combination with carboplatin and paclitaxel for relapsed ovarian cancer (Kelly *et al*., 2007).

Fenretinide (4-hydroxy(phenyl)retinamide) is a liposoluble vitamin, synthetic derivative of retinoic acid. In some cancer types, such as ovarian, mammary, renal, pulmonary carcinomas and gliomas, it was proposed to act mostly by promoting the intracellular accumulation of reactive oxygen species and mitochondrial disruption that results in cell death through apoptosis and/or necrosis (Wu *et al*., 2001).

Lonafarnib (SCH66336) and tipifarnib (R115777) are two experimental farnesyl-OH-transferase (FTase) inhibitor tricyclic compounds. By obstructing FTase in catalysing the transfer of a farnesyl group to the pre-Ras protein, they prevent the physiologic attachment of mature Ras to the cell membrane, step necessary to switch signals transferring from RTKs (Liu *et al*., 2007; Agrawal and Somani, 2009).

Targeting folate metabolism pathway: early and recent inhibitors (Table 1)

Methotrexate (MTX) and 5-fluorouracil (5-Fu), belonging to the antimetabolites anticancer class, have been administered for years to patients with several solid and systemic tumours such as colon, breast cancer and lymphomas. MTX exerts its action inhibiting dihydrofolate reductase (DHFR) an enzyme needed to reduce di-hydro- to tetra-hydrofolates. Conversely, 5-Fu exerts its activity through the inhibition of thymidilate synthase (TS), an enzyme catalysing the conversion of deoxyuridine 5′ monophosphate (dUMP) into deoxythymidine 5′ monophosphate (dTMP) by its metabolite 5-fluoro-2′-deoxyuridine-5′-monophosphate (5-FdUMP). Several designed TS-specific inhibitors, such as raltitrexed (ZD1694), were shown to have similar efficacy when compared to 5-Fu. In 1992, the multitargeted antifolate pemetrexed disodium (LY231514) was synthetized. Pemetrexed acts not only on both TS and DHFR but also on two other enzymes: glycinamide ribonucleotid formyl-transferase (GAR-FT) and aminoimidazole carboxamide ribonucleotide formyl-transferase (AICAR-FT) working in the folate cycle. Pralatrexate is an antifolate (a folate analogue metabolic inhibitor) has been designed to accumulate preferentially in cancer cells. Based on preclinical studies, researchers believe that pralatrexate selectively enters cells expressing reduced folate carrier type 1 (RFC-1), a protein that is overexpressed on certain cancer compared to normal cells (Figure 2).

Failure of single-agent and combined cytotoxic chemotherapy

As observed by Pinedo and Chabner, in their 'Cancer Chemotherapy/8, The EORTC Cancer Chemotherapy Annual', until 1985, only few reports described the use of chemotherapy for hMPM. Soresen *et al*. randomized doxorubicin and cyclophosphamide, using a crossover design, 32 previously untreated patients, but none of the patients obtained complete or partial remission (Soresen *et al*., 1985). In another trial using cisplatin in 24 patients, the response rate (RR) was of 12% (Mintzer *et al*., 1985). In 1991, a review focusing on the activity of single-agent and combination chemotherapy (Krarup-Hansen and Hansen, 1991), examining a huge number of schedules and trials of almost all the cytostatic compounds available at that time, came to the conclusion that results neither confirm any substantial activity nor justify the use of any single agent as standard therapy. Moreover, the data of combination are comparable with those of single-agent chemotherapy and no major difference was detected among the various combinations.

In the mid-1990s, De Vita, Hellman and Rosenberg in their '*Cancer: Principles and Practice of Oncology*' wrote: '. . . Response rates to standard single-agent remain difficult to define. . . Doxorubicin appears to have some activity against mesothelioma, although RRs vary considerably. MTX and 5-Fu may also have single-agent activity. Cisplatin as a single agent does not appear to be significantly active (10% RR in several phase II studies). RR for combination regimens range from 30% to 40%, in single institution series, to 0% to 14%, for cooperative group trials. RR for combinations with and without doxorubicin are similar (about 18%) to those obtained with single-agent doxorubicin'.

The main standard chemotherapic agents used were anthracyclines and related compounds (i.e. doxorubicin, epirubicin), alkylating agents (i.e. cyclophosphamide, cisplatin, carboplatin), vincas and related compounds (i.e. vincristine, vindesine), antimetabolites (i.e. 5-Fu, MTX), plus some biological agent (i.e. BCG, IFN-α, -β, -γ, IL-2). Also, combination chemotherapy regimens, tested in those years, did not offer better results: combinations of doxorubicin with either cisplatin, RR 28% (Zidar *et al*., 1983); cyclophosphamide and dacarbazine, RR 7% (Samson *et al*., 1987); or cyclophosphamide, dacarbazine and vincristine, RR 21% (Spremulli *et al*., 1977); combinations cisplatin-etoposide, RR 12% (Eisenhauer *et al*., 1988); cisplatin-vinblastine, RR 25% (Tsavaris *et al*., 1994); cisplatin-mitomycin C, RR 31% (Chahinian *et al*., 1993).

In 1998, a review of clinical data from studies using chemotherapy in patients with hMPM (Boutin *et al*., 1998) highlighted the still disappointing results. At best, objective responses after single-agent treatment were achieved in 20–30% of cases, without significant impact on overall survival. Despite their good reputation, antracyclines achieved responses in no more than 15% of cases; similarly, cisplatin alone at high doses achieved a RR of only 14–33%. High dosage (hd) MTX showed responses in 37% of cases. RR using combined-agent protocols (doxorubicin with cisplatin or cyclophosphamide, or vincristine; cisplatin with mitomycin or 5-Fu; MTX-hd with vincristine and so on), achieved 25–30% (for review, see Favoni and Florio, 2011).

Molecular targeted therapy – preclinical studies

Established cell lines, post-surgical human specimens and animal models still represent unavoidable means to identify potential new drugs for hMPM.

The largely disappointing results obtained with classical cytotoxic agents for the treatment of hMPM, prompted in the past years several preclinical studies to (i) identify additional or alternative mechanism(s) of action for known agents; (ii) provide insights into the *in vitro* activity of novel compounds;

(iii) suggest more effective clinical strategies. Moreover, all these studies represent the basis for the development of molecular targeted therapy.

In this paragraph, we report a representative selection of the most significant studies uncovering the pharmacological modulation of key molecular pathways involved in hMPM carcinogenesis.

Cytotoxic agents

In the past years, several studies demonstrated the *in vitro* cytotoxicity of cisplatin and doxorubicin on several hMPM cell lines and the potentiation of their effects when co-administered with several sensitizing agents (de Cupis *et al.*, 2003; Tagawa *et al.*, 2006; Cao *et al.*, 2007). Similar results were also obtained in xenografted tumours and now cisplatin is commonly used as front-line agent for hMPM medical therapy [for review see (Favoni and Florio, 2011)]. However, the still disappointing clinical results prompted research for novel, more effective drugs.

Second-generation drugs versus standard cytotoxic agents

The so-called second generation anticancer agents (taxanes, gemcitabine, fenretinide, topoisomerase I inhibitors) are more toxic than cisplatin for several histologically heterogeneous hMPM cell lines, through a mechanism that only partially involves the activation of apoptosis (de Cupis *et al.*, 2003). In other studies using four cell lines (M14K, M24K, M25K, M38K), it was confirmed the sensitivity of hMPM cells to docetaxel, paclitaxel, gemcitabine and to the irinotecan active metabolite SN-38, used as single agents. Although a high variability among the lines was observed, docetaxel, paclitaxel and SN-38 showed in most cases higher efficacy than gemcitabine (Ollikainen *et al.*, 2000). Pemetrexed, a multitargeted folate pathway inhibitor, induced hMPM cell toxicity in several cell lines, although showing very different IC_{50} (ranging from 22 nM to 10 μM), independently from the folate receptor expression levels (Nutt *et al.*, 2010). However, several insights into the mechanism of action of these drugs allowed the identification of the treatment sequence to be adopted to maximize the results. A study aimed to identify the optimal schedule for combination therapy of pemetrexed and gemcitabine, showed that the simultaneous exposure of MSTO-211H hMPM cells to both drugs was antagonistic, but a strong synergism was observed when pemetrexed preceded gemcitabine; the inverted sequence was again antagonistic (Nagai *et al.*, 2008). Comparable results were obtained in a study addressing the optimization of gemcitabine-cisplatin protocols using cell lines derived from pleural effusions of untreated hMPM patients (BR95 MG06, epithelioid; DE05, sarcomatoid and MM98 biphasic). Four-hour pretreatment with gemcitabine followed by 68-hour exposure to cisplatin was found to exert synergistic activity in both epithelioid and sarcomatoid hMPM cell lines, inducing a strong S-phase arrest that correlated with accumulation of double-strand breaks (Zanellato *et al.*, 2011). Thus, it was proposed that gemcitabine increases cisplatin-induced double-strand breaks by inhibiting DNA adduct repair.

EGFR family TK inhibitors

Approximately 70% of hMPMs show aberrant expression of EGFR, while in several cases, and in a subset of hMPM cell lines, both EGFR and TGF-α are expressed, suggesting an autocrine regulation of EGFR in hMPM (Destro *et al.*, 2006). In four EGFR-expressing cell lines derived from previously untreated patients with epithelial (H2461 and H2591), sarcomatoid (H2373) and biphasic (MSTO-211H) hMPMs, gefitinib significantly inhibited EGF-dependent cell signalling including phosphorylation of Akt and ERK1/2 (Janne *et al.*, 2002). Furthermore, treatment with gefitinib led to a significant dose-dependent reduction of colony formation (41–89% at 10 μM) when hMPM cells were grown in soft agar. A differential sensitivity among the cell lines was reported with MSTO-211H, H2461 and H2373 showing higher responsiveness than H2591. Gefitinib (10 μM) induced 89% of growth inhibition in H2373 hMPM cells, showing a dose-dependent arrest at the G1/S and a corresponding increase in $p27^{kip1}$ levels (Janne *et al.*, 2002). Gefitinib, erlotinib and canertinib (CI-1033, a potent inhibitor of both EGFR, HER-2 and ErbB-4), not only induced apoptosis but also inhibited migration and matrix metalloprotease production in M14K, ZL34 and SPC212 hMPM cells, confirming the potential effectiveness in targeting multiple components of EGFR family in hMPM (Liu and Klominek, 2004). In another study (Favoni *et al.*, 2010), gefitinib inhibited EGF-induced proliferation in two hMPM cell lines, derived from pleural effusion (IST-Mes2) or tumour biopsy (ZL55). Gefitinib treatment induced cell cycle arrest in both cell lines, while apoptosis was observed only for high concentrations (over the IC_{50} values that were 20 and 10 μM for IST-Mes2 and ZL55, respectively) and prolonged drug exposure (72 h) (Favoni *et al.*, 2010). As far as intracellular signalling, gefitinib inhibited both EGFR and ERK1/2 activation, being maximal at drug concentrations that induce cytostatic effects, suggesting that the proapoptotic activity of gefitinib was independent from EGFR inhibition. Interestingly, gefitinib treatment increased membrane EGFR content, through membrane stabilization of inactive receptor dimers that were shown to be induced by the drug also in the absence of EGF (Favoni *et al.*, 2010; Barbieri *et al.*, 2011). Thus, the formation of inactive EGFR dimers may represent an additional mechanism of the antiproliferative activity of gefitinib. Gefitinib also induced cytotoxic effects in MSTO, H28 and H226 hMPM cell lines with IC_{50} ranging from 5 to 20 μM (Nutt *et al.*, 2009). The possibility to obtain a synergistic effect by the co-treatment of IST-Mes2 and ZL55 cells with gefitinib in the presence of cisplatin and gemcitabine was addressed in a recent study. However, no additivity was shown by isobologram analysis (Barbieri *et al.*, 2011), confirming disappointing results recently emerged from clinical studies (Govindan *et al.*, 2005; Garland *et al.*, 2007).

Treatment with lapatinib, a dual inhibitor of EGFR/ErbB2, caused G1/S cell cycle arrest and growth inhibition in only two (H2373 and H2452) out of 10 hMPM cell lines treated, showing IC_{50} values of 1 and 0.8 μM, respectively (Mukohara *et al.*, 2005b). Moreover, lapatinib treatment caused a time-dependent decrease in active Akt and/or ERK1/2 levels and an increase in $p27^{kip1}$ expression. The combination of lapatinib with U0126, LY294002 or rapamycin (MEK, PI-3 K and mTOR inhibitors, respectively) caused greater growth inhibition

than either drug alone in the sensitive cell lines, while this did not occur in the resistant cells (Mukohara *et al.*, 2005b). These findings suggest that EGFR alone is a therapeutic target for a minority of hMPM, but combining EGFR inhibitors with signal transduction inhibitors will enhance the overall effectiveness.

PDGFR TK inhibitors

PDGF is a potent mitogen for connective tissue cells and mesothelial cells. PDGF receptors (PDGFRs) are differentially expressed in hMPM cells compared with normal mesothelium, with the former expressing PDGFR-β and the later PDGFR-α (Langerak *et al.*, 1996). However, different studies reported that, *in vivo*, PDGFR-β is expressed only in about 40% of hMPM specimens (Porta *et al.*, 2007). *In vitro* experiments demonstrated that imatinib, an inhibitor of PDGFR TK, induced apoptosis via the inhibition of the Akt/PI-3 K pathway in hMPM cell lines (IST-Mes2, REN, MMP), enhances sensitivity of hMPM cell lines to chemotherapy and selectively synergizes with gemcitabine and pemetrexed in PDGFR-β-positive mesothelioma cells (Bertino *et al.*, 2007b; 2008). Similar results were also showed *in vivo*: the combined treatment with imatinib and gemcitabine decreased tumour proliferation rate, increased the number of apoptotic cells and prolonged survival of immunodeficient mice orthotopically injected with hMPM REN cells, as compared to gamcitabine alone (Bertino *et al.*, 2008).

VEGF-VEGFR inhibitors

There is a strong rationale to inhibit VEGF signalling in hMPM since these patients show the highest VEGF levels of any solid tumour patient (Linder *et al.*, 1998). VEGF and its receptors (VEGFR-1 and VEGFR-2) are overexpressed in hMPM tissues compared with normal mesothelial cells, hMPM cell lines, pleural effusions and high levels of VEGF are detected in serum of mesothelioma patients (Kumar-Singh *et al.*, 1999; Strizzi *et al.*, 2001). In this context, VEGF may also act in a functional autocrine loop that directly stimulates the growth of hMPM cells. Indeed, VEGF production could have an impact on patient survival, not only by promoting tumour angiogenesis but also by directly stimulating tumour growth. The anti-VEGF antibody bevacizumab in association with pemetrexed inhibited the growth of different hMPM cell lines orthotopically xenotransplanted in immunodeficient mice, showing a synergistic effect. The treatment also caused the suppression of the pleural effusion and prolonged survival of the mice (Li *et al.*, 2007). VEGFR-2 inhibitors vandetanib (that also targets EGFR and RET) and sunitinib (that also targets PDGFR) showed a significant cell growth inhibition in MSTO, H28 and H226 cells showing a significantly low IC_{50} (3–7 μM) that, however, was mediated by inhibition (dephosphorylation) of VEGFR-2 only, in H226 cells (the other cell lines did not express this receptor; Nutt *et al.*, 2009). In the hMPM cell line, EHMES-10 (expressing VEGF, VEGFR-2, EGFR and RET/PTC3 oncogenic rearrangement), vandetanib induced apoptosis and inhibited cell proliferation with an IC_{50} of 0.3 μM (Ogino *et al.*, 2008). As far as *in vivo* studies is concerned [orthograft of EHMES-10 cells in severe combined immunodeficiency (SCID) mice], it was shown that once-daily oral treatment with vandetanib inhibited tumour angiogenesis and reduced significantly the growth of thoracic tumours and the production of pleural effusions, resulting in the prolonged survival of mice (Ogino *et al.*, 2008). In contrast, gefitinib showed no effects against EHMES-10 cell growth both *in vitro* and *in vivo*. These results suggest that vandetanib can target RET-dependent tumour cell proliferation and survival and VEGFR-2-dependent tumour angiogenesis (Ogino *et al.*, 2008). From studies using H2052, H2452, H28 and MSTO-211H hMPM cells treated with carboplatin, pemetrexed and several targeted compounds (gefitinib, erlotinib, sorafenib, vandetanib), vandetanib emerged as the compound with the most potent cytotoxic activity, showing a synergistic effect with both carboplatin and pemetrexed. Vandetanib effect was mediated by the blockade of Akt phosphorylation and activation of the apoptotic program. The high cytotoxic activity and the relevant synergism with carboplatin and pemetrexed, allowed the authors to propose the association of these compounds with vandetanib in clinical trials (Giovannetti *et al.*, 2011).

Two other VEGFR inhibitors (KRN633, which inhibits VEGFR-1, -2 and -3 with similar kinetics, and ZM323881, which is highly selective for VEGFR-2 (Endo *et al.*, 2003; Nakamura *et al.*, 2004) synergize with lovastatin (that affect VEGFR and EGFR ligand activation interfering with their internalization) in the inhibition of H28 and H2052 hMPM cell survival (Zhao *et al.*, 2010). Finally, the dual TK inhibitor E7080, active on both VEGFR-2 and VEGFR-3, significantly inhibited the proliferation of MSTO-211H, NCI-H290 and Y-MESO-14 hMPM cell lines *in vitro*, while *in vivo*, after hMPM cell xenograft, significantly prolonged mouse survival, which was associated with decreased numbers of tumour-associated vessels and proliferating hMPM cells within the tumour (Ikuta *et al.*, 2009).

HGF/c-MET inhibitors

HGF (also known as scatter factor) is now recognized as a critical factor for the development of a malignant phenotype, including tumour cell invasion and metastasization. c-MET, the HGF receptor, is expressed at higher level in hMPM tissues than in normal pleura (Klominek *et al.*, 1998) and result to be autocrinally activate in response to SV40 (Cacciotti *et al.*, 2001). Moreover, an autocrine HGF/c-MET loop has been detected in several hMPM cell lines. SU11274, a small molecule with c-MET TK inhibitory activity, inhibited cell proliferation in MSTO-211H, H513, H2596 and H28, but not in H2052, H2452, and in non-malignant Met-5A cells. Interestingly, the non-responding cells were also insensitive to the proliferative effects of HGF. In H28 cells SU11274 treatment also significantly affected cell migration (Jagadeeswaran *et al.*, 2006). NK4 is another antagonist of c-Met that show also antiangiogenic activity through binding to perlecan. NK4 inhibited EHMES-10 cell growth *in vitro* and *in vivo* inducing, in the latter setting, the activation of the apoptotic programme, primarily through the inhibition of tumour angiogenesis (Suzuki *et al.*, 2010). c-Met inhibitor PHA-665752 inhibits hMPM cell growth with a low IC_{50} (about 1–2 μM) only in those cell lines (for example H2461 and JMN-1B) in which an autocrine HGF/c-Met loop was present (Mukohara *et al.*, 2005a), indicating that targeting this GF/receptor system may represent a valuable therapeutic option only in particular clinical conditions.

Multiple RTK inhibitors

The discouraging results obtained in clinical trials notwithstanding the brilliant *in vitro* results, led to the hypothesis that, *in vivo*, multiple RTK are activated recruiting overlapping pathways and thus compensating the antagonism induced on individual receptors by selective molecules. In a large study on 20 hMPM cell lines, several EGFR family components, c-Met, PDGFR and VEGFR were found to be constitutively activated, often in the same cells (Kawaguchi *et al.*, 2009). Accordingly, the co-treatment with EGFR and c-Met inhibitors (PD153035 and SU11274, respectively) resulted in a highly synergic response (Kawaguchi *et al.*, 2009). Thus, newly developed multitarget molecules have been assayed in preclinical testing. One of these compounds, already in advanced stage of evaluation for multiple solid tumours is sorafenib. Sorafenib inhibits PDGFR and VEGFR-2 TK activity and the Ras/Raf/MEK/ERK signalling pathway (a common pathway involved in the proliferation of all the RTK, see Figure 1) acting at the level of the serine/threonine kinase B-Raf and MEK (Iyer *et al.*, 2010). The efficacy of sorafenib *in vitro* as monotherapy and in combination with TRAIL, the TNF-related pro-apoptotic ligand, was studied in six hMPM cell lines. Sorafenib showed pro-apoptotic effects in all the cells lines, causing, within 3 h of treatment, dephosphorylation and/or downregulation of a number of known pro-survival molecules: MCL-1L, c-FLIPL, survivin and cIAP, while no involvement of caspases was detected (Katz *et al.*, 2009). Conversely, TRAIL was not active as single agent but significantly increased sorafenib cytotoxicity. *In vivo*, the therapeutic efficacy of the combination sorafenib/TRAIL on human tumour xenografts in nude mice was confirmed, suggesting its potential development for clinical testing (Katz *et al.*, 2009). In STO cells, which express EGFR, the co-expression of the cognate ligands TGF-α, induced an autocrine/paracrine loop resulting in the constitutive activation of ERK1/2, Akt and mTOR. *In vitro*, cytotoxicity studies showed STO cell line to be resistant to gefitinib but sensitive to sequential treatment with everolimus (mTOR inhibitor) and sorafenib acting on the signalling cascade, downstream of the receptor (Perrone *et al.*, 2010). Cediranib (inhibitor of VEGFR-1–2-3, c-Kit and PDGFR) also showed efficacy *in vitro* and now is in phase I/II studies (Nikolinakos and Heymach, 2008). In three human hMPM cell lines (MSTO-211H, NCI-H28 and NCI-H2052), in which a constitutive activation of c-src is present, the treatment with dasatinib (inhibitor of Bcr-Abl, src kinase family c-KIT, ephrin receptor and PDGFβ receptor) caused cell cycle arrest and apoptotic cell death and inhibition of cell migration, effects that were ascribed to the src inhibitory activity of the drug (Tsao *et al.*, 2007). Conversely, NCI-H2452 cells showed resistance to dasatinib treatment (Tsao *et al.*, 2007).

PI-3 K/Akt/m-TOR Inhibitors

PI-3 K/Akt/mTOR pathway, which is responsible of tumour aggressiveness and chemoresistance, was targeted with rapamycin in several human and murine hMPM cell lines that displayed elevated Akt activity, causing growth arrest in G1 (Barbone *et al.*, 2008). Similarly, combined treatment with PI-3 K inhibitor LY294002 and cisplatin inhibited cell proliferation and induced apoptosis with greater efficacy than either agent alone (Altomare *et al.*, 2005). The combination of the mTOR inhibitor sirolimus with cisplatin significantly increases cell death rate versus either drug alone in 4/12 of the hMPM cell lines tested (Hartman *et al.*, 2010). In primary cells from 15 hMPM patients grown as spheroids, mTOR inhibition using rapamycin reduced the resistance to gemcitabine-induced apoptosis, an effect that correlated with elevated mTOR expression in these samples (Wilson *et al.*, 2008). The inhibition of mTOR signalling was shown to be responsible of temsirolimus antiproliferative effects on six hMPM cell lines *in vitro* and *in vivo*, after xenotransplant in SCID mice. Interestingly, MPM cells showing intrinsic or acquired resistance to cisplatin were more responsive to temsirolimus. Accordingly, cisplatin and temsirolimus exerted synergistic inhibition of mTOR signalling and enhanced the inhibition of proliferation and the activation of apoptosis in these hMPM cell lines (Hoda *et al.*, 2011). As far as another mTOR inhibitor, everolimus, two phase II studies are ongoing although no results were still disclosed (Favoni and Florio, 2011).

COX inhibitors

The treatment of three hMPM cell lines (MSTO-211H, NCI-H2052, NCI-H2452) with non-specific (sulindac and flurbiprofen) or selective (DuP-697 and NS-398) COX-2 inhibitors (COXIB), and three cytotoxic agents (cisplatin, vinorelbine and pemetrexed), showed that COXIBs increased the sensitivity of hMPM cells to pemetrexed cytotoxic effects (O'Kane *et al.*, 2010). In another study (Stoppoloni *et al.*, 2010), five hMPM cell lines (NCI-2452, MPP89, IST-Mes1, IST-Mes2 and MSTO-211H) were treated with rofecoxib (selective COX-2 inhibitor) and gefitinib (selective EGFR TK inhibitor), alone and in combination. Only MPP89, IST-Mes1 and IST-Mes2 showed sensitivity to rofecoxib and gefitinib individual treatments. Unexpectedly, co-administration of these drugs caused a synergistic cytotoxicity only in IST-Mes2, the line more sensitive to each individual drug, but was antagonistic in IST-Mes1 and MPP89 cells. Thus, it was proposed that rofecoxib and gefitinib exert cell type-specific effects that may vary among different hMPM cells (Stoppoloni *et al.*, 2010).

NF-κB pathway inhibitors

Two molecules are currently studied: bortezomib and onconase (ranpirnase), acting through different mechanisms.

Bortezomib through the blockade of 20S proteasome impairs NF-κB activity and the degradation of cdk inhibitors (Adams *et al.*, 1999). In four hMPM cell lines with different histological characteristics (MS589 and JMN, biphasic; H2052, sarcomatoid and H28, epithelial), bortezomib causes a G2/M and G1/S cell cycle arrest, though the stabilization of $p21^{waf1}$, and $p27^{kip1}$ (Gordon *et al.*, 2008). Co-treatment with cisplatin demonstrated that bortezomib induce a synergistic effect at high doses, but antagonistic effects at low doses (Gordon *et al.*, 2008). It was speculated that low doses bortezomib may affect degradation of survival/antiapoptotic proteins (x-linked inhibitor of apoptosis protein, survivin) thus antagonizing cisplatin cytotoxicity (Zucali *et al.*, 2011). However, a concentration-dependent potentiation of cisplatin and pemetrexed cytotoxicity was observed when bortezomib was administered prior to these drugs (Borczuk *et al.*, 2007; Gordon *et al.*, 2008). *In vivo*, bortezomib administration caused tumour growth inhibition in a xenografts model in

which tumours reproduce some mesothelioma clinical features (Sartore-Bianchi *et al.*, 2007). These results and, in particular, bortezomib inhibition of tumour spreading to diaphragmatic surface and formation of malignant effusions, along with its safety, supports the test of bortezomib for the treatment of hMPM. Ranpirnase (onconase) originally isolated from oocytes of the northern leopard frog (*Rana pipiens*), is a member of the pancreatic RNase A superfamily of ribonucleases (Lee, 2008). Ranpirnase exerts antiproliferative and cytotoxic effects *in vitro* and *in vivo* and has been shown to act synergistically with different cancer therapeutic agents. The cytotoxic and cytostatic effects of ranpirnase are the consequence of tRNA degradation that results in the disruption of protein translation and the induction of programmed cell death (apoptosis) (Lee, 2008). Three immortalized hMPM cell lines (H2959, H2373 and H2591) exposed to ranpirnase (20 $\mu g \cdot mL^{-1}$) significantly decreased cell count and *in vitro* invasiveness (Matrigel invasion assay) (Goparaju *et al.*, 2011). NF-κB1 (p50) expression and downstream targets were decreased after ranpirnase treatment. Ranpirnase treatment caused a significant decrease in cell proliferation, invasion and in the expression of certain miRNAs. Hsa-miR-17* was significantly up-regulated and hsa-miR-30c was significantly down-regulated by ranpirnase treatment in all cell lines. Recapitulation of this miRNA expression pattern expressing 'hsa-miR-17* mimic' and 'hsa-miR-30c inhibitor' resulted in down-regulation of NF-κB1 and reduced malignant behavior in functional assays. Thus, ranpirnase was reported to exert anti-tumour activity in hMPM cells through miRNA modulation of NF-κB activity (Goparaju *et al.*, 2011). To delve deeper in the mechanism of action of ranpirnase, microarray analysis was used to compare gene expression profiles in human hMPM cell lines before and after exposure to 5 $\mu g \cdot mL^{-1}$ onconase for 24 h (Altomare *et al.*, 2010). Ranpirnase treatment consistently resulted in up-regulation of IL-24, previously known to have tumour suppressive activity, as well as ATF3 and IL-6. Induction of ATF3 and the pro-apoptotic factor IL-24 by ranpirnase was highest in the two most responsive hMPM cell lines (M29 and M35), as defined by DNA fragmentation analysis. In addition to apoptosis, gene ontology analysis indicated that oncogenic pathways affected by ranpirnase include also MAPK signalling, cytokine-cytokine-receptor interactions and Jak-STAT signalling (Altomare *et al.*, 2010).

Peroxisome proliferator-activated receptor-γ (PPAR-γ) agonists

Troglitazone, a PPAR-γ agonist, proposed as anti-diabetic drug, was shown to have anticancer activity against several cancer cell lines *in vitro* and *in vivo* (Lehmann *et al.*, 1995). Troglitazone alone inhibited hMPM cell line (EHMES-10 and MSTO-211H) proliferation in a dose-dependent manner via induction of G1 arrest in the cell cycle and apoptosis *in vitro*, and inhibited the production of thoracic tumours and pleural effusion in EHMES-10 cell-bearing SCID mice. In both *in vitro* and *in vivo* experimental setting, the combination of troglitazone and cisplatin showed an additive inhibitory effect on hMPM cell growth (Hamaguchi *et al.*, 2010).

HDAC inhibitors

The proapoptotic activity of vorinostat was reported on three cell lines and fresh biopsies derived from hMPM patients in association with valproate (sodium salt of 2-propylpentanoic valproic acid), an antiepileptic drug known to possess cytotoxic activity to many different cancer types also through its histone deacetylase inhibitor activity (Isenberg *et al.*, 2007). Vorinostat increased apoptosis induced by cisplatin and pemetrexed so this agent was proposed to be a valid option to improve response to the standard chemotherapic regimens (Vandermeers *et al.*, 2009).

Other sensitizing agents

A different approach studied to improve hMPM cytotoxicity consists in looking for agents able to potentiate cisplatin effects. Cisplatin-induced cell death and apoptosis was greatly enhanced using two monoclonal antibodies (lexatumumab and mapatumumab) able to activate the TNF-related apoptosis-inducing ligand receptor 1 (TRAIL-R1). However, the maximal effects were obtained when the treatment with lexatumumab and mapatumumab was performed after cisplatin addition, with the reverse sequence much less effective (Belyanskaya *et al.*, 2007).

Hexamethylene bisacetamide showed *per se* high cytotoxicity for MM-B1 and MM-E1 cell lines, but highly potentiate doxorubicin cytotoxic effects and overcome doxorubicin resistance in MM-EI cells (Palumbo *et al.*, 2004).

Future perspectives: the tumour-initiating cell model

Although the newly developed targeted drugs allowed potential improvement in hMPM pharmacological approach, the sometime contrasting results obtained using established cell lines and the not always good correspondence from preclinical and clinical trials, are still a significant issue limiting the potential of traslational studies.

The high variability of cytotoxic or targeted drugs among the different cell lines but also *in vivo* in clinical trials among hMPM patients, supported the idea that individual biological differences between tumours exist, regulating the drug sensitivity. These differences found their biological correlate in the cancer stem cell theory. The recently developed protocols to isolate and expand putative cancer stem cells from several malignancies, included hMPM (Lee *et al.*, 2006; Cortes-Dericks *et al.*, 2010; Kai *et al.*, 2010; Melotti *et al.*, 2010; Clevers, 2011; Frei *et al.*, 2011; Ghani *et al.*, 2011), is opening a potential new perspective also for preclinical studies.

Recent theories propose that those tumours are organized in a cellular hierarchy maintained by a small subpopulation of cells capable of tumour initiation and maintenance. The tumour-initiating cells (TICs) can be operationally defined as cells able to give rise to a tumour when transplanted in immunodeficient mice. Tumour initiation can be explained by the stochastic model or by the cancer stem cell model. In the first, every cancer cells can initiate and propagate the tumour. The theory of the 'cancer stem cells' (CSC) suggests that a subpopulation of malignant cells with stem cell properties can give rise to a hierarchy of proliferative and progressively differentiating cells, originating the intra-tumour heterogeneity. The existence of putative CSCs has been confirmed in several types of tumours, including leukaemias,

mammary and lung cancers and brain tumours, exploiting known properties of normal stem cells, such as exclusion of the fluorescent dye Hoechst 33342 (side population) or differential expression of surface markers, such as CD24, CD34, CD38, CD44 and CD133 (Clevers, 2011). Stemness and tumourigenicity of this subpopulation have been demonstrated by injecting cells expressing these markers, in immunodeficient (NOD-SCID) mice (Figure 3). In every case, a very small number of cells are sufficient to give rise to a tumour that maintains the heterogeneity of the original neoplasia. According to the 'stem cell origin of cancer' hypothesis, stem cells, or other cells that acquired the ability to self-renew, accumulate genetic alterations over long periods of time, escape from the control of their environment and give rise to cancerous growth. The essential feature of the CSC model is that tumours are hierarchically organized such that TICs and non-TICs are phenotypically distinguishable and it should be possible to purify a population with the unique ability to generate serially transplantable tumours that re-create the heterogeneity of the patient malignancy. If tumours are maintained by quiescent CSCs, it might explain why many treatments that reduce tumour mass fail to cure cancer patients. Generally, chemotherapy target fast-growing cells and might leave the slow cycling stem cells untouched. Because they may be relatively protected from current treatment strategies, CSCs are thought to be responsible for resistance to chemotherapy and the recurrence of disease. The biological differences between primary tumours and the established cell lines derived from them limit the value of *in vitro* cellular models for the evaluation of novel therapeutic agents. The procedures used for deriving cell lines might have limited the persistence of heterogeneous cultures to rare cases, while the methods currently used for culturing CSCs derived from primary tumours and their *in vivo* expansion produce phenotypically diverse non-tumourigenic cells, recapitulating at least some of the heterogeneity in the tumours from which they derive. Moreover, after long-term cultures, cell lines may acquire additional genetic mutations selected to adapt them to the artificial environment. The resulting growth rate of human tumour cell lines is much more rapid (doubling times of one or few days) than that of primary human tumours (doubling times of 1–3 months) (Tubiana and Malaise, 1976); therefore, cell lines are more likely to respond to antiproliferative agents than the original tumours *in vivo*. In addition, most of the existing tumour cell lines induce experimental tumours only when injected in high number, suggesting that most cells within a given cell line are not able to initiate and maintain the tumour *in vivo*. Furthermore, long-term culture of cell lines often select for an homogeneous population representing the clone best fitted for the culture conditions, while in most malignancies have been recognized high intra-tumour heterogeneity with respect to genotype, gene expression profile, cell morphology, proliferation rate, ability to metastasize, sensitivity to drugs, dependence on growth signals and tumour initiation capacity (Michor and Polyak, 2010).

At least for some tumours (glioblastoma), it has been demonstrated that culturing tumourigenic stem cells in a chemically defined medium more closely mirror the phenotype and genotype of primary tumours than do serum-cultured cell lines (Lee *et al.*, 2006).

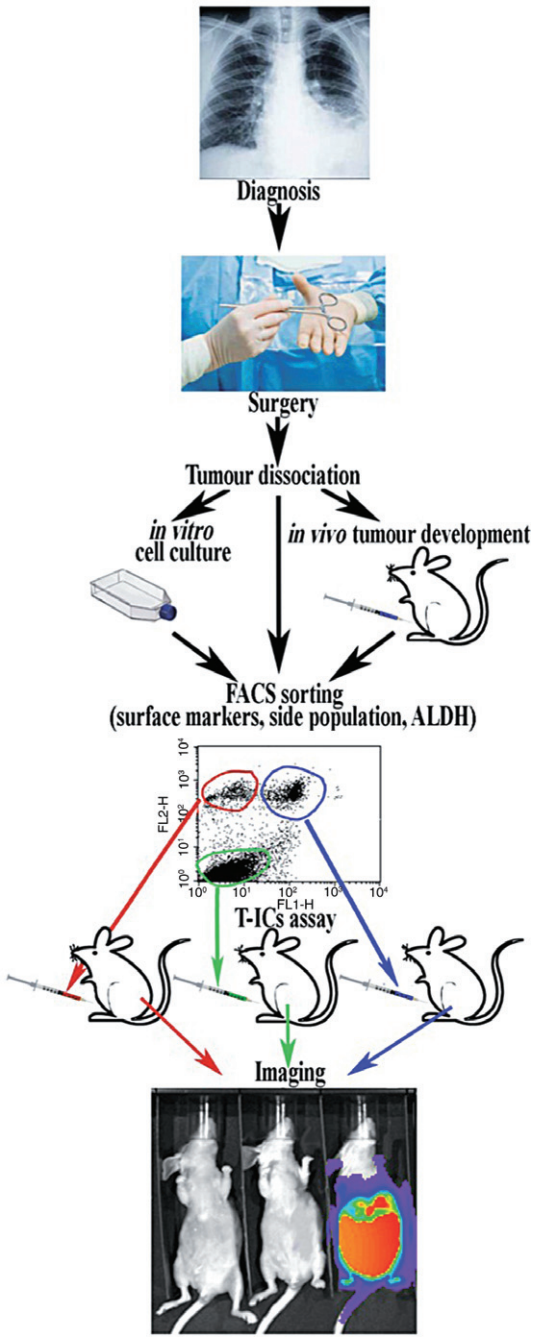


Figure 3

Diagram outlining the experimental procedures commonly used to identify the cancer stem cells in solid tumours. After the diagnosis, the tumour mass is surgically removed. The tumour sample is dissociated and then cultured *in vitro* and/or orthotopically transplanted into immunodeficient mice. Tumour cells expanded *in vitro*, recovered from xenotransplant or derived from primary tumours, are analysed. Subgroups of similar cells are identified according to their cell surface markers (side population, ALDH expression or other methods are also used) and purified using flow cytometry techniques. Isolated subpopulations are orthotopically transplanted into individual recipient mice to assess their tumour-initiating capacity. Tumour development is verified by visual inspection or by imaging techniques. ALDH, aldehyde dehydrogenase.

The derivation of cell lines with different cell morphologies from hMPM tumours has been reported. Although different hMPM cell lines currently used represent different tumour histotypes, rarely their sensitivity to specific drugs predict the *in vivo* responses in mesothelioma patients.

Several attempts have been made to verify if hMPM follows the CSC model. Melotti *et al*. (2010) established primary hMPM cell cultures able to engraft, after pseudo-orthotopic intraperitoneal transplantation, in immunodeficient mice and maintain this ability to after serial transplantation. *In vivo*, these cells mainly grow as free-floating cells that eventually aggregate, recapitulating a characteristic that is thought to be typical of normal mesothelial stem/progenitor cells. The cells were grown under low-serum conditions and extensively characterized with cytogenetic, immunohisto- and cytochemical analyses and PCR. The expression of stemness markers was also assayed and it was found that BMI-1 was positive, while Sox2, Nanog, Oct4 and CD133 were negative. The cell lines derived are also highly enriched in TIC when compared to hMPM cell lines, which require three orders of magnitude more cells to obtain the same take rate in pseudo-orthotopic intraperitoneal transplantation in immunodeficient mice. No successful attempt to enrich in tumourigenic cell has been described. Kai *et al*. (2010) evaluated the efflux of the DNA-binding dye Hoechst 33342 from different established cell lines that can be identified as a side population (SP) in flow cytometry that have been shown to be enriched for cells with stem cell properties. Sorted mesothelial SP cells exhibited enhanced proliferation potentials and higher expression of stem cell genes, compared to non-SP cells. However, *in vivo* tumourigenic assay injecting SP and non-SP sorted cells in NOD/SCID mice produced tumour mass not statistically different based on cell subpopulations injected. Cortes-Dericks and colleagues (Cortes-Dericks *et al*., 2010) characterized by PCR the expression of the CSC markers CD133, Bmi-1, uPAR and ABCG2 in three established cell lines and their enrichment response to cisplatin and pemetrexed treatments. The expression of some stem cell marker was increased in cells surviving the chemotherapeutic treatment, indicative of their potential role in chemoresistance. CSC markers were not used to select tumourigenic sub-populations. Ghani *et al*. (2011) established new hMPM cell lines from surgical samples by serial transplantation into NOD/SCID mice and analysed the expression of 106 putative CSC markers. They found that cells CD9+ and CD24+ have higher potential to generate spheroid colonies than negative cells and generate larger tumours in mouse transplantation assay. Frei *et al*. (2011) used the Dye Cycle Violet, a fluorescent dye less toxic than Hoechst 33342, in order to identify SP in hMPM primary cultures from xenografts. SP and non-SP cells were tested for self-renewal, chemoresistance and tumourigenicity. Tumourigenicity was assayed in SP and non-SP cells and only a tendency of more frequent tumour formation with the SP fraction was observed. In SP-derived tumours compared to non-SP tumours were observed an increased resistance to a high concentration of cisplatin.

Thus, although the continuum research advancement, until now, not clear evidences were still found about the hypothesis that hMPM contain a non-tumourigenic subpopulation of cells. In the absence of specific markers that can distinguish tumourigenic from non-tumourigenic cancer cells, there is no evidence that a cancer follows the CSC model. Without this evidence, it would be possible that all cancer cells have the same stochastic probability of proliferating or forming a tumour. Irrespective of the question of whether or not hMPM follows the hierarchical model that is still unresolved, the use of TICs isolated from hMPM and cultured in stem cell-permissive medium is highly advisable for pharmacological studies. Thus, TIC cultures from hMPM could represent a step ahead as experimental cell model to be added to classical hMPM established cell lines, especially as far as the evaluation of drug sensitivity and changes in pathways activation induced by pharmacological treatments. If these premises will be confirmed, TIC studies will provide useful biological information hopefully reflecting the individual characteristics of each patient's disease also in terms of drug sensitivity, an important step towards a personalized therapy.

Conclusions

With the awareness that systemic therapy is likely the only effective therapeutic option for hMPM patients, in the last decades, many cytotoxic compounds have been tested, in both single- and combined-administration regimens, in laboratory as well as in clinical trials. Phase II/III studies have identified pemetrexed or gemcitabine in combination with platinum derivatives as front-line, even if still unsatisfactory, treatments. The activity of those combinations is better if compared to historical results but, since RR > 20% is a rare event, it remain modest and further improvements in drug development are strongly needed. Advances in knowledge of molecular and biological mechanisms that regulate the growth and the spread of hMPM, as well as the identification of new tumour markers are leading to the design, synthesis and development of novel more active targeted agents. The so-called 'bio-immunological therapies' are under investigation at preclinical levels. To this goal, new experimental models of hMPM, such as TIC cultures, begin to be established to provide additional preclinical tools for development of new therapeutic modalities: some of them are promising. In other tumour models (i.e. glioblastoma), using TIC cultures the specific sensitivity of individual tumours to TKI was demonstrated (Griffero *et al*., 2009). Thus, the increasing knowledge of crucial genetic and cellular abnormalities in hMPM, in combination with better experimental models will help in developing innovative strategies and therapeutic modalities to overcome the poor current standard pharmacological protocols.

In this review, we have described the reported activity of a series of new agents, together with several well-known cytotoxic drugs; currently most of them are under *in vitro* investigation in hMPM combined with the standard 'old' drugs. Parallely, an interesting fall-out on clinical studies using the early novel compounds, together with some synergistic combination emerging from preclinical studies, is already detectable.

Conflict of interest

The authors state no conflicts of interest.

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