

Preliminary data suggestive of a novel translational approach to mesothelioma treatment: imatinib mesylate with gemcitabine or pemetrexed

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Background: Malignant mesothelioma is a cancer which is refractory to current treatments. Imatinib mesylate is a selective inhibitor of tyrosine kinases such as bcr-abl, c-Kit, c-Fms and platelet derived growth factor receptor β (PDGFR β). PDGFR β is often overexpressed in mesothelioma cells and is a therapeutic target for imatinib in some solid tumours. A study was undertaken to assess whether imatinib alone or combined with chemotherapeutic agents may be effective for treating mesothelioma.

Methods: Cultures from mesothelioma MMP, REN and ISTMES2 cell lines were treated with imatinib alone or in combination with a chemotherapeutic agent.

Results: Imatinib induced cytotoxicity and apoptosis selectively on PDGFR β positive mesothelioma cells via blockade of receptor phosphorylation and interference with the Akt pathway. Of the chemotherapeutic agents tested in combination with imatinib, a synergistic effect was obtained with gemcitabine and pemetrexed.

Conclusions: This study provides a rationale for a novel translational approach to the treatment of mesothelioma which relies on enhancement of tumour chemosensitivity by inhibition of Akt.

Malignant mesothelioma (MMe) is an asbestos-related tumour, the incidence of which is expected to rise dramatically in Europe.¹ In the USA the incidence of MMe has already increased by 90% in the last few years.² Because of its biological aggressiveness, MMe is nearly always fatal except in rare less advanced cases, with a median survival of 12.6 months.³

A number of growth factors such as hepatocyte growth factor (HGF),^{4,5} vascular endothelial growth factor (VEGF)^{6,7} and insulin-like growth factor-1 and -2⁸ have been shown to play a significant role in the development and progression of MMe. Moreover, several studies have reported a crucial role for platelet derived growth factor (PDGF) A and B in MMe cell growth.⁹ A high level of expression of PDGF receptor β (PDGFR β) was seen in MMe cells but not in normal human mesothelial cells (HMC), mostly expressing PDGFR α .¹⁰ Furthermore, increased expression of PDGF A and B was detected at higher levels in MMe cells than in HMC,¹¹ and a significant reduction in MMe cell growth or migration was observed by blocking PDGF A and B.¹² Expression of c-Kit on MMe cells has been demonstrated by some authors, although its role in this tumour is controversial.^{13–15} Production of macrophage colony stimulating factor (M-CSF) by mesothelial cells has already been reported,¹⁶ and inhibition of c-Fms receptor by imatinib has been demonstrated.¹⁷

Many cytokines are released in the microenvironment by tumour stromal cells, and PDGF paracrine stimulation has been demonstrated in human tumours, particularly MMe.^{18,19} PDGFR β activated by PDGF B can induce PI3K/Akt signalling²⁰ which seems to be crucial for the survival of MMe cells.²¹

Imatinib is a selective inhibitor for a subset of tyrosine kinases including bcr-abl, c-Kit, PDGFR β ²² and c-Fms.¹⁷ PDGF receptors are expressed by several tumour cells and have been identified as potential therapeutic targets for imatinib.²³ In mesothelioma the extent of PDGFR β positive specimens ranges from about 30% to 45% in different studies.^{24,25} Although the therapeutic inefficacy of imatinib monotherapy

for mesothelioma has recently been reported,^{25,26} combination therapies with imatinib in mice yielded successful results.^{27,28} Gemcitabine, cisplatin, etoposide, doxorubicin and, more recently, pemetrexed have been shown to be active in the treatment of MMe. Combined treatment with cisplatin/pemetrexed and cisplatin/gemcitabine have been found to be more effective than each single agent used alone.²⁹ The aim of the present study is to investigate a translational approach which assesses the possible efficacy of imatinib as a single agent and in combination treatment for MMe.

METHODS

Cell cultures

Mesothelioma cells were derived from pleural effusions and stabilised in culture as continuous cell lines. MMP cells and primary HMC were characterised and cultured as previously described.⁵ REN cells were kindly provided by Dr Albelda and ISTMES2 were from the IST cell depository of Genoa (Italy).

Drugs

Imatinib was kindly provided by Novartis (Basel, Switzerland), and gemcitabine and pemetrexed were provided by Lilly (Indianapolis, Indiana, USA). Commercially available cisplatin, doxorubicin and etoposide were obtained from Alexis (Lausen, Switzerland).

Signal transduction

Cells were grown in 0.2% fetal bovine serum (FBS) for 24 h, then pre-incubated for 90 min in the presence or absence of 10 μ M imatinib. Purified PDGF (R&D, Milan, Italy), 20 ng/ml, was added to the same medium. Immunoprecipitation and

Abbreviations: HGF, hepatocyte growth factor; HMC, human mesothelial cells; LC₅₀, lethal concentration killing 50% of cells; M-CSF, macrophage colony stimulating factor; MMe, malignant mesothelioma; PDGF, platelet derived growth factor; PDGFR β , PDGF receptor β ; VEGF, vascular endothelial growth factor

immunoblotting were performed as previously described.⁵ Antibodies used were: PDGFR β , phospho-PDGFR β , c-Kit, c-Fms (Santa Cruz Biotechnology, USA), phospho-Akt-Ser473 (Cell Signaling, USA), phosphotyrosine (UBI, USA) and phospho-Erk1/2 (Sigma, USA). Reactions were detected by the Enhanced Chemiluminescence System (ECL, Amersham, UK).

Cytotoxicity and apoptosis

Subconfluent cells in 96-well plates were exposed for 48 h to medium supplemented with 2% FBS, with or without different drugs at concentrations ranging from 1×10^{-10} M to 1×10^{-3} M. Cell viability was assessed by MTT assay³⁰ on eight replicates at each concentration point to determine single drug lethal concentration (LC₅₀) values. Normalised cytotoxicity percentages were obtained from the formula: (A₅₇₀ mean values of extracts from treated samples/A₅₇₀ mean values of extracts from untreated control samples) $\times 100$.

LC₅₀ values, calculated using Origin software (Microcal Software, USA), were used to draw the theoretical additivity isobole according to the 50% isobologram method.³¹ A series of dose-response curves were then generated for each chemotherapeutic drug as above in the presence of several fixed concentrations of imatinib. The resulting LC₅₀ values were plotted on the isobologram for assessment of the hypothetical superadditive effect.

Apoptosis was evaluated by TUNEL analysis (DeadEnd Colorimetric TUNEL System, Promega, USA) following treatment with imatinib, alone or combined with gemcitabine or pemetrexed, and the specific LC₅₀ values were determined by MTT analysis in each cell type as follows. MMP: imatinib 3×10^{-7} M, gemcitabine 5×10^{-7} M, pemetrexed 6.5×10^{-6} M; REN: imatinib 1×10^{-6} M, gemcitabine 5×10^{-9} M, pemetrexed 1×10^{-5} M; ISTMES2: imatinib 4×10^{-6} M, gemcitabine 1×10^{-9} M, pemetrexed 5×10^{-6} M. In brief, subconfluent cells plated on glass slide flaskets (NUNC, Rochester, NY, USA) were exposed to medium supplemented with 2% FBS containing the different drugs for 48 h and subsequently fixed in 10% formalin. Biotin-dU positive nuclei were counted on 10 fields with at least 100 cells in the same slide. Values are expressed as the mean (\pm SE) percentage of positive nuclei of the total counted.

Statistical analysis

For the cytotoxicity assay we performed three separate experiments for each drug and drug combination in the different cell types. Data from each experiment are expressed as mean (SE) values of eight determinations for every concentration point. All mean values from each of the three experiments were used to calculate the curve with the best fit using the Origin software and to calculate the corresponding LC₅₀ value with confidence limits by regression analysis. These LC₅₀ values were compared using the Student's *t* test with theoretically additive doses and their confidence intervals were calculated as described by Tallarida.³²

For apoptosis, statistical differences between the theoretical additive effects of the chemotherapeutic agents (gemcitabine or pemetrexed) plus imatinib vs the measured effects of imatinib/chemotherapeutic combinations were evaluated by the Student's *t* test.

In all statistical evaluations the significance threshold is specified in the text.

RESULTS

Expression of PDGFR β , c-Kit and c-Fms by MMe cells

We evaluated the expression of PDGFR β , c-Kit (tyrosine kinase receptor for stem cell factor) and c-Fms (M-CSF receptor) by

immunoprecipitation and immunoblotting analysis on a panel of eight MMe cell lines. Five of the eight cell lines were positive for PDGFR β (see fig 1 in supplementary data file available online at <http://thorax.bmj.com/supplemental>). Of the PDGF receptors, only PDGFR β (but not PDGFR α) was expressed in MMe cells examined. We selected three MMe cell lines for their different representative expression pattern. PDGFR β was expressed at a higher level in MMP and REN cells than in ISTMES2 cells, while untransformed HMC did not express the PDGFR β receptor. The expression of c-Kit and c-Fms occurred at higher levels in MMP cells and was reduced in REN and ISTMES2 cells. HMC only displayed a very low level of c-Fms expression (fig 1A).

Effect of imatinib-mediated PDGFR β inhibition on Akt

MMe cells positive for PDGFR β were also tested by immunoprecipitation with PDGFR β antibodies followed by immunoblotting with phosphotyrosine antibodies after growing cells in low serum conditions. MMe cells displayed negligible levels of tyrosine phosphorylation whereas the addition of recombinant PDGF B increased the receptor phosphorylation of all cells (fig 1B, upper panel). Neither c-Kit nor c-Fms phosphorylation was detectable in the MMe cells (data not shown).

We then determined whether treatment with imatinib could interfere with signalling pathways elicited by this receptor. In low serum conditions, only MMP cells displayed autonomous Akt activity (determined as Ser⁴⁷³ phosphorylation) whereas, on stimulation with PDGF, tyrosine phosphorylation of PDGFR β and Akt phosphorylation were increased but were markedly inhibited by 10 μ M imatinib in all MMe cells examined. Basal Erk1/2 activity was slightly enhanced after PDGF in MMP and, to a lesser extent, in REN cells, while both activities were barely affected by treatment with imatinib 10 μ M (fig 1B, lower panel).

Conversely, Akt inhibition was complete and comparable to that obtained by treatment with the phosphatidylinositol-3 kinase (PI3K) inhibitor wortmannin at a concentration of 100 nM (fig 1C). Interestingly, Akt activity in MMP cells, expressing also HGFR/Met,⁵ was increased by the addition of recombinant HGF (100 ng/ml) but was not affected by imatinib (fig 1D). This indicates a selective blockade of PDGFR β -dependent Akt signalling by imatinib.

Effect of imatinib on viability of MMe cells expressing PDGFR β

In view of the crucial role played by Akt in determining survival of HMC and MMe cells,²¹ we postulated that imatinib could negatively affect the viability of PDGFR β -positive MMe cells. After incubation for 48 h with up to 100 μ M imatinib, cell viability tested by the MTT assay was markedly decreased with a LC₅₀ of 1.84×10^{-5} M, 1.89×10^{-5} M and 2.05×10^{-5} M for MMP, REN and ISTMES2 cells, respectively. Gemcitabine and pemetrexed have already been shown to be particularly effective in combination with cisplatin for MMe chemotherapy.²⁹ We therefore tested the cytotoxic effect induced by these two agents in the presence of different concentrations of imatinib. As expected, gemcitabine and pemetrexed killed MMe cells in a dose-dependent manner. The presence of imatinib modified the profile of the dose-response curves with a shift towards lower LC₅₀ values and a decrease in the fraction of drug-resistant cells (fig 2A).

We did not observe any evidence of PDGFR β phosphorylation/activation by either gemcitabine or pemetrexed (see fig 2 in supplementary data file available online at <http://thorax.bmj.com/supplemental>), as recently reported for epidermal growth factor receptor.³³

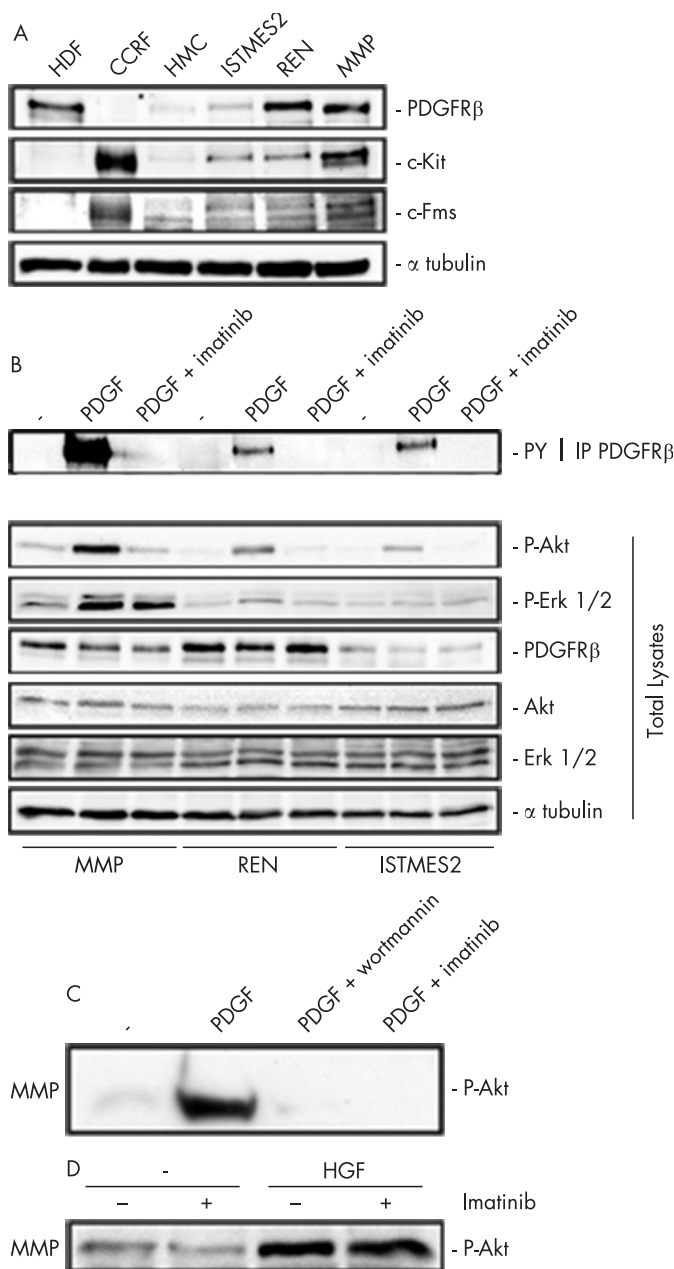


Figure 1 Expression of platelet derived growth factor receptor β (PDGFR β) in malignant mesothelioma (MME) cells. (A) Immunoblotting with PDGFR β , c-Kit and c-Fms antibodies on human mesothelial cells (HMC) and three representative MME cell lines. Controls: human dermal fibroblasts (HDF) expressing PDGFR β and CCRF, CCRF-HSB-2, human leukaemic lymphoblast cells expressing c-Kit and c-Fms. (B) Immunoprecipitation with PDGFR β antibodies followed by immunoblotting with phosphotyrosine antibodies (upper panel); immunoblotting with the indicated antibodies on whole lysates (lower panel). In both panels MMP, REN and ISTMES2 cells were in low serum (–) or stimulated with PDGF in the presence or absence of 10 μ M imatinib or (C) 100 nM wortmannin. (D) Immunoblotting with P-Akt (P-Ser 473) antibodies of MMP cells stimulated by 50 ng/ml hepatocyte growth factor (HGF) in the presence or absence of 10 μ M imatinib.

Synergy of imatinib with gemcitabine and pemetrexed in inducing MME cell death

Activation of tyrosine kinase receptors by ligands induces PI3K and Akt activities, exerting several biological effects including increased cell survival with relevant effects on human carcinogenesis.³⁴ We have recently shown that Akt plays a major survival role for MME cells.²¹ Therefore, based on the

clear-cut toxic effect induced by imatinib on MME cells mediated by the inhibition of the PI3K/Akt pathway, we hypothesised that this inhibitor may also reinforce cytotoxicity generated by other cytotoxic agents.

Combined treatments of imatinib with other chemotherapeutic drugs were therefore analysed by the isobologram plot method.³¹ Interestingly, only imatinib/gemcitabine and imatinib/pemetrexed combinations showed a significant synergism in reducing MMP ($p \leq 0.001$) and REN (ranging from $p \leq 0.01$ to $p \leq 0.001$) cell viability compared with the effects observed with single agents alone. This was revealed by inserting all LC₅₀ values on a concave upward curve below the isoeffective plot (fig 2B). In REN cells the synergistic effect is still appreciable, although to a lower extent, while in ISTMES2 cells the effect of imatinib in combination with other chemotherapeutic agents was significantly antagonistic (ranging from $p \leq 0.05$ to $p \leq 0.001$).

The effectiveness of these combined treatments was confirmed when cell death was investigated by TUNEL analysis. The combination of imatinib with gemcitabine or pemetrexed induced a significant increase in apoptosis ($p \leq 0.001$) compared with the theoretical additive effect of each chemotherapeutic agent with imatinib (table 1). No synergistic effect was observed with any of the other chemotherapeutic drugs (results not shown). Interestingly, the concentrations of the single agents used in the combined treatment were much lower than those obtained at therapeutic dosages.

DISCUSSION

We describe here some preclinical results which provide a rationale for a novel combined approach to MME treatment via inhibition of PDGFR β signalling. Our findings on cultured cells are in accordance with previous evidence of PDGFR β expression in MME cells and a lack of expression in normal HMC.¹⁰ With regard to the relevance of PDGFR β expression in vivo, the percentage of positive specimens reported is in the range of 30–45% in the different studies reported.^{24–25} These data therefore offer a rationale for testing the tyrosine kinase inhibitor imatinib on PDGFR β activity in MME cells.

Autocrine or paracrine mechanisms may explain the activation of PDGFR β in vivo. An autocrine loop has been described as an activating mechanism leading to tyrosine kinase receptor activity in MME cells,⁵ and the stromal microenvironment has been shown to be a fundamental source of activating ligands for PDGFR in human tumours.³⁵ Tyrosine phosphorylation of this receptor in MMP and REN cells is inhibited by imatinib, leading to cytotoxic effects and addressing the role of downstream PI3K/Akt survival signalling.

We and others have shown that Akt activation in MME cells is a crucial signalling pathway which contributes to the MME malignant phenotype.^{21–36} Even though this is also dependent on the activity of several other tyrosine kinase receptors, our findings show that specific interference with the PDGFR β -dependent pathway causes an increase in cell chemosensitivity.

Preclinical studies on several human solid tumours have confirmed the efficacy of imatinib as a cytotoxic agent.^{37–40} In chronic myeloid leukaemia and gastrointestinal stromal tumour the carcinogenic role of the fusion protein BCR-ABL³⁹ and activating mutations of c-Kit,⁴¹ respectively, predict a clinical response to imatinib. Conversely, in MME, two recent negative reports gave clear evidence that imatinib monotherapy is ineffective.^{25–26} On the other hand, combined treatment with imatinib and different chemotherapeutic agents has been shown to be effective in mice.^{27–28}

Our results clearly indicate that PDGFR β expression in MME cells is essential for sensitivity to imatinib and for the synergy observed between imatinib and gemcitabine or pemetrexed.

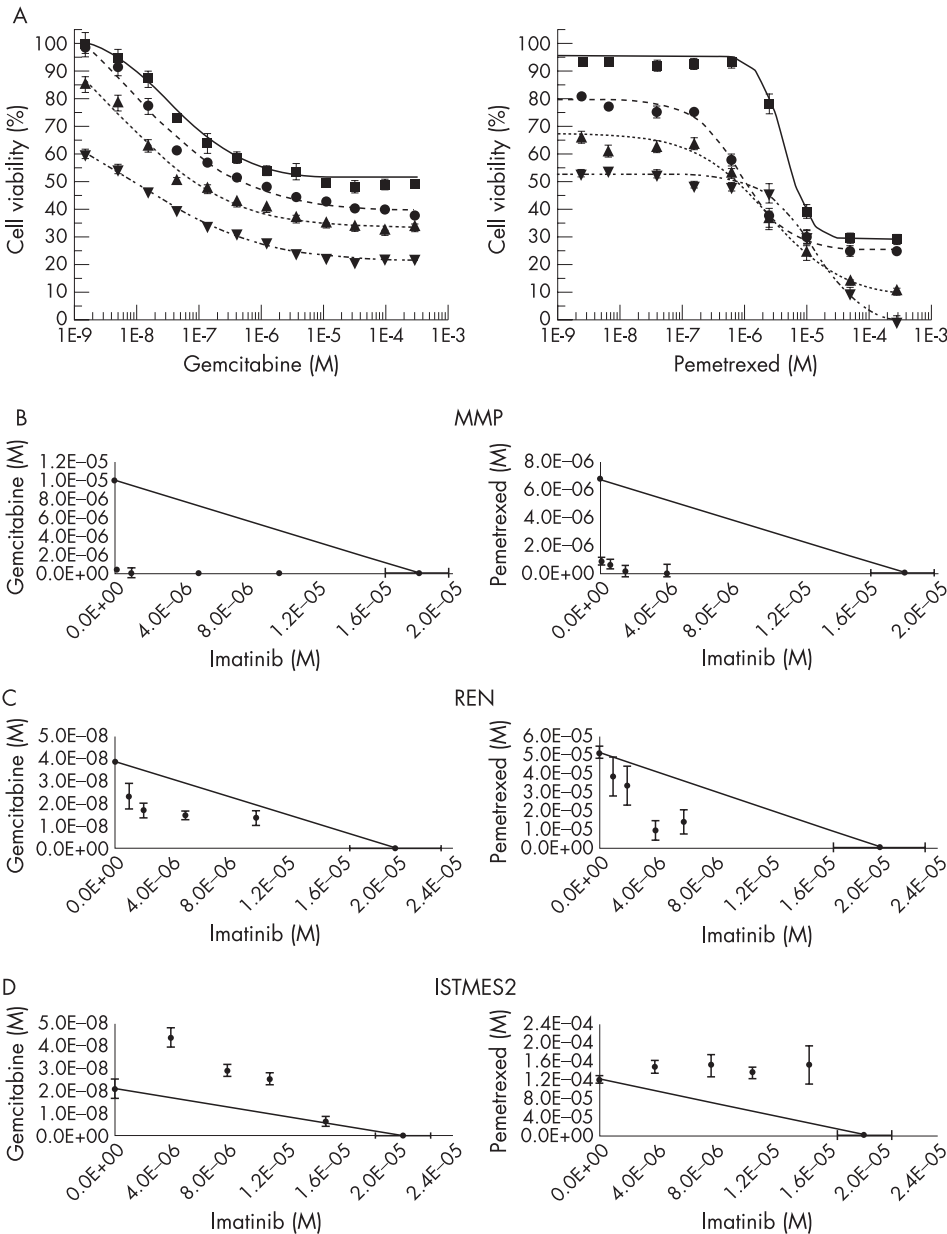


Figure 2 Synergy of imatinib with gemcitabine and pemetrexed. (A) Dose-effect curves of cell viability for gemcitabine (left) and pemetrexed (right) in the presence of different concentrations of imatinib. For the imatinib/gemcitabine combination: ●, 1×10⁻⁷ M; ▲, 2.5×10⁻⁶ M; ▼, 1×10⁻⁴ M. For the imatinib/pemetrexed combination: ●, 3×10⁻⁷ M; ▲, 6×10⁻⁷ M; ▼, 1.5×10⁻⁶ M. Representative data from three experiments conducted with eight determinations for each point. Points represent mean (±SE) percentage. (B–D) 50% isobologram plot for imatinib in combination with gemcitabine (left) and pemetrexed (right) on MMP, REN and ISTMES2 cells. Points are mean (±SE) 50% lethal concentration (LC₅₀) calculated by regression analysis.

However, when all three receptors sensitive to imatinib and upstream of the PI3K/Akt pathway are co-expressed in the same cell type, as in MMP, the synergistic effect is higher than in REN cells where only two of them are expressed (PDGFRβ and c-Kit).

Gemcitabine and pemetrexed are known to be active on MMe cells⁴² and their combination with imatinib has intriguing implications. In particular, the synergism shown in this study indicates that very low doses of chemotherapeutic agents should be sufficient to exert a therapeutic effect.

Table 1 TUNEL analysis of apoptosis induced in malignant mesothelioma (MMe) cells by single drugs or by drug combinations

Treatment	MMP	REN	ISTMES2
Imatinib	1.10 (0.35)	1.00 (0.23)	1.70 (0.19)
Gemcitabine	1.58 (0.42)	3.07 (0.51)	2.80 (0.32)
Pemetrexed	0.98 (0.47)	1.04 (0.26)	1.00 (0.26)
Imatinib + gemcitabine	5.34 (0.40)*	9.72 (0.48)*	1.02 (0.48)*
Imatinib + pemetrexed	8.48 (0.40)*	4.72 (0.26)*	0.04 (0.26)*

Data are expressed as the mean (SE) percentage of biotin-dU positive nuclei for 100 counted cells at a magnification of 100×. The values for each treatment were subtracted from untreated control values. Different concentrations of drugs were used, as described in the Methods section.

*p≤0.001, difference between theoretical additive effects of chemotherapeutics (gemcitabine or pemetrexed) + imatinib vs measured effects of imatinib/chemotherapeutic combinations.

Given our previous findings,²¹ the mechanism underlying the observed in vitro synergy is probably imatinib-dependent PDGFR β inhibition which, in turn, leads to Akt inactivation resulting in sensitisation of MMe cells to low chemotherapeutic concentrations. However, it is conceivable that other biological effects could play a role in humans. Reduction of the intratumoral interstitial fluid pressure and increased uptake of chemotherapeutics by imatinib have been shown in vivo.²⁷ In addition, imatinib has been shown to interfere with VEGF expression and associated neoangiogenesis.⁴³

Although some progress has been made in the treatment of MMe, the results are still unsatisfactory and MMe remains an ideal field in which to test new therapeutic approaches.⁴² Our work on the synergy between imatinib and chemotherapeutic drugs active in MMe provides a strong rationale for a new approach to the treatment of this disease and will be evaluated further in early phase clinical trials.

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Further details are shown in figs 1 and 2 in the supplementary data file available online at <http://thorax.bmj.com/supplemental>.

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Competing interests: None declared.

GG and LM contributed equally to the study.

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PULMONARY PUZZLE

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Answer

Transthoracic needle aspiration of the mass showed abundant small mature lymphocytes and some atypical cells with cytological abnormalities. An immunoperoxidase panel revealed a positive reaction with pankeratin, vimentin and LCA stains. Stains for MOC-31, Ber-EP4, TTF-1 and calretinin were negative. The pathologist at the local hospital suggested a possible large cell carcinoma of a lymphoepithelioma type but requested further investigation.

A CT-guided core biopsy stained for cytokeratin 5/6 and p63, supporting the diagnosis of a pleural-based thymoma. There was a separate infiltrate consistent with lymphocytic interstitial pneumonitis (LIP). This was confirmed by a video-assisted thoracoscopic surgical lung biopsy (fig 1). The diagnosis of a pleural-based thymoma was made in the absence of a mediastinal mass on initial imaging.

The thymoma was treated with six cycles of cisplatin and etoposide, with prednisone for the LIP. Follow-up imaging 8 months later showed significant improvement in both the pleural thymoma and LIP.

Pleural thymoma

Thymomas are epithelial-type neoplasms that typically occur in the anterosuperior mediastinum. Up to 4% of thymomas originate from ectopic thymic tissue.^{1,2} Diagnosis of a pleural-based thymoma is difficult, with the differential diagnosis including lymphoma, mesothelioma, sarcoma and other metastases or spread from a primary pulmonary malignancy.

The course of pleural thymoma is usually indolent with non-specific symptoms. Imaging can demonstrate a unifocal mass, multifocal masses, diffuse pleural thickening or pleural effusions. Primary pleural thymoma does not have the mediastinal involvement usually seen in lymphoma. Histological examination is required for the diagnosis and has recently been reviewed.² Briefly, thymomas consist of thymic epithelial cells incompletely surrounded by fibrous septae with infiltrating polyclonal lymphocytes. Immunohistochemistry can distinguish thymomas from other malignancies. Cytokeratin stains cells derived from an epithelial lineage, including thymoma and mesothelioma. Thymomas typically stain positive for cytokeratin 5/6, p63, CD99, CD1a and TdT.²

Our patient had a bilateral pulmonary infiltrate consistent with LIP. While thymomas often have a polyclonal lymphocytic infiltrate, they do not typically cause a diffuse pulmonary infiltrate as seen in this case. To our knowledge this is the first case of a thymoma-associated LIP.

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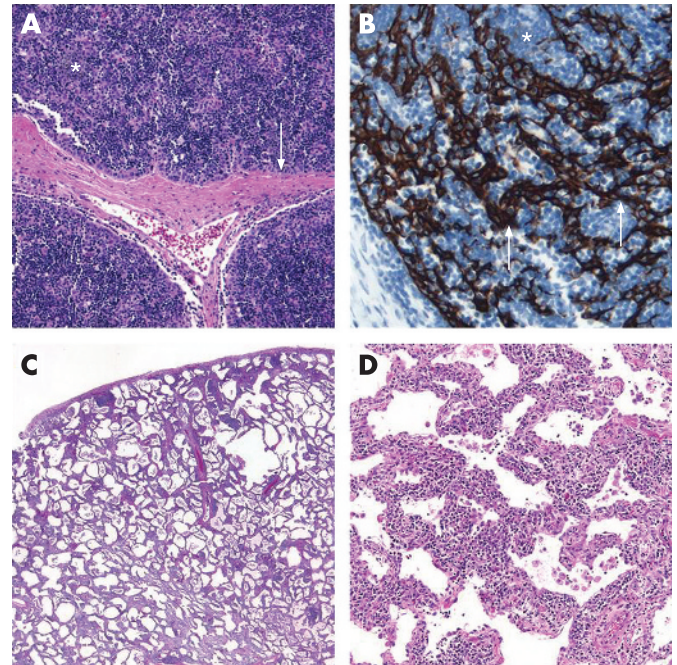
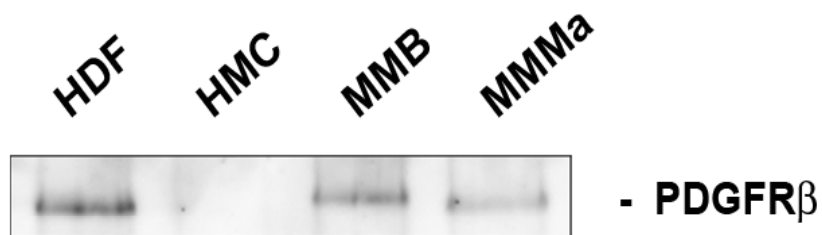
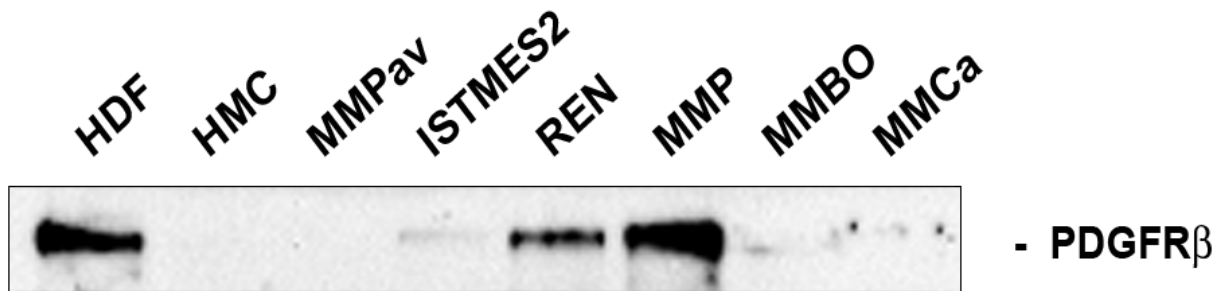


Figure 1 (A) Photomicrograph of thymoma demonstrating the typical features of a lymphoid and epithelial proliferation (*) traversed by hyalinised fibrous septae (arrow). H&E; 40× original magnification. (B) Medium power photomicrograph of the thymoma in which pankeratin immunohistochemistry (dark stain, arrows) highlights the network of neoplastic thymic epithelial cells interspersed within a benign lymphoid stroma (*). 100× original magnification. (C) Low power scanning photomicrograph of the lung wedge biopsy demonstrating diffuse interstitial infiltration of lymphocytes and plasma cells. H&E; 12.5× original magnification. (D) Medium power photomicrograph of lung wedge demonstrating expansion of the alveolar septae by mononuclear inflammatory cells. 110× original magnification.

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- 1 Minniti S, Valentini M, Pinali L, *et al*. Thymic masses of the middle mediastinum: report of 2 cases and review of the literature. *J Thorac Imaging* 2004;**19**:192–5.
- 2 Qing G, Ionescu DN, Colby TV, *et al*. A 75-year-old man with an asymptomatic pleural-based mass discovered on routine chest radiographs. Primary pleural thymoma. *Arch Pathol Lab Med* 2006;**130**:e62–5.



Supplementary Fig. 1

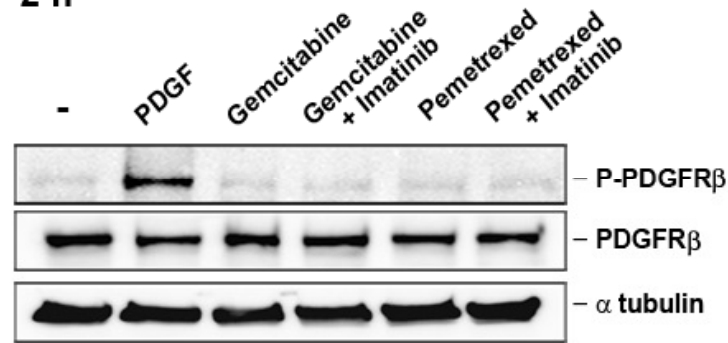
PDGFR β expression in MMe cell lines.

Immunoblotting on whole lysates with PDGFR β antibodies on HMC and eight established MMe cell lines.

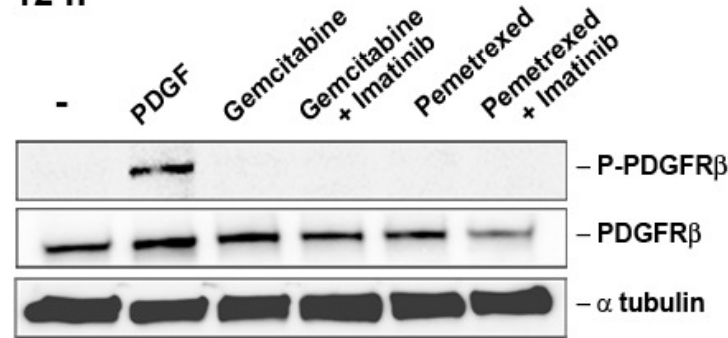
Controls: HDF, Human Dermal Fibroblasts expressing PDGFR β .

MMP

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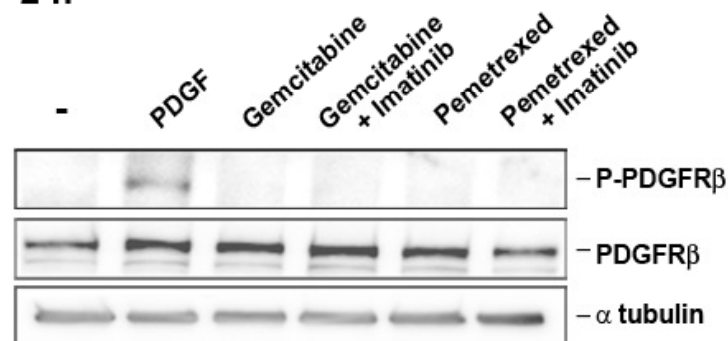


12 h

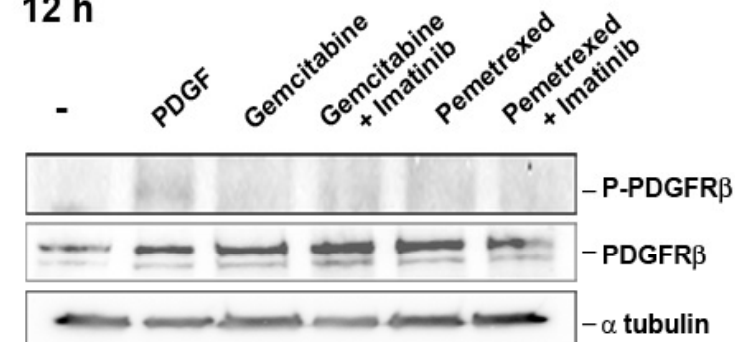


REN

2 h



12 h



Supplementary Fig. 2

PDGFR β activity in MMe cells upon Gemcitabine or Pemetrexed treatment. Immunoblotting with antibodies recognizing PDGFR β (PDGFR β) and its phosphorylated (active) form (P-PDGFR β) on whole lysates of MMP and REN cells.

α -tubulin antibodies were used as loading controls.

Cells were in low serum (-), stimulated with PDGF or treated 2 or 12 hours with LC50 concentrations of the indicated chemotherapeutics in presence or absence of 10 μ M Imatinib.

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APPROACH TO MESOTHELIOMA THERAPY:

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IMATINIB MESYLATE WITH GEMCITABINE OR PEMETREXED

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Novara, Italy

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Foundation, Pavia

these authors gave equal contribution

Financial support: Associazione Italiana per la Ricerca sul Cancro (AIRC), Buzzi
Foundation for the study of Mesothelioma, Italian Group for the Study and Therapy for
Mesothelioma (G.I.Me.).

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Keywords: Pleural Neoplasm; Protein Kinase Inhibitors; Chemotherapy; Drug Synergism.

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ABSTRACT

Malignant mesothelioma is a cancer refractory to current therapies. Imatinib Mesylate (STI571, Glivec™) is a selective inhibitor of tyrosine kinases as bcr-abl, c-Kit, c-Fms and Platelet Derived Growth Factor Receptor β (PDGFR β). PDGFR β is often overexpressed in mesothelioma cells and is a therapeutic target for Imatinib in some solid tumors. The aim of this study is to assess whether Imatinib alone or combined with chemotherapeutic agents may be successful for mesothelioma therapy. Cultures from mesothelioma MMP, REN and ISTMES2 cell lines, were treated with Imatinib alone or in combination with a chemotherapeutic. We show here that Imatinib induces cytotoxicity and apoptosis selectively on PDGFR β positive mesothelioma cells, *via* blockade of receptor phosphorylation and interference with the Akt pathway. Among chemotherapeutics tested in combination, Imatinib synergizes with Gemcitabine and Pemetrexed. We provide a rationale for a novel translational approach to mesothelioma therapy, relying on enhancement of tumor chemosensitivity, via inhibition of Akt.

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Malignant Mesothelioma (MMe) is an asbestos-related tumor, whose incidence is expected to raise dramatically in Europe [1], while in U.S.A. the MMe incidence has already increased by 90% in the last years [2]. Due to its biological aggressiveness, MMe is constantly fatal, except in rare less advanced cases, with a median survival of 12.6 months [3].

A number of growth factors such as Hepatocyte Growth Factor (HGF) [4, 5], Vascular Endothelial Growth Factor (VEGF) [6, 7], Insulin-like Growth Factor -1 and -2 [8] have been shown to play a significant role in the development and progression of MMe. Moreover, several findings underscore the crucial role of Platelet Derived Growth Factor (PDGF) A and

B in MMe cell growth. [9] High expression level of PDGFR β was demonstrated in MMe cells, but not in normal Human Mesothelial Cells (HMC), mostly expressing PDGFR α . [10]

Furthermore, increased expression of PDGF A and B were detected at higher levels in MME cells compared to HMC [11], and a significant reduction in MME cell growth or migration was observed by blocking PDGF A and PDGF B [12]. Expression of c-Kit on MME cells has been demonstrated by some authors, although its role in this tumor is very controversial [13-15]. MCSF production by mesothelial cells has been already shown [16], and inhibition of c-Fms receptor by Imatinib has been demonstrated [17].

Many Cytokines are released in the microenvironment by tumor stromal cells and PDGF paracrine stimulation has been demonstrated in human tumors and MME in particular.[18, 19] PDGFR β activated by PDGF B can induce PI3K/Akt signaling [20], which seems to be crucial for survival of MMe cells.[21]

Imatinib is a selective inhibitor for a subset of tyrosine kinases, including bcr-abl, as well as c-Kit, PDGFR β [22], as well as c-Fms [17]. PDGF receptors are expressed by several tumor cells and have been identified as potential therapeutic targets for Imatinib [23]. In mesothelioma, the extent of PDGFR β positive specimens ranges from about 30% and 45% in

different studies.^[24, 25] Although the therapeutic inefficacy of Imatinib monotherapy for mesothelioma has been recently reported ^[25, 26], combination therapies with Imatinib in mice yielded successful results.^[27, 28]

Gemcitabine, Cisplatin, Etoposide, Doxorubicin and, more recently, Pemetrexed have been demonstrated to be active for MMe treatment. Combined therapy Cisplatin/Pemetrexed and Cisplatin/Gemcitabine have been demonstrated more effective than each single agents used alone.^[29] The aim of the present study is to investigate a translational approach which assessing the possible efficacy of Imatinib, as a single agent or in combined therapy for MMe.

Methods

Cell cultures. Mesothelioma cells were derived from pleural effusions and stabilized in culture as continuous cell lines. MMP cells and primary Human Mesothelial Cells (HMC) were characterized and cultured as previously described.^[5] REN cells were kindly provided by Dr. Albelda and ISTMES2 were from the IST cell depository of Genoa (Italy).

Drugs. Imatinib was kindly provided by Novartis (Basel, Switzerland); Gemcitabine and Pemetrexed by Lilly (Indianapolis, IN). Commercially available Cisplatin, Doxorubicin and Etoposide were from Alexis (Lausen, Switzerland).

Signal transduction. Cells were grown in 0.2 % Foetal Bovine Serum (FBS) for 24 h, then pre-incubated for 90 min in presence or absence of 10 μ M Imatinib. To the same medium 20 ng/ml purified PDGF (R&D, Milan, Italy) was added. Immunoprecipitation and immunoblotting were performed as previously described.^[5] Antibodies used were: PDGFR β , phospho-PDGFR β , c-Kit, c-Fms (Santa Cruz Biotechnology, USA), phospho-Akt-Ser473 (Cell Signaling, USA), phosphotyrosine (UBI, USA) and phospho-Erk1/2 (Sigma, USA). Reactions were detected by Enhanced Chemiluminescence System (ECL, Amersham, UK).

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Cytotoxicity and apoptosis. Subconfluent cells in 96-well multiwell plates were exposed for 48 h to medium supplemented with 2% FBS, with or without different drugs at concentrations ranging from 1×10^{-10} M to 1×10^{-3} M. Cell viability was assessed by MTT assay [30] on eight replicas of each concentration point, to determine single drug LC50 values. Normalized cytotoxicity percentages were obtained according to the formula: $(A_{570} \text{ mean values of extracts from treated samples} / A_{570} \text{ mean values of extracts from untreated control samples}) \times 100$.

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LC50 values, calculated using Origin software (Microcal Software, USA), were used to draw the theoretical additivity isobole, according to the “50% Isobologram” method [31]. Afterward, series of dose-response curves for each chemotherapeutic drug were generated, as above, in the presence of several fixed concentrations of Imatinib. The resulting different LC50 values were plotted on the isobologram, for assessment of the hypothetical superadditive effect.

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Apoptosis was evaluated by TUNEL analysis (DeadEnd™ Colorimetric TUNEL system, Promega, USA), following treatment with Imatinib, alone or combined with Gemcitabine or Pemetrexed, accordingly with the specific LC50 values determined by MTT analysis in each cell type, as follows. MMP: Imatinib 3×10^{-7} M, Gemcitabine 5×10^{-7} M, Pemetrexed 6.5×10^{-6} M.

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REN: Imatinib 1×10^{-6} M, Gemcitabine 5×10^{-9} M, Pemetrexed 1×10^{-5} M. ISTMES2: Imatinib 4×10^{-6} M, Gemcitabine 1×10^{-9} M, Pemetrexed 5×10^{-6} M. In brief, sub-confluent cells plated on glass slide flaskets (NUNC, Rochester, NY, USA) were exposed to medium supplemented with 2% FBS and containing the different drugs for 48 hours and subsequently fixed in 10% formalin. Biotin-dU positive nuclei were counted on 10 fields with at least 100 cells in the same slide. Values are expressed as percentages of positive nuclei over total counted, \pm S.E.

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Statistical analysis. For cytotoxicity assay, we performed three separate experiments for each drug and combination in the different cell types. Data from each experiment are expressed as

mean percentage \pm Standard Error ($S.E.$) of eight determinations for every concentration point. All mean values from each of three experiments were used to calculate the best fit curve by the Origin software and to calculate the corresponding LC50 with confidence limits by regression analysis. These LC50 values were compared by t-Student test, with theoretically additive doses and their confidence intervals, calculated as described [by Tallarida \[32\]](#). For apoptosis, statistical differences were evaluated by t-Student test between [theoretical additive effects of chemotherapeutics \(Gemcitabine or Pemetrexed\) plus Imatinib, vs. the measured effects of Imatinib/chemotherapeutic combinations](#). In all statistical evaluations the significance threshold was specified in the text.

Results

PDGFR β , c-Kit and c-Fms are expressed by MMe cells. We evaluated the expression of PDGFR β , c-Kit (tyrosine kinase receptor for Stem Cell Factor) and c-Fms (Macrophage Colony Stimulating Factor Receptor) by immunoprecipitation and immunoblotting analysis on a panel of eight MMe cell lines. Five out of eight cell lines were positive for PDGFR β (Supplementary Fig. 1). Between PDGF receptors, only PDGFR β , but not PDGFR α , was expressed in MMe cells examined. We selected three MMe cell lines for their different representative expression pattern. In MMP and in REN cells PDGFR β was expressed at higher level than in ISTMES2 cells, while untransformed Human Mesothelial Cells (HMC) did not express the PDGFR β receptor. The expression of c-Kit and c-Fms occurred at higher levels in MMP cells, while in REN and ISTMES2 expression of these receptors was reduced. HMC only displayed very low level of c-Fms (Fig. 1A).

Imatinib-mediated PDGFR β inhibition selectively affects Akt. MMe cells positive for PDGFR β , were also tested by immunoprecipitation with PDGFR β antibodies followed by

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immunoblotting with phosphotyrosine antibodies, after growing cells in low serum conditions. MMe cells displayed negligible levels of tyrosine phosphorylation whereas addition of recombinant PDGF B increased the receptor phosphorylation of all cells (Fig. 1B, upper panel). Neither c-Kit nor c-Fms phosphorylation was detectable in all MMe cells (data not shown).

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Then, we tested if treatment with Imatinib could possibly interfere with signaling pathways elicited by this receptor. In low serum conditions, only MMP cells displayed autonomous Akt activity (determined as Ser⁴⁷³ phosphorylation), whereas upon PDGF stimulation tyrosine phosphorylation of PDGFR β along with Akt phosphorylation were increased, but markedly inhibited by 10 μ M Imatinib, in all MMe cells examined. Basal Erk1/2 activity was slightly enhanced after PDGF in MMP and, a lesser extent, in REN cells, while both activities were barely affected by treatment with Imatinib 10 μ M (Fig. 1B lower panel).

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Conversely, Akt inhibition was complete and comparable to what obtained by treatment with the PI3K inhibitor Wortmannin at concentration of 100 nM (Fig 1C). Interestingly, Akt activity in MMP cells, expressing also HGFR/Met [5], was increased by addition of recombinant HGF (100 ng/ml), but not affected by Imatinib (Fig 1D). This indicates a selective blockade of the PDGFR β dependent Akt signaling by Imatinib.

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Imatinib reduces cell viability of MMe cells expressing PDGFR β . In view of the crucial role played by Akt in determining survival of HMC and MMe cells [21], we postulated that Imatinib could negatively affect PDGFR β -positive MMe cells viability. Upon 48 h incubation with up to 100 μ M Imatinib, cell viability tested by MTT assay markedly decreased, with a LC50 of 1.84×10^{-5} M, 1.89×10^{-5} M and 2.05×10^{-5} M for MMP, REN and ISTMES2 cells respectively. Gemcitabine and Pemetrexed have been already demonstrated particularly effective in combination with Cisplatin for MMe chemotherapy [29]. Therefore we tested the

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cytotoxic effect induced by these two agents, in presence of different concentrations of Imatinib. As expected, Gemcitabine and Pemetrexed caused death of MMe cells, determined by MTT assay, in dose-dependent manner. The presence of Imatinib modified the profile of the dose-response curves, with a shift toward lower LC50 values and by decreasing the fraction of drug resistant cells (Fig 2 A).

We did not observe any evidence of PDGFR β phosphorylation/activation by any of the two chemotherapeutic agents (Supplementary Fig. 2), as recently reported for EGFR [33].

Imatinib synergizes with Gemcitabine and Pemetrexed in inducing MMe cell death.

Activation of tyrosine kinase receptors by ligands induces phosphatidylinositol-3 kinase (PI3K) and Akt activities, exerting several biological effects, including increased cell survival with relevant effects on human carcinogenesis [34]. We recently demonstrated that Akt plays a major survival role for MMe cells [21]. Therefore, based on the clear-cut toxic effect induced by Imatinib on MMe cells, mediated by the inhibition of the PI3K/Akt pathway, we hypothesized that this inhibitor may also reinforce cytotoxicity generated by other cytotoxic agents.

Thus, combined treatments of Imatinib with other chemotherapeutics were analyzed by the isobologram plot method [31]. Interestingly, only Imatinib/Gemcitabine and Imatinib/Pemetrexed combinations showed a significant synergism in reducing MMP ($p \leq 0.001$) and REN (ranging from $p \leq 0.01$ to $p \leq 0.001$) cell viability, compared to the effects observed with single agents alone. This was revealed by lining of all LC50 values on a concave upward curve, below the isoeffective plot (Fig. 2B). In REN cells the synergistic effect is still appreciable, although at lower extent, while in ISTMES2 cells the effect of Imatinib/chemotherapeutics was significantly antagonistic (ranging from $p \leq 0.05$ to $p \leq 0.001$).

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The effectiveness of these combined treatments was confirmed when cell death was investigated by TUNEL. The combination of Imatinib with Gemcitabine or Pemetrexed

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induced a significant increase in apoptosis ($p \leq 0.001$), compared to the theoretical additive

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effect of each chemotherapeutic plus Imatinib (Tab. 1). On the contrary, no synergistic effect

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was observed with any of other chemotherapeutic drugs (not shown).

Interestingly, the concentrations of the single agents used in the combined treatment were by far lower than those obtainable at therapeutic dosages.

Table 1. TUNEL analysis of apoptosis induced in MME cells by single drugs or by drug combination.

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

Data are expressed as the percentage of Biotin-dU positive nuclei for 100 counted cells at a magnification of 100X ± S.E. Values of each treatment were subtracted of untreated control values. Different concentrations of drugs were used, as described in Methods.

(*) Statistically significant ($p \leq 0.001$) difference between theoretical additive effects of chemotherapeutics (Gemcitabine or Pemetrexed) plus Imatinib, vs. measured effects of Imatinib/chemotherapeutic combinations.

Discussion

We describe here some preclinical results, providing the rationale for a novel **combined** approach to MMe therapy, **via PDGFR β signalling inhibition**. Our findings on cultured cells are in accordance with previous evidences **on PDGFR β broad expression in MMe cells and lack of expression in the normal counterpart HMC** [10]. With regard to the relevance of *in vivo* PDGFR β expression, the percentage of positive specimens reported is in the range from 30% to 45%, depending on the different studies [24, 25]. Therefore, these data offered the rationale for testing in MMe cells the tyrosine kinase inhibitor Imatinib on PDGFR β activity.

Either autocrine or paracrine mechanisms may lie beneath the activation of PDGFR β *in vivo*. An autocrine loop has been described as an activating mechanism leading to tyrosine kinase receptor activity in MMe cells [5], and stromal microenvironment has been shown to be a fundamental source of activating ligands for PDGFR in human tumors [35]. The tyrosine phosphorylation of this receptor in MMP and REN cells is inhibited by Imatinib, leading to cytotoxic effects and addressing toward the role of downstream PI3K/Akt survival signaling. We and others showed that Akt activation in MMe cells is a crucial signaling pathway for contributing to MMe malignant phenotype [21, 36]. Even though this is also dependent on several other tyrosine kinase receptor activities, our findings demonstrate that specific interference with PDGFR β dependent pathway exerts a relevant increase in cell chemosensitivity.

Preclinical studies on several human solid tumors revealed the efficacy of Imatinib as a cytotoxic agent [37-40]. In CML and GIST the carcinogenic role of the fusion protein BCR-ABL [39], and activating mutations of c-Kit [41], respectively, are predictive of clinical response to Imatinib. Conversely, for MMe two recent negative reports gave clear evidence that Imatinib monotherapy is ineffective [25, 26]. On the other hand, combined therapy of Imatinib with different chemotherapeutic agents has been shown effective in mice [27, 28].

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Our results clearly indicate that PDGFR β expression in MMe cells is mandatory for the sensitivity to Imatinib and for the synergy observed between Imatinib and Gemcitabine or Pemetrexed. However, when all three receptors sensitive to Imatinib and upstream PI3K/Akt pathway are co-expressed in the same cell type, as in MMP, the synergistic effect is higher than in REN cells, where only two of them are expressed (PDGFR β and c-Kit).

Gemcitabine and Pemetrexed are well known active agents on MMe cell [42], and their combination with Imatinib discloses intriguing implications. Particularly, the synergism revealed here indicates that very low doses of chemotherapeutic agents should be sufficient to exert therapeutic effects.

Given our previous findings [21], the mechanism underlying the observed *in vitro* synergy is most probably the Imatinib-dependent PDGFR β inhibition, which in turn leads to Akt inactivation, resulting in MMe cell sensitization to low chemotherapeutic concentrations.

However, it is conceivable that other biological effects could play a role in humans.

Reduction of the intratumoral interstitial fluid pressure and increased uptake of chemotherapeutics by Imatinib has been demonstrated *in vivo* [27], as well as the Imatinib interference with VEGF expression and associated neoangiogenesis [43].

Albeit some steps forward have been done in MMe therapy, results are still unsatisfactory and MMe remains an ideal field to test new therapeutic approaches. [42] Our work on synergisms between Imatinib and chemotherapeutics active on MMe provides a strong rationale for a new approach to MMe therapy and will be further evaluated in early phase clinical trials.

Acknowledgements

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thank the Buzzi Foundation (Casale Monferrato, Italy) for financial help. This work is part of G.I.Me. (Gruppo Italiano per lo Studio e la Terapia del Mesotelioma) and A.I.P.O. (Associazione Italiana Pneumologi Ospedalieri) network program.

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Figure legends

Fig. 1 - PDGFR β expression in MMe cells. (A) Immunoblotting with PDGFR β , c-Kit and c-Fms antibodies on HMC and three representative MMe cell lines. Controls: HDF, Human Dermal Fibroblasts expressing PDGFR β and CCRF, CCRF-HSB-2, human leukemic lymphoblast cells expressing c-Kit and c-Fms. (B) Immunoprecipitation with PDGFR β antibodies followed by immunoblotting with phosphotyrosine antibodies (upper panel); immunoblotting with the indicated antibodies on whole lysates (lower panel). In both panels MMP, REN and ISTMES2 cells were in low serum (-) or stimulated with PDGF in presence or absence of 10 μ M Imatinib or (C) 100 nM Wortmannin; (D) Immunoblotting with P-Akt (P-Ser 473) antibodies of MMP cells stimulated by 50 ng/ml HGF in presence or absence of 10 μ M Imatinib.

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Fig. 2 – Imatinib synergizes with Gemcitabine and Pemetrexed. (A) Dose-effect curves of cell viability for Gemcitabine (left) and Pemetrexed (right), in presence of different concentration of Imatinib. For Imatinib/Gemcitabine combination: ●, 1×10^{-7} M; ▲, 2.5×10^{-6} M; ▼, 1×10^{-4} M. For Imatinib/Pemetrexed combination: ●, 3×10^{-7} M; ▲, 6×10^{-7} M; ▼, 1.5×10^{-6} M. Representative data of three different experiments, conducted with eight determinations for each point. Points indicate percentage mean \pm S.E.; (B) 50% isobologram plot for Imatinib in combination with Gemcitabine (*left*) and Pemetrexed (*right*) on the indicated cells. Points are $LC50 \pm$ S.E., calculated by regression analysis.

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