PRELIMINARY DATA SUGGESTIVE OF A NOVEL TRANSLATIONAL APPROACH TO MESOTHELIOMA THERAPY: IMATINIB MESYLATE WITH GEMCITABINE OR PEMETREXED

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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 (PDGFRβ). PDGFRβ 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 PDGFRβ 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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INTRODUCTION

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 PDGFRβ was demonstrated in MMe cells, but not in normal Human Mesothelial Cells (HMC), mostly expressing PDGFRα.[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] M-CSF 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] PDGFRβ 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, PDGFRβ [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 PDGFRβ positive specimens ranges from about 30% and 45% in

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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 μM 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: PDGFRβ, phospho-PDGFRβ, 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 $1x10^{-10}$ M to $1x10^{-3}$ 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} mean values of extracts from treated samples / A_{570} mean values of extracts from untreated control samples) x 100.

LC50 values, calculated using Origin software (Microcal Software, USA), were used to draw the theoretical addictivity 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.

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 $3x10^{-7}$M, Gemcitabine $5x10^{-7}$M, Pemetrexed $6.5x10^{-6}$M. REN: Imatinib $1x10^{-6}$M, Gemcitabine $5x10^{-9}$M, Pemetrexed $1x10^{-5}$M. ISTMES2: Imatinib $4x10^{-6}$M, Gemcitabine $1x10^{-9}$M, Pemetrexed $5x10^{-6}$M. 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, ± S.E.

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

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mean percentage ± Standard Error (S.E.) of eight determinations for every concentration point. All mean values from each of three experiments were used to calculate the best fit curve by the Origin software and to calculate the corresponding LC50 with confidence limits by regression analysis. These LC50 values were compared by t-Student test, with theoretically additive doses and their confidence intervals, calculated as described by Tallarida [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.

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Results

PDGFRβ, c-Kit and c-Fms are expressed by MMe cells. We evaluated the expression of PDGFRβ, 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 PDGFRβ (Supplementary Fig. 1). Between PDGF receptors, only PDGFRβ, but not PDGFRα, was expressed in MMe cells examined. We selected three MMe cell lines for their different representative expression pattern. In MMP and in REN cells PDGFRβ was expressed at higher level than in ISTMES2 cells, while untransformed Human Mesothelial Cells (HMC) did not express the PDGFRβ 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 PDGFRβ inhibition selectively affects Akt. MMe cells positive for PDGFRβ, were also tested by immunoprecipitation with PDGFRβ 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).

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 Ser^{473} phosphorylation), whereas upon PDGF stimulation tyrosine phosphorylation of PDGFRβ along with Akt phosphorylation were increased, but markedly inhibited by 10 μM 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 μM (Fig. 1B lower panel).

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 PDGFRβ dependent Akt signaling by Imatinib.

Imatinib reduces cell viability of MMe cells expressing PDGFRβ. 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 PDGFRβ-positive MMe cells viability. Upon 48 h incubation with up to 100 μM Imatinib, cell viability tested by MTT assay markedly decreased, with a LC50 of $1.84x10^{-5}$M, $1.89x10^{-5}$M and $2.05x10^{-5}$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 PDGFRβ 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 induced a significant increase in apoptosis ($p \leq 0.001$), compared to the theoretical additive effect of each chemotherapeutic plus Imatinib (Tab. 1). On the contrary, no synergistic effect 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.

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

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

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.

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Discussion

We describe here some preclinical results, providing the rationale for a novel combined approach to MMe therapy, *via* PDGFRβ signalling inhibition. Our findings on cultured cells are in accordance with previous evidences on PDGFRβ broad expression in MMe cells and lack of expression in the normal counterpart HMC.[10] With regard to the relevance of *in vivo* PDGFRβ 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 PDGFRβ activity.

Either autocrine or paracrine mechanisms may lie beneath the activation of PDGFRβ *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 PDGFRβ 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 PDGFRβ 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 (PDGFRβ 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 PDGFRβ 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.

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Figure legends

Fig. 1 - PDGFRβ expression in MMe cells. (A) Immunoblotting with PDGFRβ, c-Kit and c-Fms antibodies on HMC and three representative MMe cell lines. Controls: HDF, Human Dermal Fibroblasts expressing PDGFRβ and CCRF, CCRF-HSB-2, human leukemic lymphoblast cells expressing c-Kit and c-Fms. (B) Immunoprecipitation with PDGFRβ 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 μM 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 μM 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: ●, $1x10^{-7}$ M; ▲, $2.5x10^{-6}$ M; ▼, $1x10^{-4}$ M. For Imatinib/Pemetrexed combination: ●, $3x10^{-7}$ M; ▲, $6x10^{-7}$ M; ▼, $1.5x10^{-6}$ M. Representative data of three different experiments, conducted with eight determinations for each point. Points indicate percentage mean ± S.E.; (B) 50% isobologram plot for Imatinib in combination with Gemcitabine (*left)* and Pemetrexed (*right*) on the indicated cells. Points are LC50 ± S.E., calculated by regression analysis.

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{Krystal, 2000 #62; Wang, 2000 #59; Druker, 2001 #61; Gonzalez, 2004 #60}

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{Mathy, 2005 #79}

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{Pietras, 2003 #80}