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# Receptor tyrosine kinase inhibitors and cytotoxic drugs affect pleural mesothelioma cell proliferation: insight into EGFR and ERK1/2 as antitumor targets

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ABSTRACT

Malignant pleural mesothelioma (MPM) is an aggressive chemotherapy-resistant cancer. Up-regulation of epidermal growth factor receptor (EGFR) plays an important role in MPM development and EGFR-tyrosine kinase inhibitors (TKIs) may represent novel therapeutic options. We tested the effects of the EGFR TKIs gefitinib and erlotinib and TKIs targeted to other growth factors (VEGFR and PDGFR), in comparison to standard antineoplastic agents, in two human MPM cell lines, IST-Mes2 and ZL55. All drugs showed $IC_{50}$ values in the micromolar range: TKIs induced cytostatic effects at concentrations up to the $IC_{50}$, while conventional drug growth-inhibitory activity was mainly cytotoxic. Moreover, the treatment of IST-Mes2 with TKIs (gefitinib and imatinib mesylate) in combination with cisplatin and gemcitabine did not show additivity. Focusing on the molecular mechanisms underlying the antiproliferative and pro-apoptotic effects of EGFR-TKIs, we observed that gefitinib induced the formation and stabilization of inactive EGFR homodimers, even in absence of EGF, as demonstrated by EGFR $B_{max}$ and number of sites/cell. The analysis of downstream effectors of EGFR signaling demonstrated that EGF-induced proliferation, reverted by gefitinib, involved ERK1/2 activation, independently from Akt pathway. Gefitinib inhibits MPM cell growth and survival, preventing EGF-dependent activation of ERK1/2 pathway by blocking EGFR-TK phosphorylation and stabilizing inactive EGFR dimers. Along with the molecular definition of TKIs pharmacological efficacy *in vitro*, these results may contribute to delve deep into the promising but still controversial role for targeted and conventional drugs in the therapy of MPM.


## 1. Introduction

Human malignant pleural mesothelioma (MPM), the most common type of mesothelioma, is causally related to asbestos inhalation, and possibly to SV40 exposure and genetic susceptibility [1]. Rare early diagnosed MPM may qualify for a combination of surgery and aggressive radio- and chemio-therapy that slightly improve the prognosis. However, the majority of patients diagnosed in advanced stages (III/IV), are not candidates for surgical cure and systemic therapy is the only treatment option. In any case the low sensitivity of this tumor to all available therapies results in a poor prognosis. Single-agent (cisplatin, doxorubicin, ifosfamide, methotrexate) and combination chemotherapy have been evaluated in clinical trials and experimental studies [2,3]. At present, pemetrexed, a multi-targeted antifolate, in combination with cisplatin represents the standard frontline therapy for unresectable MPM, showing a better response rate and overall survival than cisplatin alone [4,5]. Other active agents include gemcitabine and vinorelbine [6]. In particular, the association of pemetrexed and gemcitabine shows synergistic effects on MPM cells [7] and imatinib-mesylate, enhances the effect of gemcitabine in MPM xenografts [8].

Despite improved efficacy of novel multimodality regimens, their benefits remain poor and the development of more effective treatments for MPM are needed. Advances in knowledge of MPM biology in term of pathogenesis, progression and tumor angiogenesis mechanisms may help defining new therapeutic targets.

Platelet derived growth factor (PDGF), vascular endothelial growth factor (VEGF), hepatocyte growth factor receptor (c-MET) and epidermal growth factor (EGF) are the main growth factor receptors overexpressed in MPM cell lines and tumors, activating autocrine/paracrine loops that lead to tumor development [9]. MPM cell lines express the β isoform of the PDGF receptor (PDGFR-

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β) differently from PDGFR-α preferentially expressed in normal mesothelial cells [10]. EGFR is over-expressed in a high percentage (60–70%) of MPM tissue specimens, mainly of the epithelial subtype [11], and it may induce oncogenic phenotype increasing tumor cell proliferation and angiogenesis [12,13]. Moreover, exposure to asbestos fibers is strictly related to epidermal growth factor receptor (EGFR) aggregation leading to its autophopshorylation and activation [14]. A recent study demonstrates EGFR overexpression in 52% of epithelial MPM as a negative prognostic factor although not related to gene status alterations [15].

Signal transduction pathways activated by asbestos microfibers involve mitogen activated protein kinases (MAPKs), mainly extracellular-related kinases 1 and 2 (ERK1/2), which are also important determinants of MPM cell survival and chemoresistance. Interestingly, EGFR-dependent ERK1/2 activation has been related to increased expression of cancer resistance-related proteins, such as ABCG2, causing deregulation apoptotic process regulatory mechanisms [16].

Among targeted therapies, tyrosine kinase inhibitors (TKIs), directed against growth factor receptors, are promising agents under evaluation in clinical trials for several tumor types. EGFR is the most studied target in cancer chemotherapy, frequently overexpressed or mutated in cancer cells, driving tumor development and proliferation [11]. EGFR belongs to the EGF receptor family including, beside HER1/EGFR, HER2, HER3 and HER4. Within EGFR structure, extracellular ligand-binding and intracellular TK domains have been identified. Upon ligand binding, EGFR forms homo- or hetero-dimers with the other components of the HER family. EGFR dimerization induces structural modifications of the TK domain that causes auto-phosphorylation and triggers the activation of multiple signal transduction pathways: RAS/Raf/MEK/ERK1/2, phosphatidylinositol-3 kinase (PI3K)/Akt, and Jak/STAT cascades. Downstream signaling pathways regulate processes involved in cancer survival and growth such as proliferation, apoptosis, invasion, and angiogenesis. EGFR is overexpressed in a variety of human tumors and it is therefore a promising molecular target for TKIs, including gefitinib and erlotinib. These compounds compete with ATP binding and inhibit EGFR auto-phosphorylation.

The *in vitro* inhibition of EGFR signaling with TKI suppresses MPM cell migration and invasion and ERK1/2-dependent proliferation was abolished by gefitinib in mesothelioma cells [17,18]. Gefitinib selectively enhances the response to radiation of human MPM cells in nude mice [19].

However, despite promising pre-clinical evidences, phase II studies on pleural or peritoneal malignant mesothelioma patients with gefitinib and erlotinib report that these EGFR-TKIs, at least used as single agents, did not show striking efficacy [20,21].

Current and future therapeutic options in MPM must include combination therapies, thus, in the present study, we examined the potential antitumoral activity of TKI acting on different receptors alone or in combination with classical cytotoxic drugs, to verify the possible synergism induced by the combined therapy in MPM cell lines *in vitro* [3].

Moreover, we studied the key molecular pathways activated by EGFR signaling and the role of EGFR inhibition by gefitinib on the complex signaling network activated by EGFR, and showed that EGFR-gefitinib interaction induced the formation of inactive ligand-independent receptor dimers as an additional mechanism of action of this drug, beside ATP-binding competition.

## 2. Materials and methods

### 2.1. Cell lines and culture conditions

IST-Mes2 cells were derived from MPM effusions and ZL55 cells from bioptic samples of untreated, asbestos-exposed patients [22]. Cells were maintained in culture in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 5% fetal bovine serum (FBS) glutamine (2 mM), 100 U/ml penicillin/streptomycin (all from EuroClone, Milano, Italy), at 37 °C in a humidified atmosphere at 5% $CO_2$.

### 2.2. Drugs

Drugs were obtained as follows: gefitinib (AstraZeneca, Milano, Italy) and erlotinib (Roche, Basel, Switzerland), sorafenib (Bayer, Milano, Italy), imatinib mesylate (Novartis, Basel Switzerland), gemcitabine (Eli-Lilly, Indianapolis, IN, USA), cisplatin (*cis*-dichlorodiammine platinum(II), CDDP, Sigma–Aldrich, Milano, Italy), fenretinide (R.W. Johnson Pharmaceutical Springhouse, PA, USA).

### 2.3. Competitive binding by radioreceptor assay

Cells ($1.5 \times 10^5$) were plated in complete medium in 24-well plates; after 24 h were co-incubated with [$^{125}$I]-EGF (human recombinant 3-[$^{125}$I]-iodotyrosyl EGF, specific activity > 750 Ci/mmol, GE Healthcare, Milano, Italy) and increasing concentrations of unlabeled EGF (PeproTech, London, UK) at 0.195–50 ng/ml for standard curve, +400 ng/ml excess for determination of non-specific binding. A second set of wells was treated in parallel with the same amounts of labeled/unlabeled ligand and co-incubated with gefitinib. Plates were then incubated for 2 h at 4 °C, medium was removed and cells were washed in ice-cold HBSS + 0.1% BSA, lysed in a solution containing 20 mM Hepes, 1% Triton X-100, 10% glycerol (all from Sigma–Aldrich). Released radioactivity of the bound ligand was measured in a γ-counter (Cobra Auto Gamma Counter, Perkin-Elmer) [23]. Specific binding was calculated after subtraction of non-specific binding (excess unlabeled EGF).

### 2.4. Receptor cross-linking experiments

Cells ($2 \times 10^5$) were serum-starved for 72 h, treated with gefitinib (40 μM) and/or EGF (100 ng/ml), washed with cold PBS and incubated for 30 min in a 3 mM solution of $BS^3$ [bis(sulfosuccinimidyl) suberate, Pierce, Rockford, IL, USA], in PBS [24]. The reaction was stopped with 250 mM glycine in PBS. Monolayers were lysed with 20 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton-X100, 2.5 mM Na-pyrophosphate, 1 mM β-glycerol phosphate, 1 mM $Na_3VO_4$, 1 mg/ml leupeptin, 1 mM PMSF (all from Sigma–Aldrich) buffer for 20 min, on ice. Cell lysates were collected and proteins separated by SDS-PAGE on 6% gel, Western blot analysis was performed as described in Section 2.8.

### 2.5. Cytotoxicity and cell growth recovery analyses

Cell viability was determined by the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma–Aldrich). Cells were seeded in 96-well microplates in complete medium. After overnight incubation, medium was replaced by medium containing increasing concentrations of each drug or the respective vehicle for up to 72 h. MTT solution (2 mg/ml in PBS) was added for 4 h at 37 °C. Optical density (O.D.) was read in a spectrophotometer at 540 nm [23]. In recovery experiments, MTT assay was performed on 72 h-treated cells, incubated with drug-free fresh medium for additional 72 h.

### 2.6. Combined-treatment effects

Ist-Mes2 were seeded in 96-well plate (2500 cells/well) and after 24 h gefitinib or imatinib were added to the culture medium in the presence of cisplatin or gemcitabine. Concentrations were selected on the basis of previously performed dose–response

curves. After 72 h of incubation cytotoxicity was evaluated by MTT assay as described above. Dose–response curves of single and combined treatment were generated and compared. The resulting IC50 values were plotted on isobolograms and the combination index (CI) method [25] were applied to data to determine the nature of pharmacological interactions.

For each given endpoint, the combination index (CI)-isobologram equation allows determination of drug interactions, where $CI < 1$, =1, and >1 indicate synergism, additive effect, and antagonism, respectively. Calculations of the CI were made under the assumption that the mechanisms of action of the four drugs (gefitinib or imatinib and cisplatin or gemcitabine) were not mutually exclusive.

### 2.7. Apoptosis detection and cell cycle analysis

Cells ($1.5 \times 10^5$) were plated in 35 mm Petri dishes in complete medium, after 24 h gefitinib was added for up to 72 h; supernatants and monolayers were collected and apoptosis was evaluated by staining with propidium iodide (PI) to evidence the hypodiploid cell fraction. Samples were measured in a DAKO $CyAn_{ADP}$ fluorescence-activated cell sorter (FACS, DAKO, Glostrup, Denmark). Cell cycle analysis of asynchronously growing cells were performed staining cells with PI in 0.2% Triton-X 100, before FACS examination.

### 2.8. Western blot analysis

Untreated, exponentially growing, cells ($6 \times 10^5$) were plated in 100 mm Petri dishes in complete medium, serum-starved for 72 h, then treated with gefitinib for 30 min. EGF (40 ng/ml) was added during the last 10 min of incubation [26]. Cell were harvested, and lysed in ice-cold buffer (Tris–HCl 20 mM, NaCl 137 mM, EDTA–$Na_2$ 2 mM, glycerol 10%, NP40 0.1%, PMSF 1 mM and $Na_3VO_4$ 1 mM) containing protease inhibitors (Complete mini, Roche Diagnostics GmbH, Mannheim, Germany). Total protein amounts were determined using Bradford assay (Bio-Rad Laboratories, Milano, Italy) and separated on 10% SDS-PAGE, transferred on PVDF membrane (BioRad Laboratories) and probed with the following antibodies: anti-EGFR and phospho-EGFR ($Tyr^{1173}$); anti-HER2 (CB11), anti-HER3 (C17) and anti-phospho STAT3 ($Tyr^{705}$) from Santa Cruz Biotechnology (CA, USA); anti- ERK1/2 and phospho-ERK1/2, Akt and phospho-Akt ($Ser^{473}$) (Cell Signaling Technology, Danvers, MA, USA), anti-α tubulin (Sigma–Aldrich) [27]. Detection of immunocomplexes was performed using chemiluminescence detection system (Immobilon WesternMillipore, Merck Millipore, Vimodrone, MI, Italy). Relative expression of the protein (phosphorylated/total ratio) was obtained by densitometric analysis (QuantityOne Software, Bio-Rad Laboratories).

### 2.9. DNA synthesis analysis by [$^3$H]-thymidine incorporation assay

Cells ($2 \times 10^4$) were plated in 24-well plates, serum-starved for 72 h and then treated with EGF (40 ng/ml) for 24 h; in the last 4 h cells were pulsed with 1 μCi/ml of [$^3$H]-thymidine (GE Healthcare) [28]. Then cells were trypsinized, extracted in 10% trichloroacetic acid (TCA), filtered and washed under vacuum through fiberglass filters (Merck Millipore) with 10 and 5% TCA and 95% ethanol. TCA-insoluble fraction was measured in a scintillation counter. When indicated, cultures were pre-treated for 10 min with 10 μM PD98059 or wortmannin (Merck Calbiochem, Darmstadt, Germany).

### 2.10. Statistical analysis

Data are expressed as mean ± S.D., statistical significance was assessed by ANOVA: $P$ value $< 0.05$ were considered statistically significant. $IC_{50}$ values, scatchard analysis, $B_{max}$, $K_D$ and sites/cell number, of binding assays were calculated using GraphPad Prism 5 Software, Inc. (La Jolla, CA, USA).

## 3. Results

### 3.1. Sensitivity to antineoplastic agents in MPM cell lines

Several classical cytotoxic drugs and many novel agents, including TKIs, are under clinical evaluation for MPM treatment. Thus, we compared the *in vitro* sensitivity of human MPM cell lines (IST-Mes2, ZL55) to EGFR-TKIs with that of cytotoxic compounds or TKIs targeted to other growth factor receptors. Drugs were selected among standard therapy used or undergoing clinical trials in MPM, including cisplatin and gemcitabine that showed the best response rate in combination therapies. Moreover, we tested *N*-(4-hydroxyphenyl)retinamide (4-HPR, fenretinide), a synthetic analog of retinoic acid that induced apoptosis in various tumor cell lines, including MPM [29]. Among targeted drugs, we analyzed the mesylate salt of imatinib (hereafter imatinib, inhibiting TK encoded by the bcr-abl oncogene as well as c-kit and PDGFR) and sorafenib (VEGFR/raf inhibitor). Concentration ranges were optimized for each compound and their growth-inhibitory effects were evaluated by MTT assay. Gefitinib caused a dose- and time-dependent growth inhibition reaching the maximum after 72 h (data not shown), thus this time-point was chosen to compare drug effects.

Calculated $IC_{50}$ values for IST-Mes2 and ZL55 cells, after 72 h of treatment with the tested drugs, are summarized in Table 1.

The level of MPM cell growth inhibition was heterogeneous within tested compounds. Both cell lines showed an $IC_{50} < 1$ μM (highly sensitive) for gemcitabine, while slightly higher values were obtained for the other compounds although all in the low micromolar range. Interestingly, a cell line-dependent chemosensitivity was observed in ZL55 cells, showing a reduced sensitivity to most drugs. This difference was more evident for erlotinib (2.6-fold higher ZL55 $IC_{50}$ than IST-Mes2 $IC_{50}$), gefitinib (2.5-fold), imatinib (2-fold) and cisplatin (3.6-fold), while $IC_{50}$ values for sorafenib, gemcitabine and fenretinide were similar in the two cell lines (Table 1).

To further assess the efficacy of drugs, we report the efficacy, in term of maximal growth inhibition, and the concentration leading to the maximum inhibition rate for each cell line (Table 1). Efficacy varied from 58 to 90% of growth inhibition *vs.* controls in IST-Mes2, after 72 h of treatment. Erlotinib showed the lowest efficacy in both cell lines (58 and 50% growth inhibition at 30 μM) while the maximal response was obtained by sorafenib (90%) in IST-Mes2 cells. High inhibitory effect (>70%) was also observed with all the other drugs in this cell line. Interestingly, while gefitinib, imatinib and sorafenib elicited a similar efficacy in ZL55 to that observed in IST-Mes2, gemcitabine, cisplatin and fenretinide induced a lower growth inhibition in ZL55 than in IST-Mes2 (Table 1).

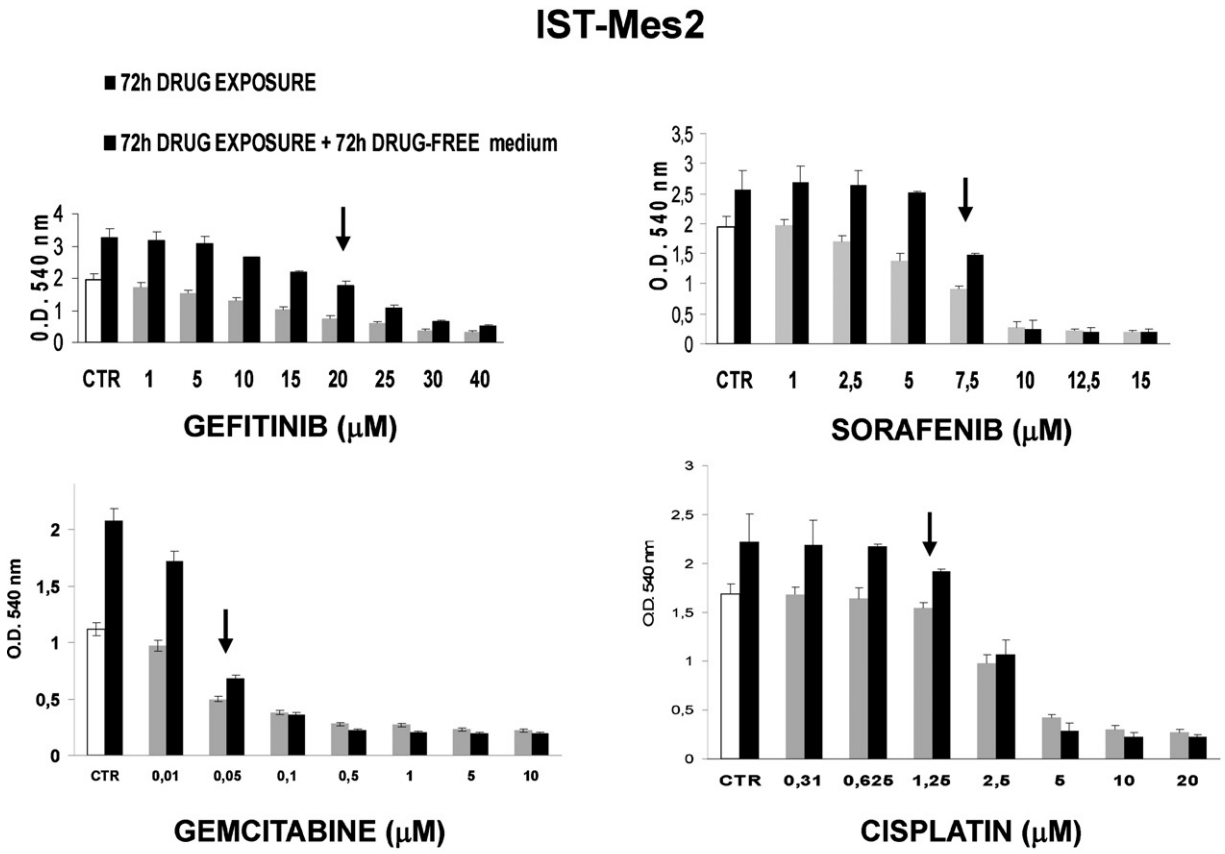
**Table 1**
Potency ($IC_{50}$) and efficacy values of targeted drugs and cytotoxic compounds on the proliferation of malignant pleural mesothelioma cell lines.

| Drug | IST-Mes2 | | ZL55 | |
|---|---|---|---|---|
| | $IC_{50}$ (μM) | [a]Efficacy ([b]μM) | $IC_{50}$ (μM) | [a]Efficacy ([b]μM) |
| Gefitinib | 5.2 | 72% (20) | 12.9 | 86% (20) |
| Erlotinib | 11.3 | 58% (30) | 30 | 50% (30) |
| Sorafenib | 6.9 | 90% (12.5) | 6.8 | 80% (15) |
| Imatinib mes. | 13.1 | 83% (20) | 26.3 | 80% (30) |
| Gemcitabine | 0.3 | 80% (5) | 0.4 | 57% (10) |
| Cisplatin | 2.3 | 82% (5) | 8.3 | 53% (40) |
| Fenretinide | 7.7 | 89% (12.5) | 9.5 | 73% (15) |

[a] Efficacy was calculated as maximal cell growth inhibition vs. untreated cells.
[b] Drug concentration that induces maximum response.

### 3.2. MPM cell growth recovery

To assess whether the growth-inhibitory effects of the studied drugs were cytotoxic or cytostatic, drug regulation of cell proliferation was analyzed by cell growth recovery experiments: cells treated with the compounds at different concentrations for 72 h were allowed to recover for furhter 72 h after removal of the drug, to evaluate the persistence of proliferating cells. In particular, from these experiments we show that, as far as TKI, low concentrations mostly induced cytostatic effects on both cell lines, evidenced by a recovery of cell proliferation after the removal of the drugs, while only at higher concentrations cytoxicity was apparent.




| Minimum cytotoxic concentration (μM) after 72h recovery-time. | | |
|---|---|---|
| | MPM cell lines | |
| DRUG | IST-Mes2 | ZL55 |
| Gefitinb | 20 | 10 |
| Erlotinib | 20 | 20 |
| Sorafenib | 10 | 7.5 |
| Imatinib mes. | 25 | 15 |
| Gemcitabine | 0.05 | 0.05 |
| Cisplatin | 2.5 | 5 |
| Fenretinide | 0.5 | 5 |

**Fig. 1.** Cell growth recovery of IST-Mes2 and ZL55 cell lines after drug exposure. Cells were treated for 72 h with increasing concentrations of drugs followed by 72 h culture in drug-free medium. Cytotoxicity was evaluated by MTT assay. Untreated cells (open column) are represented; arrows point the concentration up to cell recovery was significantly present, inducing cytostatic effects. Histograms are the mean ± S.D. Table reports the data referring to the complete panel of drug tested in both cell lines, IST-Mes2 and ZL55.

Erlotinib, imatinib and sorafenib treatment significantly prevented the recovery of cell growth starting from concentrations around $IC_{50}$ (about 20 μM for erlotinib and imatinib, and 10 μM for sorafenib in both IST-Mes2 and ZL55 cells) (Fig. 1). Conversely, treatment with gefitinib induced cytotoxic activity, for concentrations around $IC_{50}$ in ZL55 but much higher than $IC_{50}$ in IST-Mes2 ($IC_{50}$ 5.2 μM and minimum cytotoxic concentration 20 μM).

The antiproliferative effects of gemcitabine, cisplatin and fenretinide were reversible upon their removal, up to 0.05 μM, 2.5–5 μM and 0.5–5 μM, respectively, in the two cell lines (Fig. 1).

These results confirm the high cytotoxic potential of these compounds, while targeted-therapy molecules, at low doses, mainly elicit cytostatic effects.

### 3.3. Efficacy of combination treatments

Combined treatments of cytotoxic agents were proposed to achieve improved MPM response rate although the obtained results are still highly unsatisfying. We tested the possible antiproliferative activity of the concomitant exposure of IST-Mes2 cells to cisplatin and gemcitabine in the presence of gefitinib or imatinib (Fig. 2).

Dose–response curves were generated for each drug and combination using a range of concentrations including $IC_{50}$ values, selected on the basis of previous cell viability experiments.

Effects induced by interactions among the cytotoxic effects of these drugs were evaluated by isobologram plots and combination index (CI) analysis to determine possible additivity, synergism and antagonism.

However, the potential additivity of the antitumoral effects of TKIs and cytotoxic drugs was not confirmed by our *in vitro* study. In fact, only the effects induced by low imatinib concentrations (0.04 and 0.08 μM) in combination with gemcitabine were additive, being the CI ≈ 1 (1.07 and 1.17, respectively). On the contrary, combined treatments with gefitinib and cisplatin or gemcitabine, and imatinib in the presence of cisplatin produced CI values significantly lower than 1 suggesting an antagonistic activity in IST-Mes2 cells.

### 3.4. Expression and phosphorylation status of EGFR in MPM cell lines

EGFR TKIs are the most common used target molecules whose potential antitumor activity was proposed also for MPM. Thus, we deeply characterized the role of EGFR pathway and the mecha-

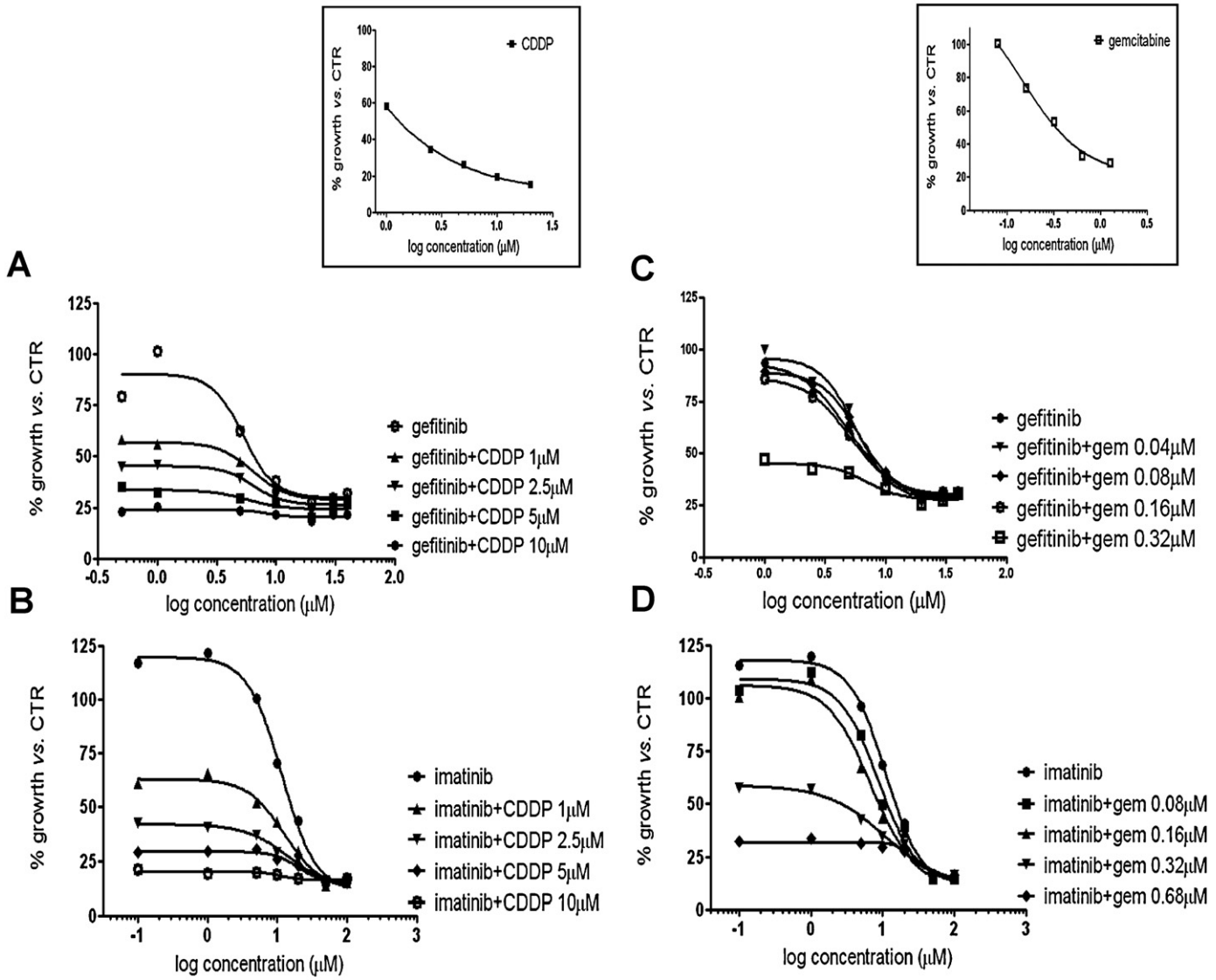



**Fig. 2.** Dose–response curves for drug combinations in IST-Mes2 cells. IST-Mes2 cultures were treated with various concentrations of gefitinib (1–40 μM), imatinib (0.1–100 μM), cisplatin (CDDP, 1–10 μM) and gemcitabine (0.04–1.68 μM) for 72 h and cytotoxicity evaluated by MTT assay. Dose–response curves of cisplatin (CDDP) and gemcitabine alone are reported in the upper insets. Panels A and B depict gefitinib and imatinib plus CDDP, panels C and D report gemcitabine combinations.

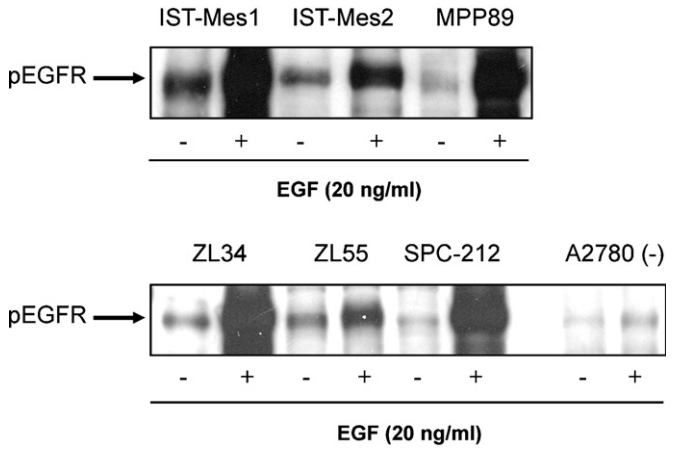



**Fig. 3.** EGF-induced EGFR activation in human malignant pleural mesothelioma (MPM) cell lines. A panel of 6 MPM cell lines exposed to EGF (20 ng/ml EGF) for 10 min showed the increase of the phospho-EGFR tyrosine kinase signal, indicating that receptor is functional. The human ovarian tumor A2780 cell line, lacking EGFR is included as negative control. Phospho-EGFR was detected by Western blotting on total cell lysates using anti-phosphorylated-EGFR (Tyr1173) antibody.

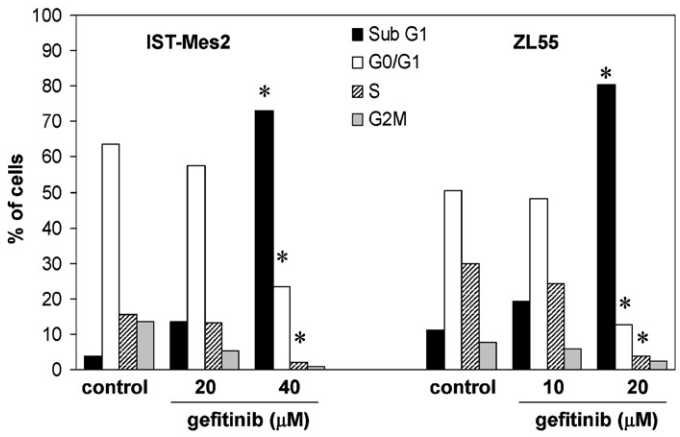



**Fig. 4.** Cell cycle analysis of IST-Mes2 and ZL55 cell lines treated with gefitinib. Cells were harvested 72 h after treatment in the absence or presence of gefitinib, stained with PI and analyzed by FACS. The percentage of cells in the S and G2/M phases was significantly inhibited by higher concentrations of gefitinib while the sub-G1 phase (hypodiploid peak corresponding to apoptotic cells) was significantly increased in response to gefitinib treatment in both cell lines ($^*p < 0.05$ *vs.* untreated control).

nisms on the cytostatic/cytoxic effects of gefitinib, taken as EGFR-TKI prototype. Preliminarily, EGFR expression levels were analyzed in a panel of 6 human MPM cell lines (IST-Mes1, IST-Mes2, MPP89, ZL34, ZL55 and SPC212) by Western blot. All cell lines expressed this receptor (data not shown), which was functional being phosphorylated after treatment with EGF (20 ng/ml,10 min) (Fig. 3). Interestingly, basal EGFR phosphorylation, index of autocrine activation, was observed in all cell lines. IST-Mes2 and ZL55 lines that displayed a marked EGFR expression and activation were chosen for further characterization.

### 3.5. Gefitinib up-regulates EGFR site number in MPM cell lines

To further characterize EGFR modulation by gefitinib and erlotinib in MPM cells, EGF-competitive binding assays were carried out in control and gefitinib pretreated cells.

Both IST-Mes2 and ZL55 cells showed basal high EGF binding site levels ($11 \times 10^4$ and $8 \times 10^4$ sites/cell, respectively) (Table 2).

Scatchard analysis of [$^{125}$I]-EGF binding to untreated MPM cells showed a binding affinity $K_D$ = 0.15 nM for IST-Mes2 and 0.19 nM for ZL55 (Table 2). After gefitinib treatment (20 μM), while $K_D$ values were slightly reduced in both cell lines, a dramatic increase in radioligand binding was observed, with $B_{max}$ values at least 2-fold higher than untreated controls. Calculated number of sites/cell increased of 100% ($22 \times 10^4$) in IST-Mes2 and 337% ($27 \times 10^4$) in ZL55 (Table 2).

Similar results were obtained with erlotinib that increased $B_{max}$ from 23.5 to 46.3 pM and the number of sites *per* cell of 80% (from $10 \times 10^4$ to $18 \times 10^4$).

The specificity of this effect was demonstrated using drugs targeting different receptors (sorafenib, imatinib) or cytotoxic molecules (gemcitabine, cisplatin, fenretinide) that affected neither EGF binding capacity nor affinity in MPM cell lines (Table 2). Thus, these results suggest that the increase in EGFR binding sites represents a specific effect of EGFR-TKIs.

### 3.6. Apoptosis in MPM cells treated with gefitinib

The dynamics of cell death in conjunction with those of cell cycle may contribute to drug cytotoxicity. To confirm the cell growth recovery experiments (see Fig. 1), we tested whether the capability of gefitinib to inhibit cell proliferation was associated to apoptotic process in mesothelioma cells. We measured apoptosis and cell cycle distribution simultaneously, by time-course (from 12 to 72 h) FACS experiments, staining the cells with propidium iodide, and evaluating apoptosis as sub-G1 fraction, represented by hypodiploid DNA content on DNA histograms. Cells were exposed to gefitinib at concentrations inducing the maximum inhibition of growth: 20–40 μM for IST-Mes2 and 10–20 μM for ZL55. Short treatments (12, 24 and 48 h) did not cause apoptosis (data not shown), while at 72 h the percentage of cells in the sub-G1 peak was dose-dependently

**Table 2**
Binding assay on malignant pleural mesothelioma cell lines treated with targeted drugs and cytotoxic compounds.

| Cell line | Drug | $B_{max}$ (pM) | | $K_D$ (nM) | | Sites/cell ($\times 10^4$) | | |
|---|---|---|---|---|---|---|---|---|
| | | C | T | C | T | C | T | T/C% |
| IST-Mes2 | Gefitinib | 40.0 | 84.0 | 0.15 | 0.13 | 11 | 22 | +100 |
| | Erlotinib | 23.5 | 46.3 | 0.12 | 0.08 | 10 | 18 | +80 |
| | Sorafenib | 36.3 | 33.5 | 0.21 | 0.15 | 15 | 13 | −9 |
| | Imatinib mes. | 27 | 25.5 | 0.25 | 0.23 | 10 | 9 | −10 |
| | Gemcitabine | 20.5 | 21.5 | 0.14 | 0.15 | 9 | 10 | +11 |
| | Cisplatin | 26 | 25 | 0.15 | 0.14 | 12 | 13 | +1 |
| | Fenretinide | 43 | 36.5 | 0.15 | 0.13 | 17 | 16 | −1 |
| ZL55 | Gefitinib | 33 | 72 | 0.19 | 0.13 | 8 | 27 | +337 |
| | Gemcitabine | 20 | 22 | 0.22 | 0.22 | 5 | 5 | – |

$B_{max}$ = binding capacity; $K_D$ = dissociation constant; C = control; T = treated.

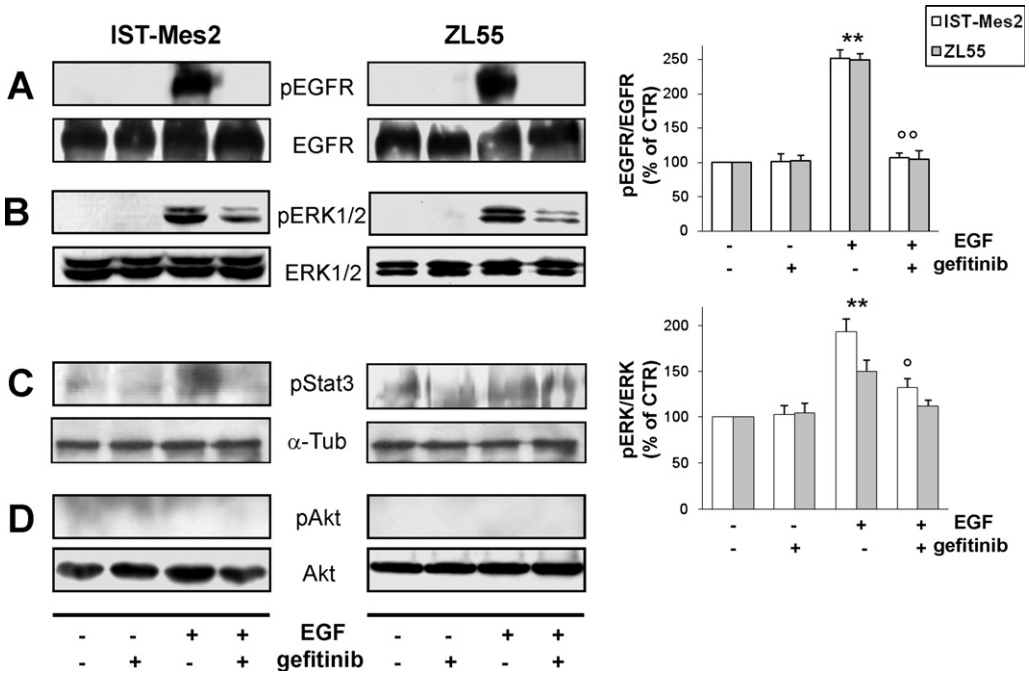



**Fig. 5.** Effects of gefitinib on the phosphorylation status of EGFR, ERK1/2, STAT3 and Akt in IST-Mes2 and in ZL55 cells. (A) EGFR phosphorylation induced by EGF is significantly abolished in both cell lines by gefitinib treatment. (B) Gefitinib significantly reverts the phosphorylation of ERK1/2 induced by EGF in both cell lines. (C) EGF activation of Stat3 is reverted by gefitinib. (D) EGF treatment did not trigger Akt phosphorylation after in both cell lines. Cell were stimulated with EGF (40 ng/ml) in the absence or presence of 20 μM (IST-Mes2) or 10 μM (ZL55) gefitinib. Activation was detected by Western blot analysis using specific antibodies against the phosphorylated forms of EGFR, ERK1/2, STAT3 and Akt. Antibodies against the total proteins or α-tubulin were used to reprobe blots and ensure the equal loading of proteins. Histograms represent the phosphorylated/total ratio of densitometric analysis values from immunoblots, reported as percentage of its own untreated controls. Bars represent the average values of three independent experiments ± S.D.; $^{\circ}p < 0.05$,$^{\circ\circ}p < 0.01$ *vs.* EGF-treated cells; $^{**}p < 0.01$ *vs.* untreated cells.

increased up to 73.2% in IST-Mes2 and 80.2% in ZL55 cells (Fig. 4). Simultaneously, the S and G2M-phase fractions of cells decreased, as a result of the concomitant significant reduction of actively cycling cells (Fig. 4).

### 3.7. *Effects of gefitinib on EGFR phosphorylation and EGFR-dependent signaling pathways*

To delve deeper into the molecular determinants of gefitinib antiproliferative activity in MPM cells, we measured EGFR phosphorylation levels after gefitinib treatment. Serum-starved cultures were exposed to EGF (40 ng/ml), gefitinib (20 μM and 10 μM, for IST-Mes2 and ZL55 respectively), or combination of the two.

EGF induced a marked activation of its receptor (Fig. 5A) in both cell lines, significantly increasing phosphorylation levels (more than 200% as evaluated by densitometric analysis), while in the presence of gefinitib this effect was completely abolished (Fig. 5A).

Then we evaluated the phosphorylation status of main downstream effectors of EGF signaling, such as ERK1/2, STAT3 and Akt. EGF significantly induced ERK1/2 phosphorylation in both cell lines (+191% and +150% *vs.* control in IST-Mes2 and ZL55, respectively) (Fig. 5B) and the previous exposure to gefitinib prevented the EGF-dependent activation of ERK1/2. Gefitinib administered alone to MPM cell lines, under the same experimental conditions, did not affect the ERK1/2 phosphorylation (Fig. 5B).

We further analyzed the phosphorylation status of STAT3: a slight constitutive activation of STAT3 was observed in untreated IST-Mes2, increasing upon exposure to EGF. When gefitinib was pre-incubated with cells prior to EGF stimulus the level of phospho-STAT3 was reduced to basal (Fig. 5C). A similar modulation of STAT3 occurred in ZL55.

In contrast, EGF-treatment did not activate Akt in both IST-Mes2 and ZL55 (Fig. 5D) and, of course, no effects were also observed in the presence of gefitinib. As a control, to test whether Akt is functionally active in these cell lines, cell stimulation with platelet-derived growth factor (PDGF), a proliferative factor for MPM cells, was performed, demonstrating that PDGF/Akt signaling was active (data not shown).

These data suggest that the effects of EGF in these MPM cell lines are mainly mediated by ERK1/2 cascade. To directly verify the role of ERK1/2 and Akt on MPM cell proliferation, EGF-stimulated cultures were pretreated with wortmannin, a specific inhibitor of

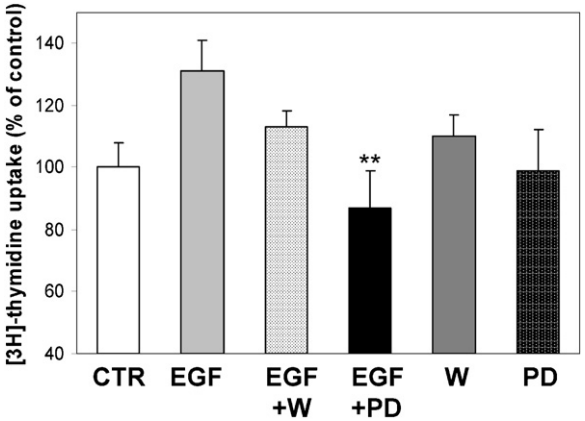



**Fig. 6.** Effects of EGF on DNA synthesis: role of ERK1/2 and Akt in IST-Mes2 cell proliferation. [$^3$H]-thymidine uptake assays were carried out after PD98059 (PD) and wortmannin (W) pretreatment to block ERK1/2 or Akt activity, respectively. MPM cell proliferation induced by EGFR is mediated by ERK1/2 while Akt activation is independent from for EGF stimulation. In the figures are reported, as % of the untreated cells (Control) the effects of EGF treatment (40 ng/ml) on IST-Mes2 cells, in the presence of a 10 min pre-treatement with 10 μM wortmannin and 10 μM PD98059. After exposure to the wortmannin, cells retain a growing state whereas the PD98059 totally abolishes the EGF dependent cell proliferation ($^{**}p < 0.01$ *vs.* EGF-treated cells).

PI-3K or PD98059 that blocks MEK activation, and tested by [$^3$H]-thymidine uptake experiments.

Wortmannin did not interfere with EGF-induced DNA synthesis thus IST-Mes2 proliferation was independent from the Akt pathway (Fig. 6). This result correlated with the lack of Akt phosphorylation observed after EGF exposure. In contrast, the proliferative role of ERK1/2 in this model was confirmed by the significant reduction (−40% *vs.* EGF) of EGF-dependent stimulation by pretreatment with PD98059 (Fig. 6).

### 3.8. Mechanisms of EGFR dimerization and phosphorylation

Increased EGF binding site number in MPM cell membranes suggest that gefitinib (and other EGFR-TKI) effects may be mediated not only through the inhibition of receptor TK activity, but also interfering with EGFR membrane dynamics. In particular we hypothesized that a stabilization of the receptor localization in membrane, may occur in the presence of gefitinib or erlotinib.

To assess this issue, the oligomeric state of EGFR was analyzed in IST-Mes2, before and after gefitinib pre-treatment by Western blot analysis after chemical cross-linking reactions. In addition, we evaluated IST-Mes2 and ZL55 for the expression of other EGFR family members as HER2 and HER3: both cell lines did not express HER2 and HER3 proteins (data not shown), thus excluding possible heterodimerization and functional interactions between EGFR and other family components.

As depicted in Fig. 7A, densitometric analysis of monomeric and dimeric EGFR amount in untreated cells did not show the presence of EGFR dimers. Importantly, EGFR dimers were detected not only after EGF treatment (100 ng/ml), as expected, but also after exposure to gefitinib alone (40 μM), and in cells treated with both compounds (Fig. 7A). The monomer/dimer percentage ratio was 1.8, 4.6 and 2.6 for EGF, gefitinib and EGF + gefitinib, respectively (Fig. 7A). Since, IST-Mes2 did not express any other ErbB family receptor (data not shown), the formation of heterodimers of EGFR with other ErbB proteins is excluded.

To explore the activation state of EGFR dimers, EGF-treated IST-Mes2 cells were analyzed using anti-phosphotyrosine EGFR antibody. Data reporting the level of EGFR phosphorylation/activation are depicted in Fig. 7B calculated, as arbitrary units, from densitometric analysis of phopho-EGFR blots. Only EGF induced EGFR phosphorylation (2-fold increase *vs.* untreated control) while gefitinib-induced EGFR dimers were not active both in the presence and in the absence of the activating ligand.

These results indicate that gefitinib promote EGFR dimerization independently from ligand binding, but dimers are inactive.

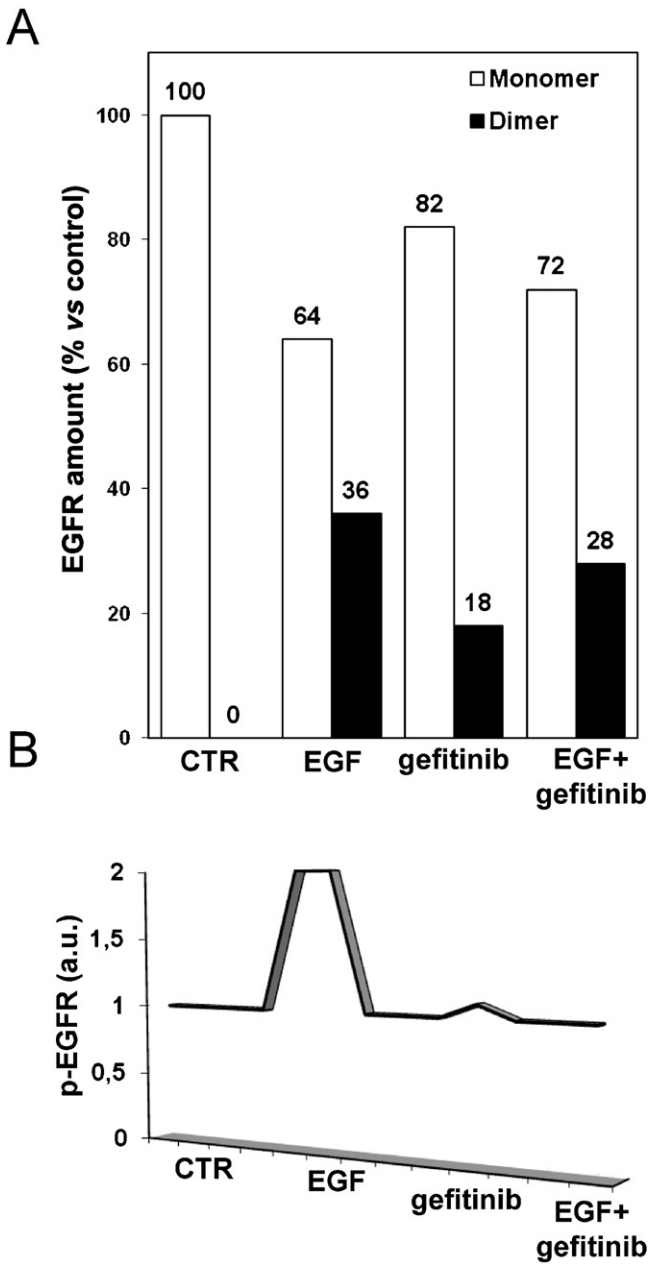



**Fig. 7.** Dimerization and phosphorylation of EGFR in IST-Mes2 cells treated with gefitinib. (A) Quantification of relative amount (%) of EGFR monomers and dimers in cells treated with EGF and/or gefitinib. EGFR dimers formation was evaluated after cells exposure to EGF (100 ng/ml) for 10 min in the presence or absence of 30 min gefitinib (40 μM) pretreatment. Cells were lysed in the presence of a chemical cross-linking reagent (BS$^3$) and Western blotting was carried out using anti-EGFR antibody followed by densitometric analysis. (B) EGFR activation profile of receptor dimers in cells treated with EGF and/or gefitinib. Gefitinib blocks EGF-dependent EGFR phosphorylation. After cross-linking reaction, EGFR dimers were detected by immunoblot with anti-phospho-EGFR: the abundance of phospho-EGFR relative to untreated control was determined by densitometry and expressed as arbitrary units (a.u).

## 4. Discussion

Generally, chemotherapic agents used in standard treatment of MPM show poor activity. At present, only the combination of cisplatin plus pemetrexed, considered the front-line regimen for this disease, has improved clinical outcome in a phase III trial that yielded a response rate of 41% and survival benefits, when compared to single agent therapies, although the outcome is still extremely unsatisfactory showing an overall survival time of about 12 months [3,5,30]. Ongoing research on novel targeted agents, translated to clinics in other neoplastic diseases, such as NSCLC [31], promotes recent investigations also in MPM.

Key growth factor signaling pathways, particularly involving receptor tyrosine kinases (e.g. EGFR), regulate tumor cell proliferation and survival and represent molecular targets in several human cancer, including mesothelioma. Pre-clinical studies inducing pharmacological EGFR signaling blockade give basis for clinical trials using targeted therapies in MPM [4].

EGF, as other growth factors, induces receptor dimerization and autophosphorylation as initial events triggering signal transduction. Different molecular mechanisms were proposed for the inhibition of EGFR by anilino-quinazolinic compounds: they may interact with receptors at the ATP-binding site inhibiting kinase activity and induce the formation of inactive membrane bound EGFR dimers after gefitinib treatment [32,33].

Indeed, we demonstrate that gefitinib forms inactive (non-phosphorylated) EGFR dimers on MPM cell membrane, indepen-

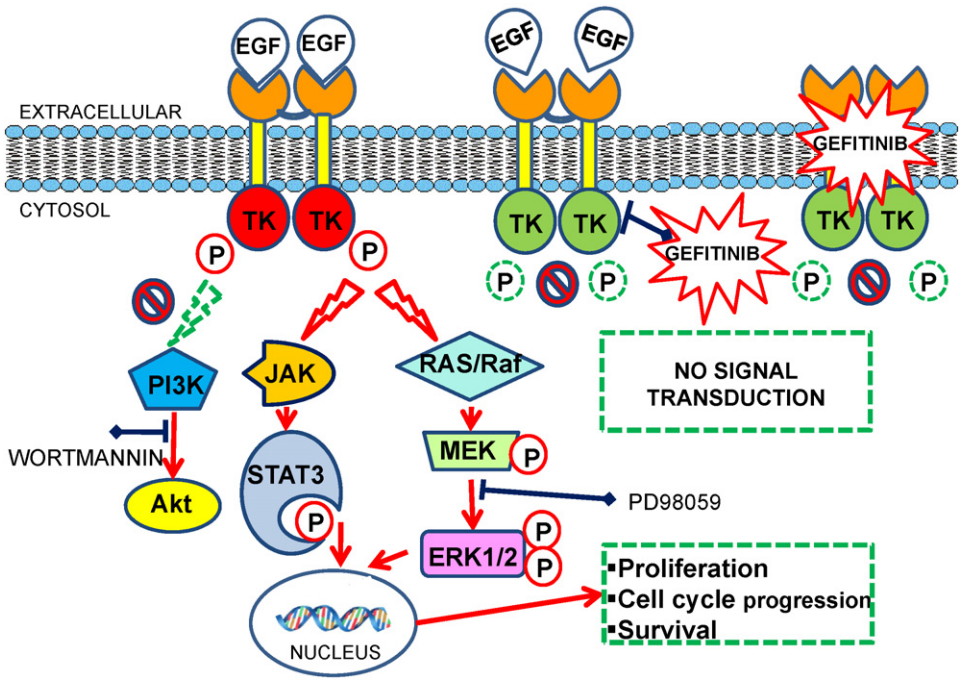



**Fig. 8.** Diagram of the key pathways for MPM cell proliferation inhibited by gefitinib. EGF binding to tyrosine kinase (TK) domain of EGFR causes receptor dimerization and phosphorylation that triggers intracellular signals mediated by PI3K/Akt, JAK/Stat3 and MAPK pathways. EGFR downstream effectors modulate cell proliferation, survival and cell cycle progression. EGF-induced MPM cell proliferation is mediated by ERK1/2 activation (blocked by PD 98059) and Stat3, it did not involve PI3K/Akt pathway (inhibited by wortmannin). Gefitinib treatment inhibits MPM cell proliferation through the block of TK phosphorylation and the stabilization of inactive EGFR dimers, also in the absence of EGF, preventing the activation of ERK1/2 proliferative pathway.

dently from ligand binding. This mechanism may explain the increased number of EGFR-binding sites observed in gefitinib-treated MPM cells by competitive binding experiments, through the unmasking of binding sites in the inactive EGFR homodimers. The stabilization of inactive gefitinib-induced EGFR dimers and ligand-independent dimerization imply that activation and dimerization of RTKs are distinct events in MPM cells (Fig. 8). This observation may also imply the presence of pre-existing EGFR oligomers activated by EGF binding [34] and stabilized by gefitinib. Dimerization of EGFR and activation of its tyrosine kinase not necessarily coincide and are not strictly consequent upon ligand or drug interaction with receptor, thus the presence of inactive dimers after gefitinib treatment may account for a further block of EGFR activity added up to TK inhibition (Fig. 8). Importantly, HER2 and HER3, potentially interacting and dimerizing with EGFR were not expressed by the cell lines (IST-Mes2 and ZL55) chosen for the study, thus avoiding possible heterodimerization as reported in other cancer cell types [32] where the gefitinib induced inactive hetero- and homodimers.

In the present study we evaluated the antiproliferative activity of conventional and targeted agents on human MPM cell lines, focusing on the effects of the EGFR-TKI gefitinib on EGFR signaling pathways. Indeed, about 70% of human malignant mesothelioma specimens show EGFR expression [11,12]. Asbestos fibers may activate EGFR and initiate signaling cascades leading to cell proliferation and carcinogenesis [35] and stimulate the MAPK pathway in rat pleural mesothelial cells after autophosphorylation of EGFR.

The screening of EGFR expression and activation on several human MPM cell lines revealed that all cultures express the receptor and, although at different levels, its phophorylated form was detected also under basal (unstimulated) conditions, suggesting that the considerable response of MPM cells to this growth factor is sustained by an autocrine/paracrine loop. However, EGF stimulation markedly increased EGFR phosphorylation/activation in all cell lines tested, independently from the basal level. In particular, among MPM cells studied in detail, the amount of phospho-EGFR in IST-Mes2 appears higher than in ZL55, and this feature is related to a higher sensitivity of the former cell line to the EGFR TKIs erlotinib and gefitinib although, $IC_{50}$ for the two cell lines are in the micromolar range.

Overactivity of PDGF and VEGF have been reported in MPM [10,36] and multitarget TKIs, inhibiting these autocrine loops (imatinib and sorafenib), have been tested in clinical trials. We observed that TKIs active on both VEGFR and PDGFR pathways exert antiproliferative activity, suggesting that constitutive activity of these receptors in these MPM cells are required for cell survival. Nevertheless, neither selective EGFR inhibitors nor multitargeted TKIs tested in clinical trials confirmed yet the promising *in vitro* results. It was proposed that antiproliferative activity of single TKIs *in vitro* are not always translatable *in vivo*, where a complex machinery of molecular alterations leading to changes in MPM growth factor receptor regulation, angiogenesis and cell survival, not yet completely understood [37].

Although, the relative low incidence of MPM make difficult to design extensive clinical trials to study new molecules, thus pre-clinical studies on established human MPM cell lines still provides reliable model to analyze the biological and molecular behaviors of this malignancy, useful for drug development.

The observation that molecularly targeted agents have limited antitumor activity as single agents has led to combine their combination with cytotoxic agents. This rationale may be more plausible in MPM where also traditional drugs often fail to demonstrate a satisfactory therapeutic role. Although gemcitabine has limited single-agent activity in this disease, response rates ranging from 12% to 48% have been reported for the gemcitabine/cisplatin combination [30,38].

In this study, targeted drugs were compared to standard cytotoxic compounds used in MPM therapy (cisplatin, gemcitabine) [4] or under clinical evaluation in a variety of cancers as fenretinide [29], as far as cytotoxicity in two MPM cell lines.

These standard drugs still represent the more effective molecules, displaying low $IC_{50}$ and high cytotoxic activity. The isobologram evaluation of combined treatment of IST-Mes2 cells with gefitinib or imatinib plus gemcitabine or cisplatin, did not show synergistic or additive effects, except for a slight additivity at low concentrations of gemcitabine plus imatinib. These data agree, at least in part, with *in vitro* and *in vivo* observations [7,8] describing enhanced MPM cytotoxicity following the combination of imatinib with gemcitabine.

EGFR-TKIs trials in MPM may be often unsatisfactory since the molecular characterization of this malignancy is still incomplete to support a strong rationale for their clinical use. Thus, we investigate the intracellular mechanisms modulated by gefitinib to exploit its antiproliferative activity.

Antiproliferative activity induced by gefitinib and erlotinib was primarily cytostatic rather cytotoxic effects, as previously described [17] and cell death and apoptosis occurred only at high concentrations, with erlotinib that shows a slightly lower efficacy than gefitinib.

The efficacy of TKIs against different molecular targets as VEGFR/Raf (sorafenib) and PDGFR/bcr-abl/c-Kit (imatinib) on the MPM cells was comparable to that of gefitinib. These data suggest that since most MPM cells show the simultaneous activation of multiple TK receptors, inhibition of two or multiple receptors might improve MPM proliferation inhibition [39].

Clinical trials have shown poor activity of gefitinib as single treatment for MPM [40], thus multi-TKI treatments may offer novel strategies. At the molecular level the unsatisfactory clinical evidence and *in vitro* efficacy of EGFR-TKIs suggest that further growth factor receptors and intracellular mediators, apart from EGFR, are involved in determining sensitivity to these agents [40].

Investigations on the intracellular pathways mediating EGFR signaling show that the inhibition of EGF-induced ERK1/2 phosophorylation represents, in our cell model, the main intracellular pathway mediating antiproliferative effects induced by gefitinib. EGFR participate in the constitutive activation of the PI3K/Akt signaling pathway in MPM cells and other solid tumors as well as in their radio- and chemo-resistance [41]. In our experimental model, Akt appears not to be involved in EGF-dependent proliferation in both cell lines since its activation upon EGF treatment is lacking, but no evidence of basal activation of Akt have been observed. This observation is in agreement with a recent paper [42] describing the lack of detectable levels of activated Akt in both basal and EGF-stimulated cultures, despite abundant expression of total Akt, in IST-Mes2 cells as well as in other MPM cell lines. It has been proposed that in these cell lines Akt pathway could be inactive, although we verified that PDGF stimulation of IST-Mes2 is able to induce Akt phosphorylation [18] demonstrating its functionality. Thus, a role of phosphatase and tensin homologue (PTEN) in the regulation of PI3K/Akt cascade could be hypothesized, since overexpression of this tumor suppressor gene is often associated with low Akt activation in MPM [43]. Moreover, continuous activation, or lack of modulation, of PI3K/Akt pathway has been observed in non-small cell lung cancer cell lines resistant to gefitinib [44]. These mechanisms should be deeply investigated in IST-Mes2 and ZL55 cells as compared to active Akt-expressing MPM cell types, investigating differences in drug sensitivity or EGF-induced signal transduction activation.

However, these findings provide a rationale for the evaluation of anticancer activity of gefitinib, either alone or in combination with conventional cytotoxic antineoplastic agents or other targeted molecules.

The molecular machinery activated by EGF and inhibited by gefitinib includes inactive dimers formation at membrane level, preventing the downstream activation of ERK1/2 pathway. The present results need to be confirmed and deepened by further investigations to characterize the molecular mechanisms of drug activity, but they start to shed light on the molecular mechanisms involved in the activity of TKIs to overcome the resistance of MPM to conventional anticancer therapeutics.

## Disclosure statement

The authors have nothing to disclose.

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