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Everolimus restores gefitinib sensitivity in resistant non-small cell lung cancer cell lines

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ABSTRACT

The epidermal growth factor receptor (EGFR) is a validated target for therapy in non-small cell lung cancer (NSCLC). Most patients, however, either do not benefit or develop resistance to specific inhibitors of the EGFR tyrosine kinase activity, such as gefitinib or erlotinib. The mammalian target of rapamycin (mTOR) is a key intracellular kinase integrating proliferation and survival pathways and has been associated with resistance to EGFR tyrosine kinase inhibitors. In this study, we assessed the effects of combining the mTOR inhibitor everolimus (RAD001) with gefitinib on a panel of NSCLC cell lines characterized by gefitinib resistance and able to maintain S6K phosphorylation after gefitinib treatment.

Everolimus plus gefitinib induced a significant decrease in the activation of MAPK and mTOR signaling pathways downstream of EGFR and resulted in a growth-inhibitory effect rather than in an enhancement of cell death. A synergistic effect was observed in those cell lines characterized by high proliferative index and low doubling time. These data suggest that treatment with everolimus and gefitinib might be of value in the treatment of selected NSCLC patients that exhibit high tumor proliferative activity.

1. Introduction

Lung cancer is the main cause of cancer deaths in the developed world in both men and women, and non-small cell lung cancer (NSCLC) accounts for about 80–85% of all lung cancers [1]. Current treatments, including chemotherapy, radiotherapy and surgery, have provided only limited improvement in the natural history of the disease. New treatments are thus needed to improve survival of NSCLC patients [2].

Inhibition of the Epidermal Growth Factor Receptor (EGFR) pathway has been proposed as a novel molecular targeted approach for NSCLC. Two main strategies targeting EGFR have been developed: small-molecule inhibitors of the tyrosine kinase domain (tyrosine kinase inhibitors [TKIs], as erlotinib and gefitinib), and monoclonal antibody (cetuximab), directed against the extracellular domain of EGFR, that inhibits its phosphorylation/activation and stimulates internalization [3].

Clinical data have shown that 10–20% of patients with refractory advanced NSCLC responded to gefitinib or erlotinib [4]. A higher probability of response appears to be associated with certain biological and clinical characteristics (such as adenocarcinoma histotype, female sex, never smoking status and Asian ethnic origin) and with biological features of the tumor [5]. In particular, molecular analysis showed that in most cases, responders harbored specific mutations in the region that encodes the catalytic domain of EGFR [6].

By contrast, acquired resistance to EGFR TKIs involves in same cases the recurrent T790M mutation, which affects the catalytic domain of the kinase weakening the interaction of the inhibitor with its target [7]. Tumor cells can develop other mechanisms of resistance to EGFR TKIs such as the activation of alternative tyrosine kinase receptors (IGF-1R), amplification of the *MET* gene and constitutive activation of signaling pathways downstream of EGFR (i.e. *K-RAS* mutations) [8,9].

Two major intracellular signaling pathways activated by EGFR, the PI3K/AKT/mTOR and the RAS/RAF/MAPK cascades, have a central role in controlling cell survival and modulating cell growth and proliferation [10]. These pathways represent potential novel therapeutic targets.

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NSCLC cell lines responsive to EGFR TKIs with growth arrest or apoptosis show a down-regulation of the PI3K/AKT/mTOR pathway [11]. However, deregulation of the AKT-dependent pathway has been well documented in a variety of human tumors [12] and has been associated with resistance to EGFR TKIs in NSCLC cell lines [13]. Deregulation of the PI3K/AKT/mTOR pathway could result from several alterations, including PI3K isoform gene amplification, activating mutations of PI3K subunits, *AKT* gene amplification and overexpression, as well as loss of function of PTEN [8,14]. Frequent AKT activation and mTOR phosphorylation were found in 51% of NSCLC patients and in 74% of NSCLC cell lines [15].

The inhibition of mammalian target of rapamycin mTOR signaling pathway could be a promising therapeutic option. Everolimus (RAD001) is an orally bioavailable derivative of rapamycin that is in current use as a post-transplant immunosuppressive agent and has been shown to inhibit the proliferation of tumor cell growth in preclinical studies, and phase I/II clinical trials in lung cancer are under way. In particular, at a daily dose of 10 mg, Everolimus was reported to be active and safe in pretreated advanced NSCLC patients [16,17]. The crucial role of the constitutive activation of the PI3K/AKT pathway in the development and maintenance of an EGFR-resistant phenotype supports the hypothesis that a combination of EGFR TKIs and mTOR inhibitors may be the best choice for treatment. Clinical trials testing this strategy in advanced NSCLC are ongoing and only preliminary results are available [18–20].

To better support *in vivo* studies and to further clarify their interaction, we assessed the effects of combining the mTOR inhibitor everolimus with gefitinib on an extended panel of NSCLC cell lines characterized by gefitinib resistance.

2. Materials and methods

2.1. Cell culture

The human NSCLC cell lines H292, H322, H460, H1299, H1975, H596, H1650, Calu-1, Calu-3, SKMES-1 and SKLU-1 were cultured as recommended. All media were supplemented with 2 mM glutamine, 10% fetal bovine serum (FBS Gibco, Life Technologies). Cell lines were from the American Type Culture Collection (Manassas, VA, USA) and were maintained under standard cell culture conditions at 37 °C in a water-saturated atmosphere of 5% CO_2 in air.

2.2. Drug treatment

Gefitinib (ZD1839/Iressa) was synthesized as described elsewhere [21]. Everolimus (RAD001) was provided by Novartis International AG (Basel, Switzerland). In all assays, the drugs were dissolved in DMSO immediately before the addition to cell cultures. The concentration of DMSO never exceeded 0.1% (v/v) and equal amounts of the solvent were added to control cells.

2.3. Antibodies and reagents

Monoclonal anti-EGFR, polyclonal anti-phospho-EGFR (tyr1068), polyclonal anti-phospho-mTOR (ser2248), polyclonal anti-phospho-AKT (ser273), polyclonal anti-AKT, polyclonal anti-phospho-S6K (thr389), polyclonal anti-S6K, monoclonal anti-phospho-p44/42 MAPK, monoclonal anti-p44/42 MAPK, polyclonal anti-PI3K, polyclonal anti-PTEN antibodies were from Cell Signaling Technology (Beverly, MA, USA). Monoclonal anti-actin (AC-40) antibody was from Sigma–Aldrich (St. Louis, MO, USA). Horseradish peroxidase-conjugated (HRP) secondary antibodies and the enhanced chemiluminescence system (ECL) were from Millipore (Millipore, MA, USA). Reagents for electrophoresis and blotting analysis were obtained from BIO-RAD Laboratories.

2.4. Western blot analysis

Procedures for protein extraction, solubilization, and protein analysis by 1D PAGE are described elsewhere [22]. Briefly, 30–50 μg proteins from lysates were resolved by 5–15% SDS-PAGE and transferred to PDVF membranes. The membranes were then incubated with primary antibody, washed and then incubated with HRP-anti-mouse or HRP-anti-rabbit antibodies. Immunoreactive bands were visualized using an enhanced chemiluminescence system.

2.5. Determination of cell growth

Cell number was evaluated by cell counting in a Burker hemocytometer by trypan blue exclusion and by crystal violet staining as described [23]. In brief, cells were fixed with 1% formaldehyde and stained with 0.1% crystal violet in PBS. The unbound dye was removed by washing with water. Bound crystal violet was solubilized with 0.2% TritonX-100 in PBS. Light extinction which increases linearly with the cell number was analyzed at 570 nm. Proliferation rates were estimated by the growth rate quotient as calculated by the equation: $GRQ = (N - N_0)/t \times 1/N_0$; doubling time (h) was calculated according to the formula $t \times \ln(2)/\ln(N/N_0)$, where N is the final cell number; N_0 is the initial cell number and t is the time elapsed between the two counting.

MTT and colony formation by viable cells were determined as described elsewhere [24].

2.6. Detection of apoptosis

Apoptosis was assessed by: (a) morphology on stained (Hoechst 33342, PI) or unstained cells using light-, phase contrast- and fluorescence-microscopy; (b) activation of caspase-3 (detection of cleavage products), by Western Blotting procedure as previously described [24].

2.7. Statistical analysis

Using a non-linear regression fit method (Levemberg–Marquardt algorithm), we calculated the dose–response curves of single compounds from the Hill function (1):

$$\%\mathrm{IN} = \%\mathrm{IN}_0\left(\frac{x^n}{k^n + x^n}\right) \quad (1)$$

where %IN is the % growth inhibition, $\%IN_0$ the amplitude of the signal (e.g. the saturation growth inhibition value), k is the value where %IN was the half of $\%IN_0$, and n the cooperativity index, which takes into account of possible affinity changes of target sites [25]. In the case that $\%IN_0$ did not reach 100%, we calculated from the fitting parameters the value of IC_{50} as that point in which 50% growth inhibition was expected by fitting curve.

Under the hypothesis that everolimus and gefitinib bind at different target sites and can be considered as independent, to study the combined effects of drugs we used the Bliss independence criterion for *in vitro* coexposure.

The Bliss criterion is expressed by the following equation:

$$E(x,y) = E(x) + E(y) - E(x) * E(y), \quad (2)$$

where $E(x)$ is the effect of the concentration x of the first compound (between 0 and 1), $E(y)$ the same for the second compound at concentration y, and $E(x,y)$ is the combined effect.

Table 1
Characterization of EGFR, K-RAS, PI3K and PTEN in a panel of NSCLC cell lines.

Cell lines	EGFR	K-RAS	PI3K	PTEN	IC_{50} for gefitinib (μM)
SKLU-1	WT	Mutant	WT	+	20
CALU-1	WT	Mutant	WT	+	18
H1650	Mutant	WT	WT	−	17
H596	WT	WT	Mutant	±	15
H1975	Mutant	WT	Mutant	+	10
H1299	WT	WT	WT	+	8
SKMES-1	WT	Mutant	WT	+	8
H460	WT	Mutant	Mutant	+	7
H322	WT	WT	ND	ND	0.1
H292	WT	WT	WT	+	0.06
CALU-3	WT	WT	WT	+	0.2

If the combination effect is higher than the expected value from (2), the interaction is synergistic, while if this effect is lower, the interaction is antagonistic. Otherwise, the effect is additive and there is no interaction between drugs.

To test the differences between experimental and theoretical points, we applied the method suggested by Goldoni and Johansson [26]. Briefly, the theoretical value was calculated by the experimental points of single dose–response curves [$E(x)$ and $E(y)$, respectively], and its SD was estimated by error propagation of experimental SD calculated separately on $E(x)$ and $E(y)$. Finally, the significance of the differences between theoretical and experimental values was assessed by Student's t-tests [26]. For all non-linear fits, Origin 6.0 (Originlab, Northampton, USA) was used. For all the statistical tests, SPSS 15.0 (SPSS, Chicago, USA) was used. A p-value of 0.05 was always considered as significant.

3. Results

The IC_{50} values for gefitinib in the 11 NSCLC cell lines were determined by MTT assay as previously reported [24,27]; cells showing $IC_{50} < 1$ μM were considered sensitive (H322, H292, Calu-3) and cell lines with $IC_{50} > 7$ μM (SKLU-1, Calu-1, H1650, H596, H1975, H1299, SKMES-1, H460) were considered resistant (Table 1).

Cell lines were also characterized for *EGFR*, *K-RAS*, *PI3K* gene and PTEN protein status. The H1975 cell line carrying the L858R and T790M mutations was resistant to gefitinib [7]; the H1650 cell line, despite harboring a delE746_A750 activating mutation in the exon 19 of the EGFR gene, was resistant to gefitinib and erlotinib as previously reported [28–30]; K-RAS mutations were found in four of the tested resistant cell lines.

We did not observe any basal expression of PTEN protein in the H1650 cell line as also reported by Janmaat et al. [31].

We first evaluated the effect of gefitinib on EGFR autophosphorylation on Calu-3 (sensitive) and H1650 and SKLU-1 (both resistant) cell lines. As shown in Fig. 1A, in all the cell lines tested, EGF stimulation induced a significant phosphorylation of tyr1068 as evaluated by Western blot analysis. Gefitinib inhibited EGFR autophosphorylation starting from 0.01 μM in Calu-3 and H1650 and from 0.1 μM in SKLU-1 cells. At 1 μM the inhibition was almost complete in both sensitive and resistant cell lines indicating that other mechanisms were involved in the different sensitivity to gefitinib. Moreover, EGFR levels were not related to gefitinib responsiveness, for example Calu-3, a sensitive cell line showed similar EGFR levels to those of SKMES-1, a highly resistant cell line (see Fig. 1B).

We then analyzed (Fig. 1B) the AKT/mTOR and MAPK pathways in cells exposed to 1 μM gefitinib, a concentration that completely inhibits EGFR autophosphorylation. In the sensitive cell lines Calu-3, H292, H322, gefitinib inhibited the phosphorylation of p44/42 MAPK, AKT and S6K; in contrast, in the H460, SKMES-1, H1299, H596 and H1650 cell lines, gefitinib inhibited the phosphorylation of p44/42 MAPK but had little influence on S6K phosphorylation. The T790M mutation in the H1975 cell line conferred resistance to gefitinib enhancing the receptor catalytic activity [32], indeed in this cell line no difference was observed between untreated and gefitinib-treated cells. Surprisingly, in both Calu-1 and SKLU-1 (resistant cell lines) gefitinib was effective in reducing the activity of the two signaling pathways analyzed.

We selected the five cell lines (H460, H596, H1299, H1650, SKMES-1) characterized by maintaining S6K phosphorylation after gefitinib treatment, and we evaluated the effect of everolimus on the AKT/mTOR pathway and on cancer cell growth.

The effect of everolimus on the mTOR signaling pathway is shown in Fig. 2A. In cells exposed for 24 h to 100 nM everolimus, S6K phosphorylation was completely inhibited, whereas p-p44/42 MAPK were not affected. A concomitant increase in AKT phosphorylation was observed in H460, H596 and SKMES-1 cells. We then determined cell number by crystal violet staining on cells treated for 6 days with increasing concentrations of drug from 0.01 to 100 nM. As shown in Fig. 2B, everolimus inhibited in a dose-dependent manner cell proliferation, although to different extents in the different cell models. No apoptotic morphology was observed even when the concentration of everolimus was increased to 1 μM (result not shown). It is of note that SKMES-1 and H1650, showing higher basal level of p-AKT, were highly sensitive to everolimus.

To further explore the relationship between the inhibition of AKT/mTOR signaling and the different sensitivity to everolimus, we examined p-AKT, p-S6K, and p-p44/42 MAPK in H1299 (IC_{50}: 2.3 nM) and in SKMES-1 (IC_{50}: 0.14 nM) exposed to different concentrations of everolimus, ranging from 0.01 to 100 nM (Fig. 2C). The phosphorylation of S6K was inhibited by everolimus in a dose-dependent manner with an almost complete inhibition at 1 nM. A significant increase in the level of p-AKT was observed only in the SKMES-1, this cell line also displayed a higher basal level of p-AKT. No modulation on the p-p44/42 MAPK level was detected in both cell models tested.

In an attempt to restore sensitivity to gefitinib of resistant cell lines we evaluated the effect of a combined treatment with gefitinib with everolimus. As shown in Fig. 3, gefitinib inhibited MAPK signaling, but failed to prevent the activation of S6K, whereas everolimus caused a complete suppression of S6K phosphorylation without influencing MAPK activation. Combined treatment suppressed both signaling pathways in all the cell models tested.

The pure dose–response curves of growth inhibition of H460, SKMES-1, H1299, H596 and H1650 in the presence of gefitinib or everolimus are illustrated in Fig. 4. We also show in this figure, the dose–response curves of gefitinib in the presence of a fixed concentration of everolimus for all cell lines. The fixed concentration of everolimus was 0.01–1 nM depending on the cell line.

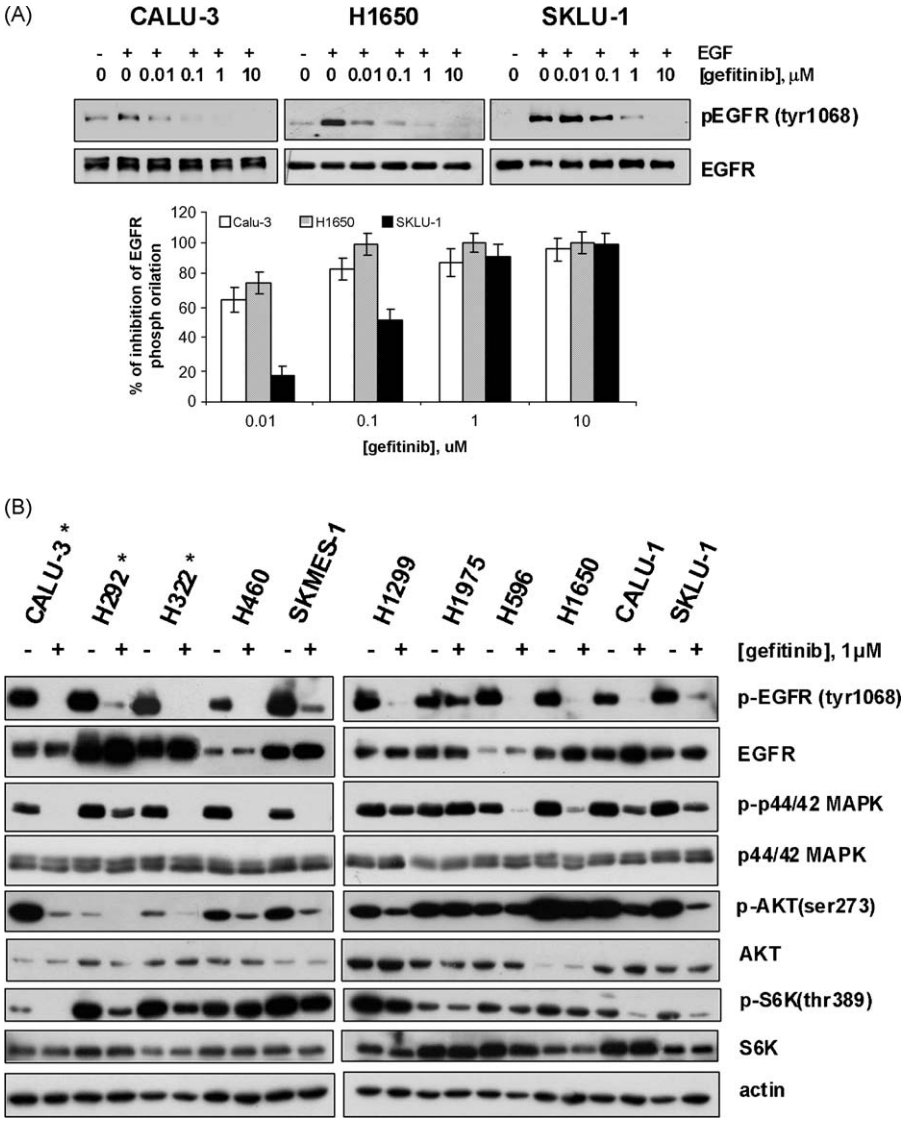

Fig. 1. Effects of gefitinib on EGFR signaling pathways. (A) Calu-3, H1650 and SKLU-1 cells were incubated for 2 h with the indicated concentrations of gefitinib before stimulation with 0.1 μg/ml EGF for 5 min. Western blot analysis was performed by using monoclonal antibodies directed to p-tyr1068 and to total EGFR. The immunoreactive spots at each point were quantified by densitometric analysis, ratios of phosphotyrosine/total EGFR were calculated and values are expressed as percentage of inhibition *versus* control. (B) NSCLC cell lines were pre-incubated for 2 h with gefitinib 1 μM and then stimulated with 0.1 μg/ml EGF for 5 min. The cells were lysed and Western blot analysis was performed on lysate proteins by using monoclonal antibodies directed against p-EGFR (tyr1068), EGFR, p-p44/42 MAPK, p44/42 MAPK, p-AKT (ser273), AKT, p-S6K (thr389), S6K, and actin. Data are from a representative experiment. The experiment, repeated three times, yielded similar results.*Cell lines sensitive to gefitinib.

However, different concentrations of everolimus combined with a fixed gefitinib concentration gave similar results in term of synergism or additivity (results not shown).

Comparing the experimental combination points with that expected by the Bliss criterion, only additive effects were observed with the H596 and H1650 cell lines. In fact, no significant differences between experimental and theoretical points were observed. By contrast, synergistic effects on growth inhibition were observed for H1299 at gefitinib 1 μM ($p < 0.05$), 5 μM ($p < 0.01$) and 10 μM ($p < 0.01$), for H460 with all tested gefitinib concentrations (1 μM $p < 0.05$, 2.5–5–10–20 μM, $p < 0.01$) and finally for SKMES-1 at gefitinib concentrations of 2.5–5 μM ($p < 0.05$). Clonogenic assay performed on H460 cells confirmed the results obtained by crystal violet staining (Fig. 5).

In the three cell lines sensitive to gefitinib (H322, H292 and Calu-3) as well as in the resistant Calu-1 and SKLU-1 cell lines (not maintaining S6K phosphorylation following gefitinib treatment) Bliss analysis showed that gefitinib is additive with everolimus (data not shown).

The effect of the combination of gefitinib and everolimus as a function of the protocol was then analyzed. The efficacy of the two drugs in combination was measured after 6 days of treatment adopting different schedules of administration (“simultaneous”, “gefitinib before”, “gefitinib after”, “gefitinib before simultaneous” and “everolimus before simultaneous”). In SKMES-1 and H1650 cells, the “simultaneous” schedule was the only one producing synergistic or additive effects on cell proliferation (data not shown).

Considering that the cell lines analyzed showed a different growth rate quotient with significantly different doubling times (Fig. 6A), we checked whether different proliferation rates of the cells affected the growth inhibition induced by the combined treatment. Growth rate was varied by modifying the number of SKMES-1 cells initially plated. SKMES-1 were seeded at different cell densities, treated with everolimus and gefitinib for 3 days and growth inhibition was then evaluated and plotted as a function of growth rate quotient. As shown in Fig. 6B, cells having a higher value of GRQ were more responsive to the combined treatment.

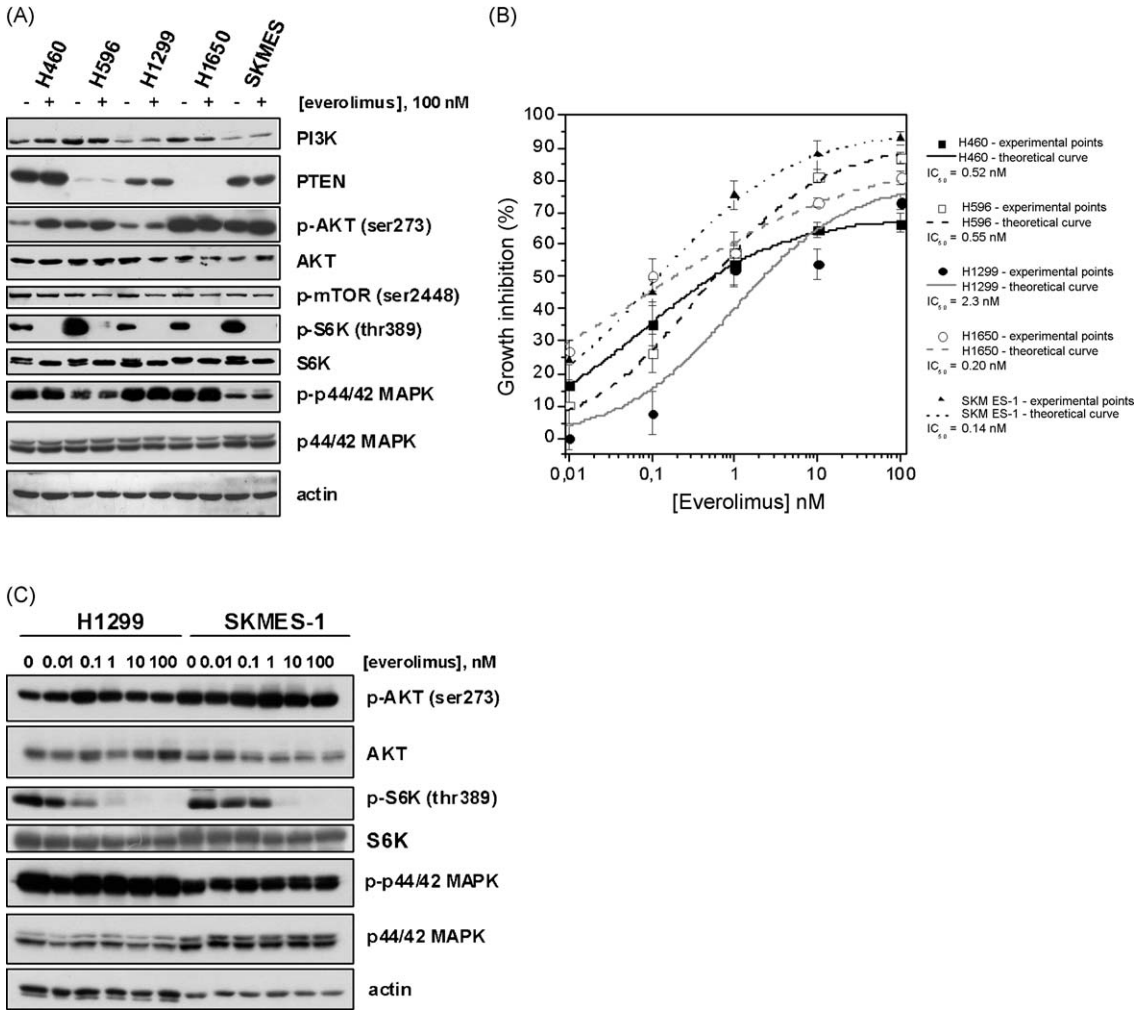

Fig. 2. Effect of everolimus on PI3K/AKT/mTOR, MAPK signaling pathways and on cell proliferation. (A) H460, H596, H1299, H1650 and SKMES-1 cells were treated with 100 nM everolimus for 24 h and then cells were lysed and Western blot analysis was performed on lysate proteins by using monoclonal antibodies directed against PI3K, PTEN, p-AKT (ser273), AKT, p-mTOR (ser2448), p-S6K (thr389), S6K, p-p44/42 MAPK, p44/42 MAPK and actin. Data are from a representative experiment. The experiment, repeated three times, yielded similar results. (B) H460, H596, H1299, H1650 and SKMES-1 cells were exposed for 6 days to different concentrations of everolimus (from 0.01 to 100 nM) and then cell number was assessed using crystal violet staining as described in Section 2. Data are expressed as percent inhibition of cell proliferation *versus* control cells. IC_{50} values are also indicated. The mean values of three independent measurements (±S.D.) are shown. (C) H1299 and SKMES-1 cells were treated with different concentrations of everolimus (from 0.01 to 100 nM) for 24 h and then cells were lysed and Western blot analysis was performed on lysate proteins by using monoclonal antibodies directed against p-AKT (ser273), AKT, p-S6K (thr389), S6K, p-p44/42 MAPK, p44/42 MAPK and actin. Data are from a representative experiment. The experiment, repeated three times, yielded similar results.

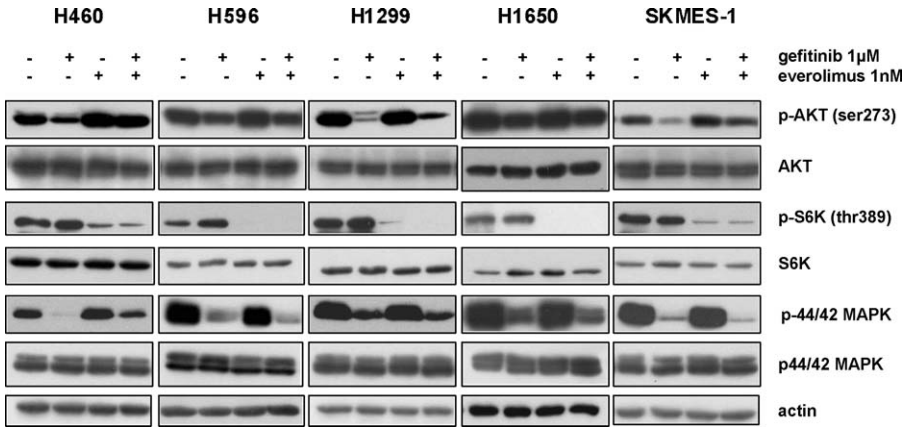

Fig. 3. Effect of gefitinib–everolimus combined treatment on PI3K/AKT/mTOR, MAPK signaling pathways. H460, H596, H1299, H1650 and SKMES-1 cells were treated with 1 μM gefitinib, 1 nM everolimus or combination of both for 24 h and then cells were lysed and Western blot analysis was performed on lysate proteins by using monoclonal antibodies directed against, p-AKT (ser273), AKT, p-S6K (thr389), S6K, p-p44/42 MAPK, p44/42 MAPK and actin. Data are from a representative experiment. The experiment, repeated three times, yielded similar results.

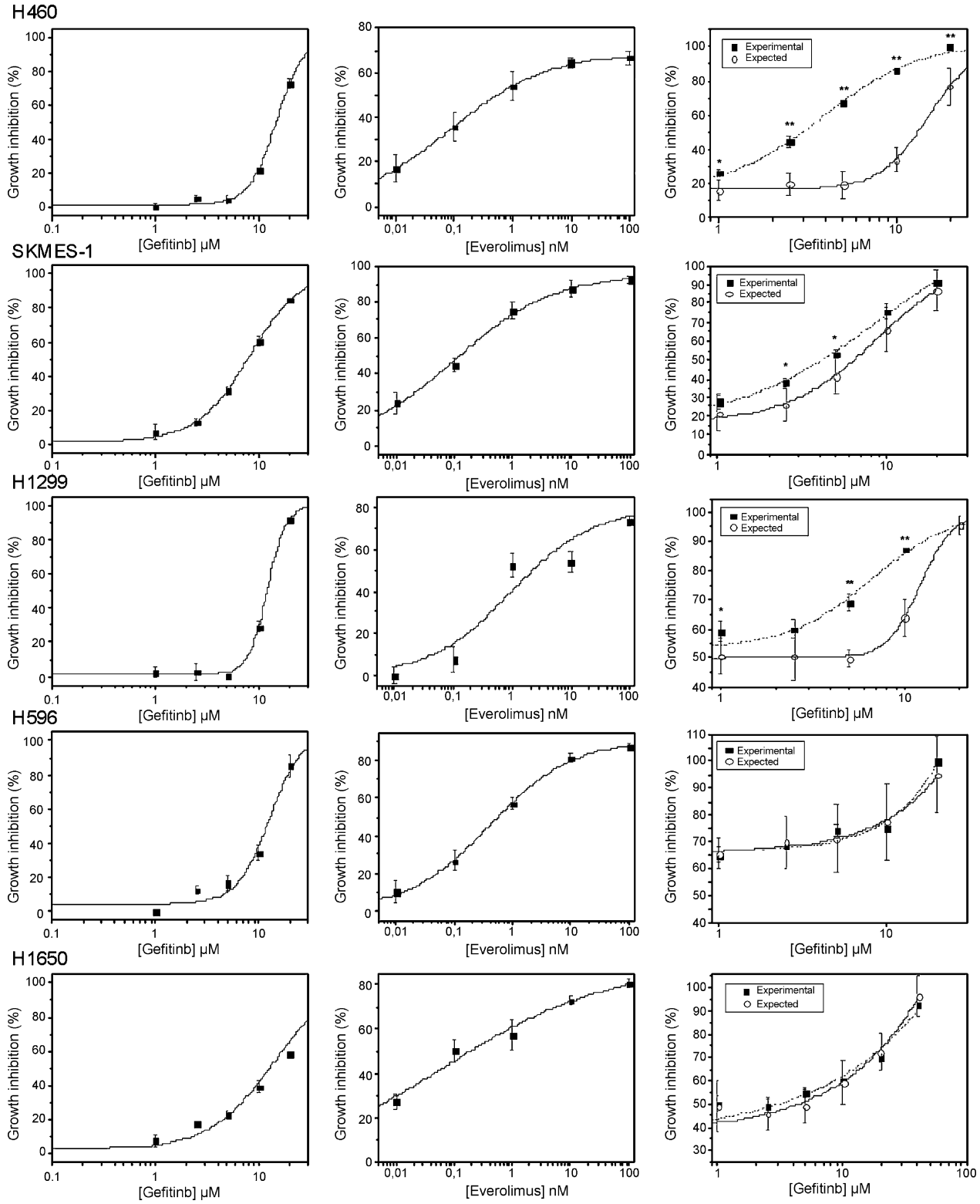

Fig. 4. Interaction between gefitinib and everolimus on the growth inhibition dose–response curves. Curves of growth-inhibitory effects of gefitinib (left), everolimus (middle) and combined treatment *versus* theoretical Bliss additivity curve (right) are reported. In the combined treatment, everolimus was 0.01 nM in H460 and SKMES-1, 0.1 nM in H1650 and 1 nM in H1299 and H596. Cells were treated with the drugs for 6 days and then cell number was assessed using crystal violet staining as described in Section 2. Data are expressed as percent inhibition of cell proliferation *versus* control cells. The experiments, repeated three times, yielded similar results. $^{*}p < 0.05$ and $^{**}p < 0.01$.

This result is consistent with the lack of apoptosis (evaluated by caspase-3 activation and morphological analysis) under any of the tested treatments at all time points analyzed suggesting that the combined everolimus–gefitinib treatment exerts, at least until 10 days, a cytostatic and not a cytotoxic effect. Taken together, these results indicate that in selected high proliferating non-small cell lung cancer cell lines the combination of gefitinib with everolimus increases the growth-inhibitory effect.

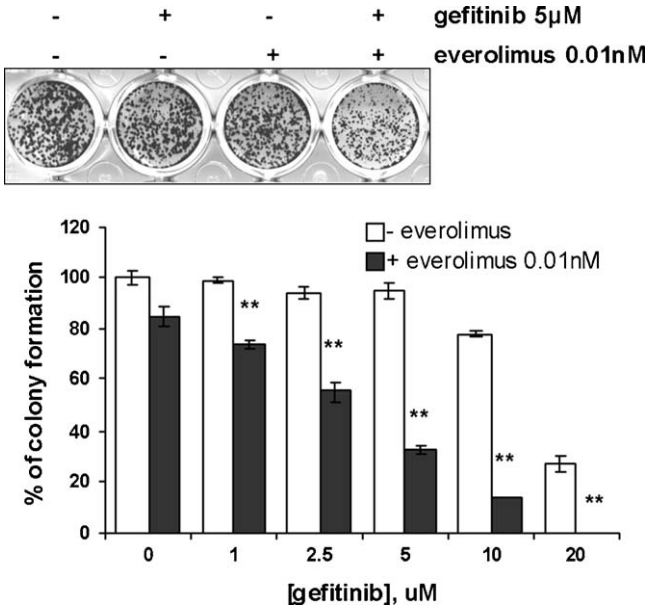

Fig. 5. Effect of gefitinib–everolimus combined treatment on colony formation ability. H460 cells were treated with gefitinib and/or 0.01 nM everolimus and the ability of individual cell to form >50 cell colonies was assessed after 10 days. All data on viability are given as percentage *versus* untreated control. A representative picture of the colonies taken using a digital camera was shown. $^{**}p < 0.01$ *versus* gefitinib-treated cells; $n = 3$.

(A)

Cell line	GRQ	DT hr
H460	0.375	15
H1299	0.278	17
SKMES-1	0.173	20
H596	0.024	52
H1650	0.010	70

(B)

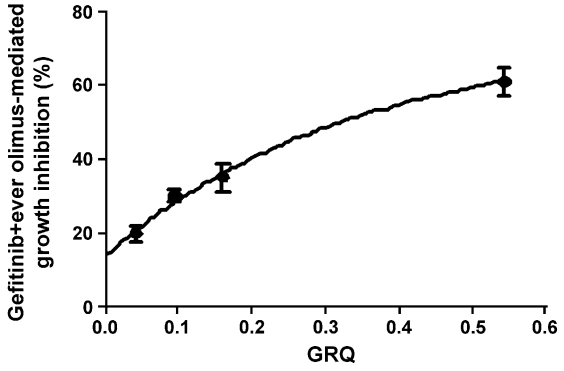

Fig. 6. Effect of proliferative index on growth inhibition induced by gefitinib–everolimus combined treatment. (A) Proliferation rates were estimated by the growth rate quotient as calculated by the equation: $GRQ = (N - N_0)/t \times 1/N_0$. Doubling time (h) was calculated according to the formula $t \times \ln(2)/\ln(N/N_0)$, where N is the final cell number; N_0 is the initial cell number and t is the time elapsed between the two counts. (B) SKMES-1 were plated at different density (2500, 5000, 10,000, 25,000/cm^2) and exposed for 3 days to 5 μM gefitinib and 0.01 nM everolimus and then cell number was assessed using crystal violet staining as described in Section 2. Percent inhibition of cell proliferation *versus* control cells was plotted as function of growth rate quotient. The experiments, repeated three times, yielded similar results.

4. Discussion

Intrinsic and acquired resistance to EGFR-targeting agents is an important issue in clinical practice. Clear evidence exists for the involvement of constitutive activation of the PI3K/AKT signaling pathway in lung carcinogenesis and in resistance to tyrosine-kinase inhibitors [15].

Of the eleven NSCLC cell lines tested, three (H322, H292, Calu-3) having an $IC_{50} < 1$ μM were considered highly sensitive to gefitinib, while the others, with an $IC_{50} > 7$ μM were considered resistant. The gefitinib concentration of 1 μM was used as cutoff for sensitivity because this concentration approaches levels observed in serum from patients under treatment with 250 mg/day [6]. In line with other reports [28,33,34] no association between responsiveness to gefitinib and EGFR level was observed.

The response of sensitive cell lines to gefitinib was previously correlated with dependence on AKT and MAPK activation in response to EGFR signaling [34] and on inhibition of both PI3K/AKT/mTOR and MAPK pathways following gefitinib treatment [28,35,36]. In the three highly sensitive cell lines we observed a marked reduction on p-p44/42 MAPK, on p-AKT, and on p-S6K following gefitinib treatment (Fig. 1B). In contrast, in five resistant cell lines (H460, SKMES-1, H1299, H596, H1650) there was no reduction in the phosphorylation of S6K suggesting that the maintenance of this survival pathway is related to resistance to gefitinib.

The presence of a mutation in the PI3K gene in H596 and the deletion of PTEN in the H1650 cells could justify the persistent phosphorylation of AKT after treatment with gefitinib (Fig. 1B and Fig. 3). However in H460, SKMES-1 and H1299 p-AKT was significantly reduced. This result is in contrast to that reported by Engelman et al. [11] on the reduction of p-AKT levels solely in sensitive NSCLC cell lines in which gefitinib inhibited growth.

Although H460, SKMES-1, Calu-1 and SKLU-1 harbor mutations in the *K-RAS* gene, gefitinib was able to reduce the phosphorylation of p44/42 MAPK as previously reported for the H460 cell line [31] indicating that K-RAS mutations cannot sustain an EGFR-independent MAPK pathway activity.

The rapamycin derivative everolimus is a potent inhibitor of tumor cell proliferation *in vitro* and in animal models of cancer [37]. In general, rapamycin and everolimus are cytostatic. In KLN-205 and A549 NSCLC cells, rapamycin induced cell cycle arrest and blocked proliferation but did not induce apoptosis [38]. In H460, H596, H1299, H1650, SKMES-1 characterized by maintaining S6K phosphorylation after gefitinib treatment, everolimus inhibited phosphorylation of S6K and slowed proliferation, albeit to different extents especially at the lower doses. This result is in agreement with that reported by Sun et al. on H460 and H1299 cell lines treated with rapamycin [39]. The high basal level of p-AKT observed in H1650 and SKMES-1 may be associated with the higher sensitivity to everolimus as reported in ovarian cancer cells [40] and in breast cancer cells [41].

An increase in AKT phosphorylation after everolimus treatment was observed in H460, H596 and SKMES-1 cells. It is recognized that mTOR inhibition induces insulin receptor substrate-1 expression and inhibits a normally negative feedback loop resulting in AKT activation in some cancer cell lines [39,42].

It is noteworthy that up-regulation of pAKT induced by everolimus in H596 and SKMES-1 cells was reduced in the presence of gefitinib, but to a lesser extent in H460 cells.

In vitro studies with rapamycin and erlotinib in the gefitinib-resistant H460, Calu-6 and SW1573 NSCLC cell lines [43] showed a synergistic growth inhibition associated with a down-modulation of rapamycin-stimulated AKT activity.

However, in H460 treated with rapamycin and gefitinib, Janmaat et al. [31] did not observe an inhibition of AKT

phosphorylation, and an enhanced cytotoxicity was obtained only when gefitinib was associated with the PI3 kinase inhibitor LY294002.

The association between mTOR inhibitors (rapamycin, everolimus or temsirolimus) and anti-EGFR drugs (gefitinib, erlotinib or cetuximab) has also been tested in human cancer cell lines derived from different types of tumors [43–47].

In particular Bianco et al. [44] showed that in resistant human GEO colon, PC3 prostate and MDA-MB-468 breast cancer cells the everolimus and gefitinib combined therapy restored the anti-proliferative effect induced by anti-EGFR drugs and induced, in colon cells, a strong reduction of AKT activation. Moreover, the combined treatment potentiated the anti-angiogenic effect of everolimus alone. This result was also confirmed on pancreatic cancer cells [45] with a combination of gefitinib and rapamycin.

In our experimental models, we demonstrated in a panel of NSCLC cell lines that the everolimus and gefitinib combination exerted a cytostatic and not a cytotoxic effect independently from inhibition of AKT phosphorylation. The effect of the combination was deeply studied on the basis of a plausible interaction model (e.g. Bliss) and we showed that the combination was exactly additive for H596 and H1650 and synergistic for H460, SKMES-1 and H1299 with suppression of proliferation rather than enhancement of cell death in contrast to the findings of Buck et al. [43] on a single NSCLC model (Calu-6 cells).

The authors suggested that the synergy for rapamycin with erlotinib was linked to the capability of erlotinib to promote apoptosis by reducing the rapamycin-stimulated AKT activity, although this conclusion was supported by observations on only one of the models responsive to the double treatment. On the other hand, we did not detect cell death, although AKT levels were partially reduced by the combined treatment.

Preliminary clinical data on combining mTOR inhibitors with EGFR-TKIs might be promising on NSCLC patients. In a phase I trial, 10 advanced NSCLC patients received the combination of everolimus and gefitinib administered orally and daily and the maximum-tolerated dose of everolimus was 5 mg daily when combined with gefitinib at a dose of 250 mg [19]. To date, only preliminary results from the subsequent phase II trial are available. In that trial, untreated or platinum-based pretreated advanced NSCLC patients, all current or former smokers, were enrolled. The results are promising; in particular, positive responses were observed in male former smokers and in one K-RAS mutated patient, all features negative for response to EGFR-TKIs [48].

In this study we provide new insights regardless a rationale applicability of the combined treatment based on tumor growth rate. Indeed cells showing higher proliferation index are more responsive to treatment than are cells with a low level of proliferation. We propose that proliferation index might be a good marker for selecting those patients who will benefit from the combined treatment.

Tumor proliferative activity, in general evaluated by Ki-67 expression, is a prognostic marker in different type of tumors included NSCLC and it is considered of potential interest in defining populations at high or low risk of recurrence [49]. No data are available about the possible correlation between high tumor proliferation and response to EGFR inhibitors. Nevertheless in this context, it has been recently reported that 18F-FDG PET, which reflects glucose metabolism and proliferative activity of tumor cells [50], may predict response to gefitinib in the treatment of advanced NSCLC patient [51].

In conclusion, our observations on combinations of everolimus and gefitinib on various NSCLC lines *in vitro* may help devise new therapies for selected high Ki-67 expressing lung cancer patients by targeting the AKT/mTOR pathway in an attempt to overcome gefitinib resistance.

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