Synergistic Antitumor Activity of Epidermal Growth Factor Receptor Tyrosine Kinase Inhibitor Gefitinib and IFN-α in Head and Neck Cancer Cells \textit{In vitro} and \textit{In vivo}

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Abstract

Purpose: Epidermal growth factor receptor (EGFR) overexpression has been implicated in the development of head and neck squamous cell carcinomas (HNSCC) and represents a potential therapeutic target for this disease. We have reported previously that growth inhibitory concentrations of IFN-α enhance the expression and activity of EGFR and that this effect could represent an escape mechanism to the growth inhibition and apoptotic cell death induced by IFN-α. In this study, we investigate whether the combination of IFN-α and gefitinib (Iressa, AstraZeneca Pharmaceuticals, Macclesfield, United Kingdom), a selective EGFR tyrosine kinase inhibitor, might have a cooperative antitumor effect on HNSCC-derived cell lines.

Experimental Design: The interaction of IFN-α and gefitinib was evaluated \textit{in vitro} on HNSCC-derived cell lines by median drug effect analysis calculating a combination index with CalcuSyn software and \textit{in vivo} by using HNSCC xenografts in nude mice. The mechanism of gefitinib and IFN-α interactions was also studied by analysis of cell cycle kinetics, apoptosis assays, and Western blotting of EGFR signal transduction components.

Results: Simultaneous exposure to gefitinib and IFN-α produced synergistic antiproliferative and proapoptotic effects compared with single drug treatment. Furthermore, daily treatment of gefitinib (50 mg/kg p.o.) in combination with an IFN-α regimen (50,000 units s.c. three times weekly) induced tumor growth delay and increased survival rate on established HNSCC xenografts in nude mice. Moreover, the concomitant treatment with gefitinib suppressed the stimulation of extracellular signal-regulated kinase phosphorylation/activity induced by IFN-α both \textit{in vitro} and \textit{in vivo}.

Conclusion: The observed cooperative antitumor effects could be, at least in part, explained by the inhibition exerted by gefitinib of an IFN-α-induced EGF-dependent survival pathway, which involves extracellular signal-regulated kinase activation. These results provide a rationale for the clinical evaluation of gefitinib in combination with IFN-α in HNSCC.

Despite advances in our understanding, prevention, and treatment of head and neck squamous cell carcinoma (HNSCC), the 5-year survival rates for affected individuals remain low. This poor prognosis for HNSCC reflects a limited understanding of the mechanisms of local and regional metastasis together with unsatisfactory responsiveness to conventional systemic therapy in recurrent/advanced disease (1).

Epidermal growth factor (EGF) receptor (EGFR) overexpression has been associated with advanced stage and poor prognosis in HNSCC (1–4); therefore, EGFR has been considered a tumor-specific therapeutic target in this neoplasm (5, 6).

EGFR, a transmembrane tyrosine kinase receptor, is an important regulator of critical events occurring during oncogenesis and tumor progression, such as cell proliferation, inhibition of programmed cell death, angiogenesis, invasion, and metastasis (7). On ligand binding, receptor dimerization activates tyrosine kinase activity and tyrosine autophosphorylation recruiting multiple signaling proteins that initiate a cascade of several signal transducers/pathways, including the Ras-Raf-mitogen-activated protein kinase (MAPK) pathway (8, 9), the phosphatidylinositol 3-kinase pathway (8, 9), and the signal transducers and activators of transcription (STAT) pathway (10), all regulating mitogenesis, survival, and other cellular functions (11).

Gefitinib (Iressa, AstraZeneca Pharmaceuticals, Macclesfield, United Kingdom) is an orally active, selective EGFR tyrosine kinase inhibitor that blocks the signal transduction pathways involved in the proliferation and survival of cancer cells and other host-dependent processes promoting cancer growth. Gefitinib has shown antitumor activity at low doses in...
preclinical studies and we have shown recently that it is potentially active in the treatment of HNSCC because it is able to arrest the growth of HNSCC-derived cell lines by inhibiting both basal and EGF-induced EGFR-mediated mitogenic signaling (12). Gefitinib has also shown antitumor activity with an acceptable tolerability profile in phase I clinical studies (13). A large phase II study has confirmed clinically meaningful antitumor activity and symptom relief of gefitinib monotherapy in patients with recurrent non–small cell lung cancer (14, 15). It has, however, to be considered that all trials exploring gefitinib combination with cytotoxic chemotherapy have been negative to date (INTACT 1 and 2 trials; refs. 16, 17), suggesting that gefitinib-based combined treatment should be designed based on a specific molecular rationale.

A novel and compelling scenario is emerging from recent findings. Groundbreaking studies have shown recently that kinase domain mutations of EGFR gene in the non–small cell lung cancer cells could predict significant clinical responses to both gefitinib and erlotinib, another EGFR tyrosine kinase inhibitor (18–20). All of the EGFR mutations affect amino acids close to the ATP-binding pocket that is targeted by gefitinib. Interestingly, in vitro, EGFR mutants showed enhanced tyrosine kinase activity in response to EGF and increased sensitivity to inhibition by gefitinib.

In addition to non–small cell lung cancer, there is evidence that gefitinib could be a potential agent for the treatment of other tumors, including HNSCC (21). In a phase II study, Cohen et al. reported that 11% of the HNSCC patients with gefitinib monotherapy had an objective tumor response to gefitinib monotherapy. On this regard, a recent article described that also HNSCC harbors the EGFR gene mutations, suggesting the rationale for the clinical applicability of gefitinib to HNSCC treatment (22).

IFN-α is a cytokine with pleiotropic biological activity mediated by the activation of intracellular pathways after binding to a specific surface receptor (23). Although IFN-α has clearly shown antitumor activity, the mechanism of such effects are presently unknown. Direct antitumor activity as well as immunostimulatory and antiangiogenic activity have been described in a variety of experimental systems (24, 25). IFN-α has been widely used in the therapy of several neoplasms, including head and neck tumors (26–29); however, contrasting data have generated concern regarding the clinical effectiveness of IFN-α monotherapy of solid tumors. In fact, the benefit of IFN-α treatment is limited to some neoplasms (30, 31) and mechanisms of tumor resistance to IFN-α have been extensively described. In details, alteration of Janus-activated kinase/STAT components of the IFN-α-induced signaling has been indicated as potential resistance mechanism for IFN-α antiproliferative effects (32). Interestingly, alterations in STAT signaling have been correlated to head and neck carcinogenesis (33).

We have shown that IFN-α, at growth inhibitory concentrations, enhances the expression and signaling activity of the EGFR in HNSCC-derived cell lines (34, 35). We have speculated that the enhanced expression and function of EGFR by the tumor cells could represent a stress response that is activated to provide an escape mechanism to the growth inhibition induced by IFN-α (36). Moreover, we have shown recently that EGF has a protective effect on IFN-α-induced apoptosis that is targeted on the stress pathway terminal kinases (37), suggesting that EGFR signaling suppresses apoptosis behaving like a survival pathway. Furthermore, we have also reported that a specific hyperactivation of EGFR-dependent Ras/extracellular signal-regulated kinase (ERK) 1/2 pathway counteracts IFN-α-mediated apoptosis and could be further stimulated by the addition of EGF (38). All these observations suggested that the selective targeting of this survival pathway might enhance the antitumor activity of IFN-α. In fact, either the transfection of a dominant form of Ras, RASN17, or the treatment of tumor cells with a specific MEK1 inhibitor (PD098056) strongly potentiated the apoptosis induced by IFN-α (38).

Because the EGF tyrosine kinase inhibitor gefitinib might induce a selective blockade of IFN-α-induced EGF-dependent survival pathways, this study explored the possibility of achieving more potent antitumor activity through combination therapy of gefitinib and IFN-α on HNSCC both in vitro and in vivo in a xenograft model.

### Materials and Methods

#### Materials

Clinical-grade gefitinib was provided by AstraZeneca Pharmaceuticals. Stock solutions were prepared in polyethylene glycol 400 and diluted to appropriate concentrations in culture medium before addition to the cells. Equivalent dilutions of polyethylene glycol 400 without the inhibitor were used as control. Human recombinant IFN-α2b was from Schering-Plough S.p.A. (Kenilworth, NJ). The IFN-α preparation had a specific activity of 2.6 × 10⁶ units/mg and >99% purity and was diluted to appropriate concentrations in culture medium before addition to the cells. Phospho–p44/p42 MAPK (Thr202/Tyr204) and p44/p42 MAPK antibodies were obtained from New England Biolabs (Beverly, MA). Phospho-STAT1 (Tyr701) and STAT1α antibodies were obtained from Santa Cruz Biotechnology, Inc. (San Jose, CA). Phospho-EGFR (Tyr1068) antibody was obtained from Santa Cruz Biotechnology. Enhanced chemiluminescence immunodetection reagents were from Amersham (Buckinghamshire, United Kingdom). All media, serum, antibiotics, and glutamine were from ICN Biomedicals (Irvine, CA).

#### Cell culture and cell proliferation assay

KB and HEP-2 HNSCC-derived cell lines (American Type Culture Collection, Rockville, MD) and CAL 27 cell line (kindly provided by Dr. J.L. Fischel, Centre Antoine Lacassagne, Nice, France) were grown in DMEM supplemented with 10% heat-inactivated fetal bovine serum, 50 units/mL penicillin, 500 μg/mL streptomycin, and 4 mmol/L glutamine in a humidified atmosphere of 95% air and 5% CO₂ at 37°C. The cells throughout all the experiments have been maintained in serum-containing medium. Cellular survival/proliferation was measured in 96-well plates by a spectrophotometric dye incorporation assay (sulforhodamine B) as described previously (39). Briefly, 1 × 10⁵ cells per well were seeded in quadruplicate, and gefitinib was added at indicated concentrations after 24 hours of seeding and daily thereafter, whereas IFN-α was added at indicated concentrations after 24 hours of seeding and every 48 hours thereafter.

#### Drug combination studies

The combination index (CI) was calculated by the Chou-Talalay equation, which takes into account both potency (D₅₀ or IC₅₀) and the shape of the dose-effect curve (40). The general equation for the classic isobologram (CI = 1) is given by

\[
CI = \frac{D_1}{D_{1x}} + \frac{D_2}{D_{2x}} + \frac{[D_{1x} + D_{2x}]}{[D_1 + D_2]}
\]

where \(D_{1x}\) and \(D_{2x}\) in the denominators are the doses (or concentrations) for drug 1 (drug 1) and drug 2 (drug 2) alone that gives x% inhibition, whereas \(D_1\) and \(D_2\) in the numerators are the doses of drug 1 and drug 2 in combination that also inhibited x% (i.e., isoeffective). CI < 1, CI = 1, and CI > 1 indicate synergism, additive effect, and antagonism, respectively (40).
The linear correlation coefficient (r) of the median-effect plot is considered the first line of statistics to measure the conformity of the data with the mass-action law principle when the experimental measurement is assumed to be accurate. A r value equal to 1 indicates perfect conformity. A poor r value may be the result of biological variability or experimental deviations.

Dose reduction index (DRI) representing the measure of how much the dose of each drug in a synergistic combination may be reduced at a given effect level compared with the doses of each drug alone.

Drug combination studies in vitro were based on concentration-effect curves generated as a plot of the fraction of unaffected (surviving) cells versus drug concentration after 72 hours of treatment. To explore the relative contribution of each agent to the synergism, combinations with different gefitinib/IFN-α molar ratios, three mixtures were tested for each schedule: equipotent doses of the two agents (IC50), higher relative doses of gefitinib (IC75 of gefitinib/IC50 of IFN-α), and higher relative doses of IFN-α (IC50 of gefitinib/IC75 of IFN-α). Assessment of drug interaction was done calculating Cl. CI/fractional effect curves represent the CI versus the fraction of cells affected/killed by gefitinib and IFN-α in combination.

**Protein extraction and Western blotting.** Cells grown and treated as indicated were washed and scraped with ice-cold PBS without Ca2+/Mg2+ and centrifuged. The cell pellets were lysed for 1 hour at 4 °C in NP40 lysis buffer [0.5% NP40, 50 mmol/L HEPES (pH 7), 250 mmol/L NaCl, 5 mmol/L EDTA, 0.5 mmol/L sodium pyrophosphate, 0.5 mmol/L sodium orthovanadate, 50 mmol/L NaF, 1 mmol/L DTT, 0.5 mmol/L phenylmethylsulfonyl fluoride, 5 μg/mL aprotinin, 5 μg/mL leupeptin, 5 μg/mL benzamidine, 1 μg/mL pepstatin] and clarified by centrifugation at 14,000 × g for 10 minutes.

Equal amount of protein, monitored by Lowry assay using bovine serum albumin as standard, was separated on SDS-PAGE. After electrophoresis, proteins were transferred to nitrocellulose paper, immunoblotted with specific antibody, and probed with the appropriate horseradish peroxidase–linked IgG. Immunoreactive bands were detected by enhanced chemiluminescence system.

**Apoptosis assays.** FACScan flow cytometer analysis was done on KB cells treated with gefitinib and/or IFN-α at the indicated concentrations. Cells were harvested with trypsin at the indicated times. Cells were washed in PBS without Ca2+/Mg2+, pooled with floating cells, then fixed in 70% ethanol and stored at -20°C until analysis. Nuclear DNA staining was done by propidium iodide. Briefly, after a washing in PBS without Ca2+/Mg2+, 106 cells were stained in 2 mL propidium iodide. DNA flow cytometry was done in duplicate by a FACScan flow cytometer (Becton, Dickinson, San Jose, CA) coupled with a CICERO signal interface module (Cytomation, Fort Collins, CO). For each sample, 20,000 events were stored in the list mode file by Cyclops SUMMIT software (Cytomation). Cell cycle analysis was done by the ModFit LT software (Verity Software House, Inc., Topsham, ME). FL2 area versus FL2 width gating was done to exclude doublets from the G2-M region. Flow cytometric analysis of apoptotic cell death was done as reported previously (41). The percentage of apoptotic cells was calculated in the subdiploid region of the DNA content, registered as FL3 signals in a log scale. To avoid cell debris contamination due to necrotic cell death, cells were selected by side scatter (SSC) versus DNA log signals (FL3) gating.

Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling assay was done on KB cells treated with gefitinib and/or IFN-α at the indicated concentrations, and cells, after washing in PBS supplemented in 0.1% bovine serum albumin, were treated with in situ detection kit according to the manufacturer's instructions (Boehringer Mannheim Biochemicals, Indianapolis, IN). Nuclei with fragmented DNA were visualized by a fluorescence microscope. In each experiment carried out twice, quantification of apoptosis was done by scoring 300 to 400 cells and evaluating the percentage of cells in apoptosis compared with control untreated cells. Mean values reported differed by <10%.

**In vivo xenograft assay.** Female BALB/c athymic (nu+/nu+) mice, 8 to 10 weeks of age, weighing 24 to 32 g, were purchased from Charles River Laboratories (Milan, Italy). The research protocol was approved, and mice were housed and maintained under specific pathogen-free conditions in the Animal Care Facility of National Cancer Institute G. Pascale in accordance with the institutional guidelines of the Italian Ministry of Health Animal Care and Use Committee. Mice were acclimatized for 1 week before being injected with cancer cells. Mice were injected s.c. with 5 × 106 KB cells that had been resuspended in 200 μL PBS. After 5 days when established tumors of ~0.3 cm3 in diameter were detected, mice were randomized and divide in four groups. Ten mice per group were treated s.c. on days 1, 3, and 5 of each week with 2 × 105 IU/kg/dose, IFN-α diluted in saline solution, and/or 50 mg/kg/dose gefitinib p.o. daily diluted as described before in polyethylene glycol 400 for 4 weeks. Control animals received equal volume of polyethylene glycol 400 p.o. as animal receiving gefitinib and were injected with equal volume of saline solution as animal receiving IFN-α. Tumor size were measured twice weekly by the modified ellipsoid formula: (π/6) × A × B × C, where A is the longest and B is the shortest perpendicular axis of an assumed ellipsoid corresponding to tumor mass (42). Body weight was measured twice weekly as control for treatment toxicity. Two-sided Student’s t test was used to compare the volume of xenograft tumors. Survival analysis was computed by the Kaplan-Meier method and statistical analysis was carried out by the χ2 test. In vivo drug combination studies were evaluated by CalcuSyn. For the calculation of CI, the values of % cell kill (CK) for a fixed tumor volume were considered [determined by the log CK (LCK)], LCK was determined as LCK = (T - C) / (3.3 × TD). For EGFR expression, proteins (50 μg) were electrophoresed on 8% SDS-PAGE, transferred to nitrocellulose membranes, immunoblotted with anti-EGFR, and detected by enhanced chemiluminescence. γ-Tubulin was used as loading control (inset).
group and control group, respectively. CK was determined as %CK = \left[ \frac{1}{C_0} \left( \frac{1}{C_0} - 10LCK \right) \right] / C_2 \times 100 \) (43).

Tumor growth delay (TGD) was determined as %TGD = \( \frac{(T - C)}{C} \times 100 \), where \( T \) and \( C \) are the same values as described above (43).

**Results**

**Antiproliferative effect of gefitinib and IFN-α in HNSCC cell lines.** We firstly evaluated the effects of both gefitinib and IFN-α on the growth of KB, CAL 27, and HEP-2 HNSCC-derived cell lines expressing different levels of EGFR (Fig. 1) and with a different p53 status (Table 1; ref. 44). In all cell lines, a dose-dependent growth inhibition was observed following treatment with IFN-α for 72 hours (Fig. 1) and the IC\(_{50}\) were 5,000 IU/mL for KB cells, 3,000 IU/mL for CAL 27 cells, and 27,000 IU/mL for HEP-2 cells (Table 1). Moreover, there was no correlation between either p53 status or EGFR expression and the effect of IFN-α. HEP-2 cell line seems to be the most resistant to IFN-α antiproliferative effect.

On the other hand, in all cell lines, the sensitivity to the antiproliferative effect of gefitinib was also dose dependent and independent to the status of p53 and interestingly to the expression of EGFR. In details, the IC\(_{50}\) at 72 hours was 5 μmol/L for KB cells, 1 μmol/L for CAL 27 cells, and 30 μmol/L for HEP-2 cells, which seem to be the most resistant, as shown also for IFN-α (Table 1).

**Synergistic antiproliferative effect of IFN-α/gefitinib combination.** We have shown previously that the IFN-α-induced antiproliferative and proapoptotic effect was paralleled by the induction of a survival escape mechanism activated by an increased EGFR expression and function (34, 37). On this basis, we have examined if the cooperative antitumor effect on HNSCC cells could be obtained combining IFN-α with gefitinib treatment. Simultaneous exposure of gefitinib and IFN-α on KB, CAL 27, and HEP-2 cells resulted in strong synergistic antiproliferative effect in all cell lines as shown by median drug effect analysis calculating a CI according to the method of Chou and Talalay (ref. 45; Fig. 2). To explore the relative contribution of each agent to the synergism, combinations with different gefitinib/IFN-α molar ratios were compared. Serial dilutions from three different mixtures were tested using equipotent doses of the two agents (at the ratio of their IC\(_{50}\)s), higher

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>p53 status</th>
<th>Gefitinib ED(_{50}) (μmol/L)</th>
<th>IFN-α ED(_{50}) (IU/mL)</th>
<th>IC(_{50}) (r)</th>
<th>DRI at IC(_{50})</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAL 27</td>
<td>Mutant</td>
<td>1 ± 0.7</td>
<td>3,000 ± 800</td>
<td>0.45 ± 0.1 (0.99 ± 0.001)</td>
<td>4.01 ± 0.7</td>
</tr>
<tr>
<td>KB</td>
<td>Null/low wild-type</td>
<td>5.4 ± 0.6</td>
<td>5,000 ± 1,000</td>
<td>0.45 ± 0.9 (0.99 ± 0.001)</td>
<td>5.24 ± 0.4</td>
</tr>
<tr>
<td>HEP-2</td>
<td>Wild-type</td>
<td>30 ± 3.5</td>
<td>27,000 ± 8,000</td>
<td>0.25 ± 0.8 (0.99 ± 0.001)</td>
<td>18.30 ± 4.5</td>
</tr>
</tbody>
</table>

NOTE: Mean ± SD of at least three different experiments done in quadruplicate. CIs were calculated for 50% cell survival (ED\(_{50}\)) by isobologram analysis done on CalcuSyn software. DRI represents the order of magnitude (fold) of dose reduction obtained for ED\(_{50}\) effect in combination setting compared with each drug alone. \( r \) is the coefficient of correlation for the fitting between CIs and fractional effects.

![Fig. 2](https://example.com/fig2.png) **In vitro** synergistic antiproliferative effect of IFN-α and gefitinib. Effect of treatment with combination of gefitinib + IFN-α in KB, CAL 27, and HEP-2 cell lines. CI/fractional effect curves showed the CI versus the fraction of cells affected/killed by gefitinib and IFN-α in combination for the different cell lines. For each cell line, two molar ratios were used: equipotent doses of the two agents (at the ratio of their IC\(_{50}\)) and higher relative doses of IFN-α (IC\(_{25}\) of gefitinib/IC\(_{75}\) of IFN-α), respectively. Combination analysis was done using the method described by Chou and Talalay (see Materials and Methods). Combinations were synergistic when CIs were < 0.8. Representative experiment (carried out at least twice for each cell line; mean CIs were reported in Table 1).
relative doses of gefitinib (IC_{50} of gefitinib/IC_{25} of IFN-α), and higher relative doses of IFN-α (IC_{25} of gefitinib/IC_{75} of IFN-α), respectively.

Typical examples of CI/fractional effect curves showing the CI versus the fraction of cells affected/killed by gefitinib and IFN-α in combination, for the different cell lines, are illustrated in Fig. 2. The combination of ineffective doses of IFN-α and high dose of gefitinib produced additive or antagonistic effect (data not shown). The best synergistic antiproliferative effect was observed when equipotent doses of IFN-α and gefitinib were combined (Fig. 2; Table 1), whereas increasing doses of IFN-α and low doses of gefitinib produced a relatively reduced synergistic effect with a higher CIs (Fig. 2). DRIs, which represent the order of magnitude (fold) of dose reduction obtained in combination setting compared with each drug alone, were shown in Table 1 for the three cell lines. IC_{50} ranging from 4- to 18.3-fold reduction were observed for both gefitinib and IFN-α.

Furthermore, we have explored different schedules of treatment combining equipotent concentrations of gefitinib and IFN-α. Twenty-four hours of sequential exposure to either agent produced similar effect on the IC_{50} or DRIs, indicating that the synergistic effect observed was not schedule dependent (data not shown).

**Gefitinib potentiates the proapoptotic effect of IFN-α.** We have shown previously that treatment with 1,000 IU/ml IFN-α for 48 hours induced apoptosis in KB cells and that EGF antagonized this effect (37). Based on this consideration, we further evaluated whether the mechanisms of the synergistic interaction between gefitinib and IFN-α involve cell killing mediated by apoptosis. We found that low doses of either IFN-α (100 IU/ml) or gefitinib (1 μmol/L) as single agent produced only slight perturbation of cell cycle kinetics and no apoptotic effects as shown by flow cytometry analysis after propidium iodide labeling of DNA (Fig. 3A). In details, a partial accumulation in G₁ phase and a decrease in S phase were observed in KB cells treated with either gefitinib or IFN-α. On the other hand, combined treatment with gefitinib and IFN-α resulted in a potent induction of apoptosis (40% compared with control) as measured by the appearance of hypodiploid population (sub-G₀, Fig. 3A). Interestingly, G₁-phase accumulation observed in cells treated with gefitinib or IFN-α as single agent disappeared in the combined treatment (Fig. 3A). The increase of IFN-α-induced apoptosis by gefitinib was also confirmed by a distinct approach using terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling technique and a higher concentration of IFN-α (1,000 IU/ml; Fig. 3B-E). The percentage of cells in apoptosis was also evaluated showing ~50% of apoptotic cells scored in the IFN-α/gefitinib combination (Fig. 3E) versus 15% and 21% in gefitinib-treated cells (Fig. 3C) and IFN-α-treated cells (Fig. 3D), respectively.

**Gefitinib suppresses the activation of the EGF signaling induced by IFN-α.** In the aim to investigate the mechanism of gefitinib and IFN-α interactions on signal transduction, we firstly evaluated the effect of IFN-α on ERK1/2 activation, a crucial event in the EGFR-dependent mitogenic pathway. As shown in Fig. 4A, acute treatment with 1,000 IU/ml IFN-α (5 minutes up to 1 hour) of KB cells does not affect neither activity nor expression of ERK1/2, whereas within 1 minute of EGF treatment a marked activation of both ERK1 and ERK2 was observed (Fig. 4A). On the other hand, phosphorylation of STAT1 proteins, which represent early downstream targets of IFN-α, was induced within 1 minute of IFN-α exposure, indicating that IFN-α-dependent pathways are active in these cells (Fig. 4A). Activation of STAT1 was not affected by EGF, whereas STAT1α protein expression was not regulated by either IFN-α or EGF treatment.

Moreover, as shown in Fig. 4B, an activation of both ERK1 and ERK2 was observed only after prolonged incubation times with IFN-α. In fact, ERK1/2 activation was evident after 24 hours with a further substantial increase after 48 hours, a timing consistent with the kinetics of EGFR up-regulation induced by IFN-α as we have shown previously (34) and with the induction of EGFR phosphorylation as shown in Fig. 4C. Chronic treatment with EGF up to 48 hours resulted in a progressive down-regulation of at least ERK2 phosphorylation compared with early time points. Concomitant treatment with gefitinib (5 μmol/L) for 24 hours antagonized the stimulation of both EGFR and ERK1/2 phosphorylation induced by either IFN-α or EGF (Fig. 4C).

**In vivo cooperative antitumor effect of IFN-α in combination with gefitinib.** To examine the in vivo interaction between gefitinib and IFN-α and to further evaluate their combinatory potential therapeutic effects, KB engrafted tumors were allowed to grow in athymic mice before randomization in four treatment groups: control, gefitinib, IFN-α, and gefitinib plus IFN-α.

Based on pilot studies (data not shown) and previous reports (46, 47), doses of both agents were specifically selected so that their independent effect on tumor growth inhibition would be modest. Mice were given with gefitinib at the dose of 50 mg/kg p.o. daily for 4 weeks and/or IFN-α at the dose of 50,000 units s.c. thrice weekly for 4 weeks.

As shown in Fig. 5, in IFN-α-treated group, the average tumor volume was even slightly increased compared with untreated mice, reaching size not compatible with life after 36 days, whereas no effect on TGD was observed (Figs. 5A and 6A, respectively). The average of tumor volume in untreated mice reached the same size after 42 days (Fig. 5A). Gefitinib treatment produced measurable but modest tumor volume reduction and TGD compared with untreated mice (Figs. 5A and 6A, respectively). In particular, ~40 days after the beginning of treatment, the average tumor volume in the gefitinib-treated group was reduced by 25% if compared with control groups (P < 0.005; Fig. 5A). On the other hand, a marked inhibition of tumor growth was found in the group treated with IFN-α plus gefitinib (Fig. 5A) with a consequent increasing TGD (Fig. 6A). In details, tumor volume in the gefitinib plus IFN-α combined treatment group reached a plateau after 40 days resulting in ~60% reduction if compared with control group (P < 0.001 for gefitinib/IFN-α versus control; P = 0.003 for gefitinib/IFN-α versus gefitinib). The resulting TGD reached a peak of ~50%, indicating that the rate of tumor growth in the control, at that point, was almost doubled compared with the combination setting. Combinatory treatment of gefitinib plus IFN-α was well tolerated as shown by maintenance of body weight (Fig. 5B) and by the absence of other signs of acute or delayed toxicity. Survival of mice in each group was also compared: gefitinib-treated mice survived longer than the mice in the control group, but a substantial increase in survival was observed in the group treated with both gefitinib and IFN-α compared with either control or gefitinib-treated mice. In fact, all mice died within 5, 6, and 8 weeks after tumor cell injection in the IFN-α, control, and
gefitinib groups, respectively. In contrast, four of seven mice treated with the combination of gefitinib and IFN-α were still alive after 10 weeks from the beginning of treatment (P = 0.018; Fig. 5C).

Furthermore, the in vivo synergistic effect between IFN-α and gefitinib described above was also confirmed by the evaluation of CIs evaluated as CI versus %CK, as described in Materials and Methods, by CalcuSyn software (Fig. 6B).

We finally evaluated whether the effect of gefitinib and IFN-α on ERK1/2 activation observed in vitro could be also observed in vivo. As shown in Fig. 6C, evaluation of ERK1/2 activation in KB tumor xenografts showed that gefitinib treatment inhibited constitutive active as well as IFN-α-induced ERK1/2 activity, confirming in vitro data. ERK1/2 protein expression levels were not affected by the different treatments.

**Discussion**

In the present study, we have shown that the combination of IFN-α with the selective EGFR tyrosine kinase inhibitor gefitinib induces cooperative antitumor effect on HNSCC-derived cell lines both in vitro and in vivo. In details, cell growth assays done in vitro showed a clear synergistic antiproliferative effect as shown by the use of the median drug effect analysis and calculating CIs. Interestingly, in all cell lines, the sensitivity to the antiproliferative effect of either gefitinib or IFN-α, as a single drug, was independent to the expression of EGFR. Campigli et al. also reported that the effects of gefitinib were...
independent of EGFR expression levels in breast cancer cells (48). Moreover, a discrepancy between EGFR expression and the antitumor effect of EGFR-targeting agents was reported recently in clinical studies and it is still considered a debated important issue (49).

We have reported cooperative antitumor effect of IFN-α and gefitinib combination also in vivo on nude mice carrying HNSCC cell line xenografts. In particular, enhanced inhibition and delay of tumor growth and increased survival rate in the combination setting compared with single drug treatments were reported. Furthermore, we have shown that gefitinib suppress the IFN-α-induced phosphorylation/activation of EGFR and ERK1/2 both in vitro and in vivo showing that it may overcome the previously described mechanisms of the IFN-α-induced EGFR-mediated survival escape (34, 37, 38). In details, we have shown that IFN-α induced a time-dependent ERK1/2 activation with maximal effect after 48 hours of treatment, timing consistent with the maximal effect of IFN-α-induced EGFR up-regulation reported previously (34, 37) and with the induction of EGFR phosphorylation. Interestingly,

![Fig. 5. Antitumor activity of gefitinib and IFN-α on established HNSCC KB xenografts. A, growth inhibition of KB xenografts in nude mice. KB cells (5 x 10^6) were s.c. injected in athymic mice as described in Materials and Methods. After 5 days (average tumor size, 0.2-0.3 cm³), mice were treated with gefitinib on days 1 to 5 of each week for 4 weeks and s.c. 3 days weekly with IFN-α or with both drugs. Points, average tumor volume measured in each mouse of the group; bars, SD. Student’s t test was used to compare tumor size among different treatment groups at day 40 after KB cell injection. B, effect of the treatment of gefitinib + IFN-α on nude mice body weight. The body weight was measured three times weekly. C, effect of IFN-α and/or gefitinib on the survival of KB xenograft mice. Survival was analyzed by Kaplan-Meier method and compared with χ² test (P = 0.018 compared with control).]

![Fig. 6. Effect of IFN-α and gefitinib in vivo interaction on xenograft TGD, CI, and ERK activity modulation. A, effect of IFN-α and/or gefitinib on TGD. TGD was determined as %TGD = [(T - C)/C] x 100, where T and C are the mean times expressed in days for the treated or control groups, respectively, to reach a defined tumor volume as described in Materials and Methods. B, in vivo IFN-α + gefitinib combination studies evaluated by CalcuSyn. For the calculation of CI, the values of %CK for a fixed tumor volume were considered. LCK was determined as LCK = [(T - C)/(3.32 x Td)], where Td represents the control group tumor volume doubling time, expressed in days, whereas T and C are the same as described above. CK was determined as %CK = [1 - (1 - 10^(%CK))] x 100 as described in Materials and Methods. C, Western blotting analysis of ERK1/2 activation and expression evaluated in tumors from treated and untreated KB xenografts. Whole-cell tumor protein lysates (50 μg) were separated by 10% SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted with specific anti-phospho-MAPK or anti-MAPK antibodies.]
this effect was confirmed for the first time in vivo where ERK1/2 activation in the IFN-α-treated group of tumor engrafted mice was also accompanied by increased tumor growth and reduced survival rate compared with control untreated mice. The potentiation of IFN-α-induced apoptosis by simultaneous treatment with gefitinib, as shown by fluorescence-activated cell sorting analysis as well as terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling assay, confirmed the hypothesis that the increased expression and function of EGFR could be part of a survival response that protects tumor cells from the apoptosis triggered by IFN-α. Moreover, the cooperative in vivo interaction between IFN-α and gefitinib are consistent with our recent observations showing the potentiation of IFN-α-induced apoptosis through the selective inhibition of Ras/ERK signaling by a dominant-negative Ras plasmid or the MEK1 inhibitor PD098059.

Relevant to our findings is the recent observation that IFN-α-induced increased expression of the EGFR has been also described in different experimental models like bladder as well as colon cancer cells (50, 51). Furthermore, we and others have shown that the Ras/MEK1/ERK and the Akt/nuclear factor-κB pathways can mediate a survival response in cells exposed to IFN-α (34, 38, 52). These results and the notion that IFN-α is a physiologic moiety has suggested that tumor cells can indeed escape from the antiproliferative and proapoptotic effects induced by this cytokine through the hyperactivation of physiologic survival pathways.

However, the compelling results we have achieved on the synergistic antiproliferative effect together with the fold of dose reduction obtained in combination setting for both gefitinib and IFN-α compared with each drug alone, both in vitro and in vivo, indicate a direct and effective cross-talk between EGFR-mediated and IFN-α-induced pathways that need to be further investigated.

In fact, a novel potential mechanism of interaction between IFN-α and EGFR signaling may include the STAT family proteins, which are activated by both transduction pathways (53). We have shown that IFN-α can induce STAT1 activation as well as, at later time points, its expression as reported by others (54). On the other hand, STAT3 is persistently activated in almost all head and neck cancers due to the dysregulation of the EGF-dependent pathway and its overexpression has been associated with poor prognosis in this neoplasm (33). A suggestive hypothesis that should be experimentally addressed is the formation of STAT1 and STAT3 heterodimer complexes on activation of both IFN-α- and EGF-mediated pathway leading to mitogenesis and survival.

Therefore, both single and multiple pathway inhibitors might be useful to increase the antitumor activity of IFN-α and reducing also its in vivo antitumor concentrations.

In this view, it is also of particular interest that in our study the best synergistic effect was obtained in vitro on HEP-2 cells that seem to be the most resistant to both drugs. To our knowledge, this is the first report on the effect of combination treatment of IFN-α with an agent targeting EGFR both in vitro and in vivo.

Another important issue to point out is the relative resistance of human tumors, including HNSCC, to the antisignaling approaches based on small molecules or monoclonal antibodies (31). This observation could be explained based on pathway redundancy in human tumor cells characterized by the existence of multiple and often compensatory survival and proliferative signals. Therefore, the definition of new strategies to switch off these antiprolongative pathways and allow the therapeutic goals becomes even more urgent. In this view, IFN-α can be of help. In fact, the exposure of tumor cells to IFN-α could result in the activation of tumor- or tissue-specific survival responses based on few transducers that assure the protection of tumor cells from the apoptosis and growth inhibition induced by the cytokine. This way, tumor cells exposed to IFN-α become highly sensitive to specific signaling inhibitors (“target prioritization”), avoiding the need of a wide inhibition of multiple survival signals. In other words, by treating the cells with IFN-α, we could mimic the effect of the kinase domain mutations of EGFR described in non–small cell lung cancer and HNSCC (18–20, 22). Mutated EGFR showed enhanced tyrosine kinase activity in response to EGF and increased sensitivity to inhibition by gefitinib. On this regard, a recent article showing that synergistic interaction between chemotherapy and gefitinib is determined by chemotherapy-induced activation of EGFR is of particular interest (55).

Overall, our study adds new insights into the molecular events underlying IFN-α effects on cell signaling. In addition, these results show that the combination of IFN-α with specific inhibitors of EGFR, such as gefitinib, may improve the efficacy of both agents in head and neck cancer and should be further clinically explored.

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References


Synergistic Antitumor Activity of Epidermal Growth Factor Receptor Tyrosine Kinase Inhibitor Gefitinib and IFN-α in Head and Neck Cancer Cells \textit{In vitro} and \textit{In vivo}

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