Response of endothelial cells to a dual tyrosine kinase receptor inhibition combined with irradiation

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Abstract

Recent studies suggest the possibility of a direct antiangiogenic effect of anti–epidermal growth factor receptor (EGFR) drugs due to the presence of EGFR on endothelial cells. The aim of this study was to analyze the direct effect on endothelial cells of associating EGFR targeting, vascular endothelial growth factor receptor (VEGFR)-2 targeting, and irradiation. We examined both the cytotoxic effects and the effect on molecular markers resulting from the combined action of gefitinib (Iressa; anti-EGFR), ZM317450 [VEGFR tyrosine kinase inhibitor (VTKI); anti-VEGFR-2], and irradiation (radiation therapy) on HMME7 cells, an immortalized microvascular endothelial cell of human origin. The presence of a functional EGFR pathway sensitive to gefitinib was shown in HMME7 cells (gefitinib-induced decrease in phospho-EGFR, phospho-p42/p44, and phospho-Akt). The stimulation of VEGFR-2 pathway led to an increase in Akt phosphorylation that was inhibited by VTKI. Of note, a post–radiation therapy induction of phospho-p42/p44 was observed on HMME7 cells, and this effect was inhibited by a pretreatment with gefitinib. Based on combination indexes (Chou and Talalay analyses), the associations gefitinib-radiation therapy, VTKI-radiation therapy, VTKI-gefitinib, and gefitinib-VTKI-radiation therapy were found to be additive, slightly synergistic, and markedly synergistic, respectively, for the cytotoxicity on HMME7 cells. Among molecular explanatory factors that were examined, the combination gefitinib-radiation therapy totally abolishes DNA-dependent protein kinase expression, and gefitinib attenuates the radiation therapy–induced enhancement of ERCC1 and augments the VTKI-induced CD95 enhancement. The existence of a radiation therapy–dependent neoangiogenesis may be related to the induction of EGFR pathway in endothelial cells, a phenomenon that can be attenuated by anti-EGFR drugs like gefitinib. In complement to the direct antitumor effects of radiation therapy and anti-EGFR drugs, a strong antiangiogenic effect may be obtained with therapeutic strategies combining radiation therapy with EGFR and VEGFR-2 targeting agents. [Mol Cancer Ther 2005;4(12):1962–71]

Introduction

The clinical development of epidermal growth factor (EGF) receptor (EGFR) targeting agents is currently expanding in the treatment of cancer (1–3). EGFR inhibition by either tyrosine kinase inhibitors or monoclonal antibodies is accompanied by changes in tumoral cell physiology affecting cell proliferation, invasion, and angiogenesis (4–7). The antiangiogenic effect of anti-EGFR therapy may be explained by a dose-dependent inhibition of the tumoral release of proangiogenic factors like vascular endothelial growth factor (VEGF), transforming growth factor-α, basic fibroblast growth factor, and interleukin-8 (8–11). Recent reports indicate that treatments combining anti-EGFR therapy and VEGF receptor (VEGFR)-2 (KDR) targeting increase tumor cell apoptosis (12). Dual inhibitors, combining the same molecule anti–tyrosine kinase activity against EGFR and VEGFR, have been developed and show promising preclinical activity in xenograft models (13, 14). A recent experimental study by Hirata et al. (15) concludes that the antitumor effects of the EGFR tyrosine kinase inhibitor gefitinib (Iressa) may be mediated, in part, by the inhibition of tumor angiogenesis through a direct action on endothelial cells that express EGFR. Their data show that gefitinib almost completely inhibited the EGF-dependent EGFR autophosphorylation in microvascular endothelial cells and completely inhibited signaling downstream of the EGFR, including Akt phosphorylation (15).

Research on radiation therapy in cancer has been mainly focused on the cancer cell itself. A recent study reported that radiation therapy has strong antiangiogenic potential with effects on endothelial cell survival, proliferation, tubule formation, and invasion (16). This latter study also pointed out an enhancement of the direct antiangiogenic effect of radiation therapy when combining radiation therapy with inhibition of VEGFR-2, fibroblast growth factor receptor-1, and platelet-derived growth factor

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receptor-β (16). The aim of the present study was to investigate the direct effect on endothelial cells resulting from the association between EGFR targeting, VEGFR-2 targeting, and radiation therapy. To do so, primary cultures of human umbilical vein endothelial cells (HUVEC) and a microvascular endothelial line of human origin (17) were used. Together with the analysis of cell survival, the status of intracellular signaling pathways was examined.

Materials and Methods

Chemicals

Gefitinib and ZM317450 [VEGFR tyrosine kinase inhibitor (VTKI)] were kindly provided by AstraZeneca (Macclesfield, United Kingdom). VTKI is a specific inhibitor of VEGFR-2 belonging to the anilinocinnoline family of compounds with an IC_{50} of 0.05 ± 0.01 μmol/L for VEGFR-2 and 0.5 ± 0.04 μmol/L for VEGFR-1 (18).

Stock solutions were prepared at 50 and 10 mmol/L for gefitinib and VTKI, respectively, and stored at −80°C in DMSO. DMSO, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, and BCA protein colorimetric test were purchased from Sigma Chemical (Saint-Quentin Fallavier, France). EGM-2 medium and its additives (fetal bovine serum, hydrocortisone, ascorbic acid, heparin, gentamicin plus amphothericin B, EGF, VEGF, basic fibroblast growth factor, and insulin-like growth factor-I) were purchased from Clonetics (Walkersville, MD). PBS was from Bio-Whittaker (Verviers, Belgium), and polyvinylidene difluoride membrane was from Bio-Rad (Marnes la Coquette, France). Enhanced Chemiluminescence Hyperfilm was purchased from Amersham Pharmacia Biotech (Little Chalfont, United Kingdom).

Cell Lines

Primary cultures of HUVEC (provided by Dr. Grall, Centre National de la Recherche Scientifique UMR6543, Nice, France) and the immortalized human mammary microvascular endothelial cell line HMME7 (kindly provided by Prof. Mike O'Hare, Royal Free and University College London Medical School, London, United Kingdom) were routinely cultivated in EGM-2 medium at 37°C and 33.5°C for HUVEC and HMME7 cells, respectively, in a fully humidified incubator (Sanyo, Osaka, Japan) in an atmosphere containing 8% CO_2. Both cell types express comparable levels of CD31 (platelet/endothelial cell adhesion molecule) and P1H12 endothelial cell markers as examined by immunofluorescence staining.

Exposure to Agents

Cells were seeded in microplates (2.5×10^3 in 100 μL medium per well) to obtain exponential growth for the entire duration of experiment. Forty-eight hours later, cells were exposed to the indicated agents. The dose-effect curves for gefitinib or radiation therapy alone or in association, as well as EGFR signaling inhibition by gefitinib, were done on both HMME7 and HUVEC; all other experiments were done on the HMME7 cell line only.

Single-Agent Dose-Effect Measurement. Forty-eight hours after cell seeding, cells were exposed to either gefitinib (5×10^{-7}–5×10^{-5} mol/L) and VTKI (1×10^{-8}–1×10^{-4} mol/L) for 72 hours or to one dose of radiation therapy (1–15 Gy). Cytotoxicity was measured 120 hours after cell seeding.

Combination Dose-Effect Measurement. Combinations of agents (gefitinib-VTKI, gefitinib-radiation therapy, and gefitinib-VTKI-radiation therapy) at equitoxic doses were added to cell cultures 48 hours after cell seeding. Doses (six per agent) were 2 to ≤20 μmol/L gefitinib and 10 to ≤100 μmol/L VTKI. Forty-eight hours after drug exposure, radiation therapy (1–10 Gy) was applied once. Cytotoxicity was measured 120 hours after exposure to radiation therapy.

Cell Growth Measurement. Growth inhibition was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide test (19) described below. Cells were washed with PBS and incubated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide. After 2 hours of exposure, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide was released and formazan blue formation was revealed by the addition of 100 μL DMSO. Absorbance at 450 nm was measured using a microplate reader (Labsystems, Helsinki, Finland). Results were expressed as the relative percentage of absorbance compared with controls without drug. Experimental conditions were tested in sextuplicate (6 wells of the 96-well plate per experimental condition), and experiments were done at least thrice. The dose-effect curves were analyzed using Prism software (GraphPad Software, San Diego, CA). Antiproliferative activity was expressed as the EC_{50} value (50% effective drug concentration) and ED_{50} value (50% effective irradiation dose).

Functional Study of EGF- and VEGF-Dependent Cell Signaling Pathways in Endothelial Cells

HMME7 and HUVEC were seeded in complete EGM-2 medium in 12-well plates (1.6×10^5 per well).

Forty-eight hours later, cells were rinsed with PBS and changed to EGM-2 medium without growth factors. After 24 hours, drugs gefitinib (1 μmol/L) and/or VTKI (10 μmol/L) were added 2 hours before growth factors (either 20 ng/mL EGF or 30 ng/mL VEGF). These drug concentrations were selected according to previous data (15, 18). Cells were stimulated by growth factors for 10 or 20 minutes, lysed in Laemmli buffer, and analyzed by Western blotting for levels of phospho-EGFR, phospho–extracellular signal-regulated kinase-1/2 (phospho-p42/p44), and phospho-Akt.

Intracellular Molecular Factors

Cells were seeded in complete EGM-2 medium in 12-well plate (2.5×10^5 per well). Forty-eight hours later, drugs were added gefitinib (1 μmol/L) and/or VTKI (10 μmol/L). Forty-eight hours later, cells were irradiated (6 Gy in one go), and 30, 60, 90, and 120 minutes and 24, 48, and 72 hours after irradiation, cells were lysed using Laemmli buffer.

The following variables were measured using Western blot analysis on cell lysates: EGFR or VEGFR signaling-related

Table 1. Characterization of primary antibodies

Proteins	MW (kDa)	Gel % acrylamide	Antibody	Source	Supplier	Dilution
Phospho-EGFR	175	7.5	Phospho-EGFR (Tyr^{1173})	Mouse	Euromedex (Mundolsheim, France)	1:1,000
Phospho-p42/p44	42/44	12	p42/p44 Mitogen-activated protein kinase	Mouse	Centre National de la Recherche Scientifique	1:5,000
Phospho-Akt	60	12	Phospho-Akt (Ser^{473})	Rabbit	Ozyme (St. Quentin en Yvelines, France)	1:1,000
p21	21	12	p21 (Cip1/WAF1)	Mouse	PharMingen (San Diego, CA)	1:1,000
p27	27	12	p27 (KIP1) HRPO	Mouse	PharMingen	1:1,000
Bax	21/25	12	Bax (B-9)	Mouse	Tebu (Le Perray en Yvelines, France)	1:1,000
Bcl-2	26	12	Bcl-2 (C-2)	Mouse	PharMingen	1:1,000
ERCC1	33/36	12	ERCC1	Mouse	NeoMarkers (Fremont, CA)	1:500
XRCC1	85	12	XRCC1	Mouse	NeoMarkers	1:500
DNA-PK	460	5	DNA-PK catalytic subunit Ab-2 (clone 25-4)	Mouse	NeoMarkers	1:500
ATM	350/370	5	ATM (B-12)	Mouse	Tebu	1:100
Actin	42	12	Actin	Mouse	Sigma	1:5,000
Caspase-3	32	12	Caspase-3/CPP32	Mouse	Transduction Laboratories (San Jose, CA)	1:1,000
Caspase-9	45	12	Caspase-9/M054-3	Mouse	MBL (Woburn, MA)	1:1,000
Cleaved poly(ADP-ribose) polymerase	89	7.5	Cleaved poly(ADP-ribose) polymerase Asp^{214}	Rabbit	Ozyme	1:1,000

Abbreviation: MW, molecular weight.

factors (phospho-p42/p44 and phospho-Akt), cell cycle–related factors (p21 and p27), apoptosis-related factors [Bax, Bcl-2, cleaved poly(ADP-ribose) polymerase, caspase-3, and caspase-9], DNA repair–related factors [ERCC1, XRCC1, DNA-dependent protein kinase (DNA-PK), and ATM].

Western Blot Analyses

Cells were rinsed twice with PBS and underwent lysis in Laemmli buffer [100 mmol/L Tris-HCl (pH 6.8), 2.5% SDS, 10% glycerol]. After heating at 95°C for 10 minutes, lysates were sonicated and protein concentration was determined using BCA protein colorimetric test.

β-Mercaptoethanol (100 mmol/L) and bromophenol blue (0.01%) were added and equal amounts of proteins (50 μg/well) were separated by electrophoresis on SDS-PAGE (5%, 7.5%, or 12% depending on protein molecular weight) and transferred on a polyvinylidene difluoride membrane overnight in a buffer containing 25 mmol/L Tris, 192 mmol/L glycine, and 20% ethanol. Prestained molecular weight markers were included in each gel. Membranes were blocked for 1 hour in TBS [10 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl] with 5% milk. After blocking, membranes were incubated overnight with the appropriate specific anti-human monoclonal antibody in TBS and 5% milk at 4°C. After washing thrice with TBS plus 0.1% Tween (5 minutes each), the membranes were incubated with horseradish peroxidase–conjugated secondary antibodies for 1 hour at room temperature. A chemiluminescence reaction was done and the membranes were exposed to Enhanced Chemiluminescence Hyperfilm according to the manufacturer's instructions. Actin has been used as a loading control protein throughout the experiments. Quantitative analysis of activities was done by imaging the autoradiograms and quantitating relative band densities using scan imaging software (Image Master, Pharmacia Biotech Sweden, Uppsala, Sweden). For primary antibodies, the list and origin of antibodies are provided in Table 1. Secondary antibodies are as follows: antibody anti-mouse, horseradish peroxidase–conjugated, 1:2,000 dilution (DAKO, Glostrup, Denmark); antibody anti-rabbit, horseradish peroxidase–conjugated, 1:2,000 dilution (DAKO).

Detection of the CD95 (Apo1-Fas Receptor)

After exponentially growing cells have been exposed for 48 hours to 1 μmol/L ZD1839 alone, 10 μmol/L VTKI alone, or combination, they were (or not) irradiated at 6 Gy. Twelve hours after irradiation, cells belonging to the different treatment groups were trypsinized, washed, and exposed to 4 ng/μL CH11 anti-Fas monoclonal antibody for 45 minutes at 4°C. After two washing steps,

cells were resuspended in DMEM containing 1:200 (v/v) goat anti-mouse IgM (Immunotech, Marseilles, France) and incubated for an additional 30 minutes at 4°C. Cells were then washed twice, and cell surface expression of the CD95 was assessed by FACScan. Analysis was carried out in a FACScan flow cytometer using CellQuest software. Cells exposed to goat anti-mouse IgM only served as negative FITC control. Relative Fas expression was defined as the ratio of fluorescence CH11 Fas monoclonal antibody to isotype-matched negative control monoclonal antibody. Relative Fas expression in untreated cells was considered as 0%. Values are mean ± SD of three experiments.

Statistical Analysis

Differences between mean values were evaluated using either one-way ANOVA with Tukey test or one-way ANOVA on ranks with Dunnett's or Student's-Newman-Keuls' test according to data distribution. $P = 0.05$ was regarded as statistically significant. All analyses used SPSS software (SPSS, Paris, France).

Measure of interaction between the effects of cytotoxic agents were done by calculating the combination indexes (CI) according to Chou and Talalay (20); briefly, CI > 1.2 means antagonism, 0.8 < CI < 1.2 means additivity, and CI < 0.8 means synergism.

Results

Growth Inhibitory Effect of Gefitinib, VTKI, or Radiation Therapy Alone or in Association in Endothelial Cells

Single-Agent Activity. First, we set out to establish the growth inhibitory effect of the receptor tyrosine kinase inhibitors or radiation therapy when applied alone on exponentially growing HUVEC or HMME7 cultures. Figure 1A shows the dose-response curve in the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay of HMME7 cells treated with radiation therapy and concentration-response curves for gefitinib and VTKI, and Fig. 1B shows the dose-response curve of HUVEC to radiation therapy and concentration-response curve for gefitinib. Gefitinib (with EC_{50} at 1.42×10^{-5} mol/L for HMME7 and 2×10^{-5} mol/L for HUVEC) and radiation therapy (with ED_{50} at 6.6 Gy for HMME7 and 7.5 Gy for HUVEC) both have a clear growth inhibitory activity on the tested endothelial cells; VTKI was less active than gefitinib.

Activity of the Combinations. Figure 2A shows the dose/concentration-response curves of HMME7 cells to the double-agent combinations (gefitinib-VTKI, gefitinib-radiation therapy, and VTKI-radiation therapy) and to the triple combination (gefitinib-VTKI-radiation therapy). Figure 2B displays the dose/concentration-response curves of

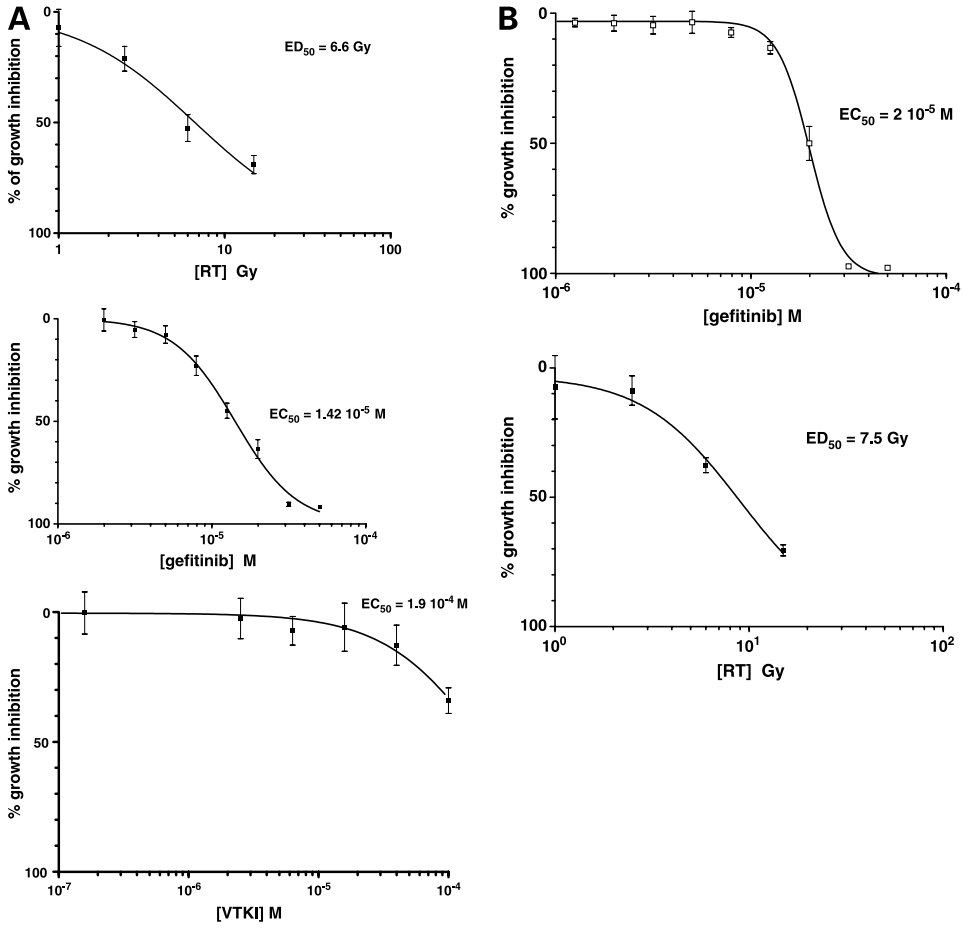

Figure 1. **A,** growth inhibitory effect of radiation therapy (*RT*), gefitinib, and VTKI on HMME7 cells. **B,** growth inhibitory effect of radiation therapy and gefitinib on HUVEC.

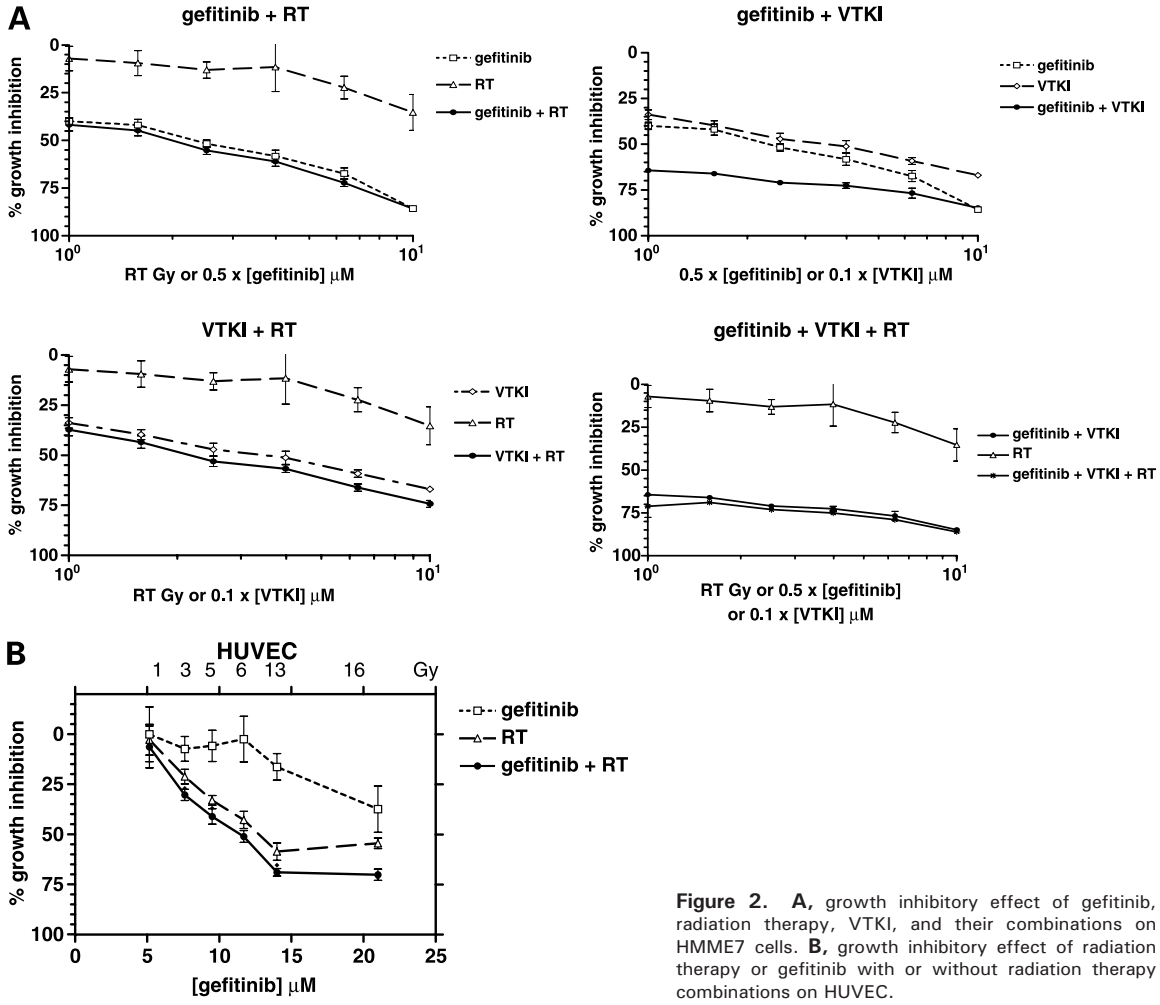

Figure 2. **A,** growth inhibitory effect of gefitinib, radiation therapy, VTKI, and their combinations on HMME7 cells. **B,** growth inhibitory effect of radiation therapy or gefitinib with or without radiation therapy combinations on HUVEC.

HUVEC to the double-agent combination (gefitinib-radiation therapy) only. A simple additivity was resulting from gefitinib-radiation therapy combination for both HMME7 and HUVEC with CIs around 1. A CI at 0.77 was in favor of a slight synergy for the tandem VTKI-radiation therapy. A CI at 0.53 was suggesting a marked synergy for the tandem VTKI-gefitinib and the triple combination gefitinib-VTKI-radiation therapy and this is for HMME7 cells only (Table 2).

Table 2. CI (mean ± SE) values for the different cytotoxic combinations

Agent combination	Cell line	
	HMME7	HUVEC
Gefitinib-radiation therapy	1 ± 0.19 AD	1.19 ± 0.21 AD/AN
VTKI-radiation therapy	0.77 ± 0.09 AD/SY	ND
VTKI-gefitinib	0.53 ± 0.2 SY	ND
VTKI-gefitinib-radiation therapy	0.53 ± 0.12 SY	ND

NOTE: CIs were resulting from all experimental points shown in the dose/concentration-response curves. AD, additivity; AN, antagonism; SY, synergy; ND, not determined.

Functional Study of EGF- and VEGF-Dependent Signaling Pathways

EGF Effect. There is a marked increase in the levels of phospho-EGFR, phospho-p42/p44, and phospho-Akt for HMME7 cells (Fig. 3A) and phospho-p42/p44 and phospho-Akt only for HUVEC (Fig. 3B) after EGF application. This augmentation is maximal at 10 minutes, still present but attenuated 20 minutes after cell exposure to EGF. A pretreatment of HMME7 and HUVEC with gefitinib before EGF application abrogates EGFR signaling. VTKI has no effect on EGF-induced signaling pathway (Fig. 3C).

VEGF Effect on HMME7 Cells. When applied on HMME7 cells, VEGF has only a moderate effect on phosphorylation of p42/p44. On the contrary, phospho-Akt is clearly augmented with a maximum effect observed 20 minutes after VEGF stimulation. This effect

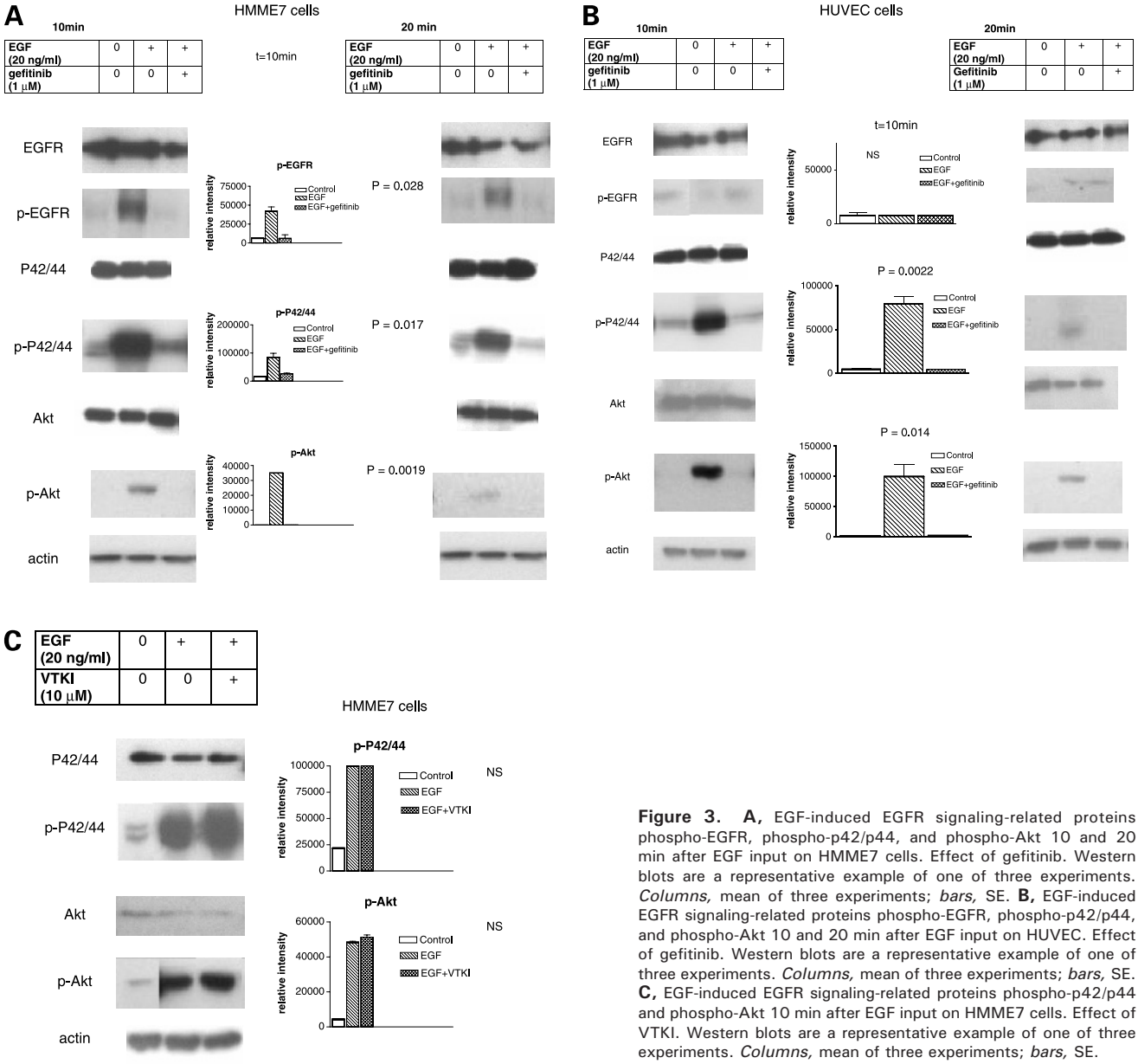

Figure 3. **A,** EGF-induced EGFR signaling-related proteins phospho-EGFR, phospho-p42/p44, and phospho-Akt 10 and 20 min after EGF input on HMME7 cells. Effect of gefitinib. Western blots are a representative example of one of three experiments. *Columns,* mean of three experiments; *bars,* SE. **B,** EGF-induced EGFR signaling-related proteins phospho-EGFR, phospho-p42/p44, and phospho-Akt 10 and 20 min after EGF input on HUVEC. Effect of gefitinib. Western blots are a representative example of one of three experiments. *Columns,* mean of three experiments; *bars,* SE. **C,** EGF-induced EGFR signaling-related proteins phospho-p42/p44 and phospho-Akt 10 min after EGF input on HMME7 cells. Effect of VTKI. Western blots are a representative example of one of three experiments. *Columns,* mean of three experiments; *bars,* SE.

is significantly inhibited by VTKI but not by gefitinib. Of note, VTKI-gefitinib combination totally inhibited the effects of VEGF on phosphatidylinositol 3-kinase-Akt pathway (Fig. 4).

Combined Effect of Gefitinib, VTKI, and Radiation Therapy

Mitogen-Activated Protein Kinase and Phosphatidylinositol 3-Kinase-Akt Pathway. The application of radiation therapy increases phospho-p42/p44 levels on endothelial cells. This significant change appears as early as 60 minutes after radiation therapy, reaching a maximum after 90 minutes, decreases after 120 minutes, and has totally disappeared 24 hours after (Fig. 5A). A pretreatment with gefitinib suppresses this increase (Fig. 5B) and such a suppressive effect is not observed with VTKI. In contrast to the effects of radiation therapy on phospho-p42/p44, radiation therapy does not modify the phospho-Akt signal (data not shown).

Cell Cycle–Related Factors. Gefitinib enhances the expression of both p21 and p27. Radiation therapy has no effect on p21 expression but decreases the cellular levels of p27. The combined effect of gefitinib and radiation therapy on p21 does not differ from the effect of gefitinib alone. Gefitinib moderates the decrease of p27 expression induced by radiation therapy (Fig. 6).

DNA Repair–Related Factors. DNA-PK expression was totally abolished by the combination of gefitinib plus radiation therapy. Although ERCC1 expression was enhanced

2 hours after exposure to radiation therapy, these levels were decreased by addition of gefitinib to radiation therapy. Neither gefitinib nor radiation therapy had any effect on XRCC1 expression. ATM was not detectable in HMME7 cells (Fig. 7).

Apoptosis. Intrinsic and extrinsic related factors were tested. Among the intrinsic pathway factors [Bax, Bcl-2, caspase-3, caspase-9, and cleaved poly(ADP-ribose) polymerase], none of them could be related to the growth inhibition observed. In contrast, CD95 (Apo1-Fas receptor) enhancement by the growth inhibitory agents corroborated the synergistic effects observed with the gefitinib-VTKI combination (P = 0.0082, paired t test; Fig. 8). There was no difference in relative expression of CD95 between gefitinib-VTKI and gefitinib-VTKI-radiation therapy, in agreement with the growth inhibition effects (Fig. 2A).

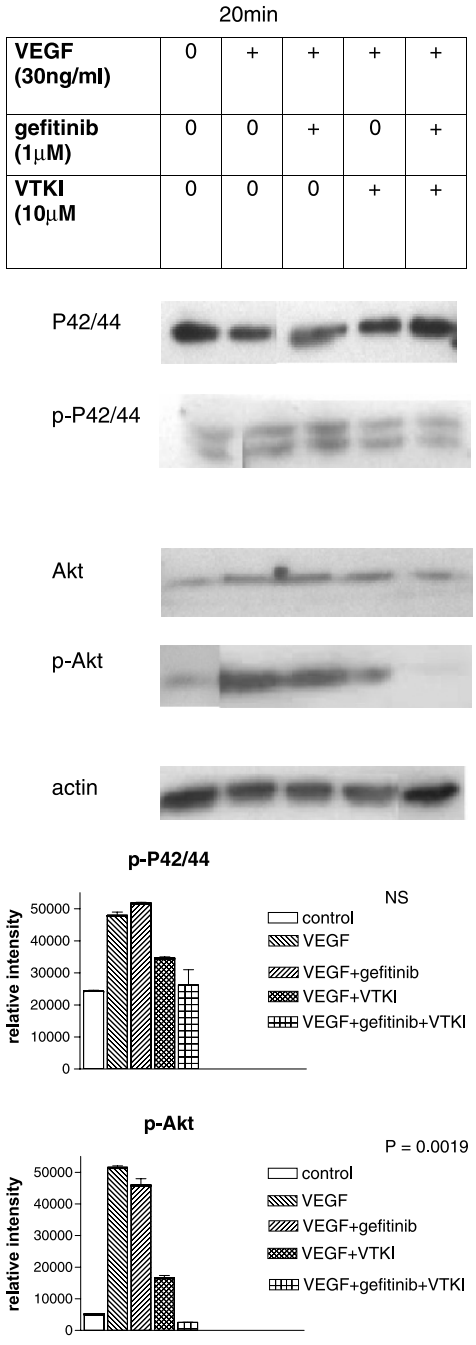

Figure 4. VEGF-induced VEGFR signaling-related proteins phospho-p42/p44 and phospho-Akt 20 min after VEGF input on HMME7 cells. Effect of VTKI. Western blots are a representative example of one of three experiments. *Columns,* mean of three experiments; *bars,* SE.

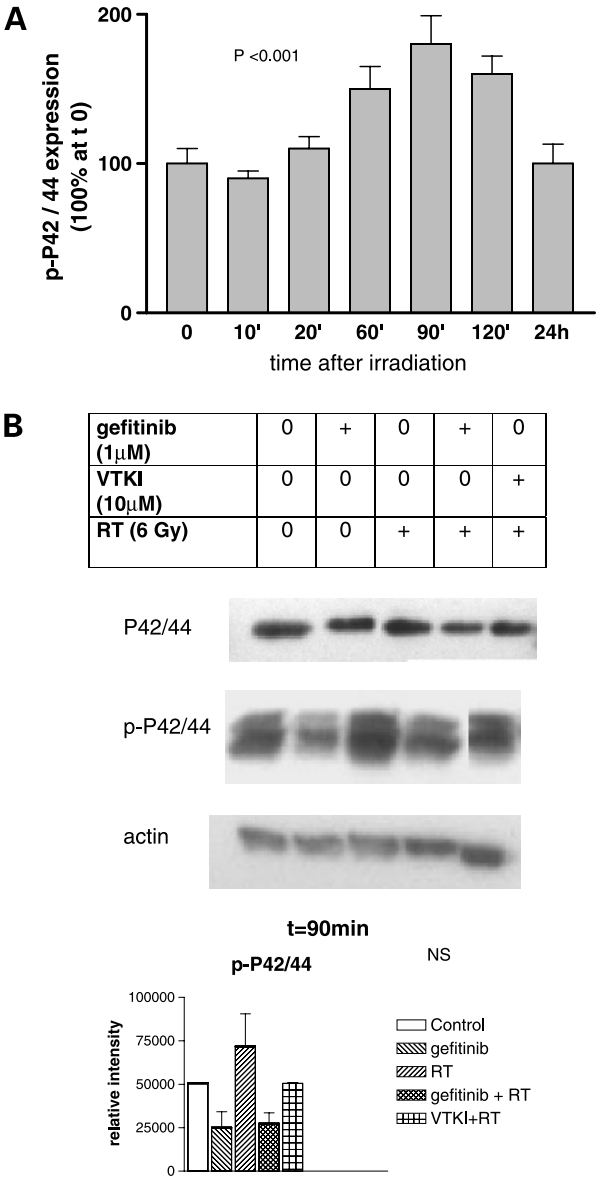

Figure 5. **A,** time-dependent increase of phospho-p42/p44 after radiation therapy application on HMME7 cells. Western blots are a representative example of one of three experiments. *Columns,* mean of three experiments; *bars,* SE. **B,** gefitinib-induced modulation of the increase of phospho-p42/p44 90 min after radiation therapy application on HMME7 cells. Western blots are a representative example of one of three experiments. *Columns,* mean of three experiments; *bars,* SE.

Discussion

The combination of biological therapeutics targeting two or more growth-controlling pathways is of current interest as innovative treatment options for the management of cancer. The association between antiangiogenic agents and anti-EGFR drugs seems particularly attractive with promising preclinical data. This dual targeting can be achieved with different drugs, such as the combination between an antivascular agent ZD6126 and gefitinib (21), or with drugs able to affect on both tyrosine kinases related to EGFR or VEGFR with interesting preclinical data obtained with drugs like ZD6474 (13) or AEE788 (14).

The induction of migration by EGF/transforming growth factor-α on endothelial cells (22) and the effects of EGFR targeting on these cells (15) point the endothelial cell itself as being a target for a dual tyrosine kinase receptor inhibition. The present study aimed to examine this particular aspect of the combination of biological therapeutics. HUVECs are frequently used as an *in vitro* model for human endothelial cells. However, this model is subject to variability due to the source of preparation (umbilical vein). A recently described endothelial cell line HMME7 of human origin was chosen for the present study to limit the sources of variability (17). The microvascular nature of these cells is important to underline. A karyotype analysis indicated that HMME7 cells exhibit a normal model number of chromosomes (17). We confirmed that HMME7 cells as well as our HUVEC cultures expressed typical endothelial markers, including CD31 and P1H12. To validate the results obtained with HMME7 cells, several experiments were conducted in parallel with both HUVEC and HMME7. Similar data were obtained with the two endothelial cell sources (Table 1), although the intrinsic sensitivity to EGFR targeting and radiation therapy might differ (Fig. 1A and B). When VEGF was applied to HMME7 cells, there was no change in p42/p44 activation but a marked increase in phospho-Akt inhibited by a preexposure to VTKI. In addition, a functional EGFR pathway, inhibited by gefitinib, was found to be present in the HMME7 cells (Fig. 3A). Here again, a comparison between HMME7 and HUVEC revealed that both endothelial cells exhibit similar EGFR signaling and inhibition by gefitinib of this biochemical pathway (Fig. 3B). This presence of EGFR signaling in endothelial cells is in agreement with previous data (23–25). Thus, the strategy of EGFR tumor targeting may involve not only the antiproliferative/cytotoxic effects on tumoral cells themselves but also those on endothelial cells.

A part of the study was orientated to the application of radiation therapy on HMME7 cells. Of particular interest was the finding that radiation therapy was able to induce p42/p44 activation, indicating a stimulation of the mitogen-activated protein kinase pathway by radiation therapy on endothelial cells. A pretreatment with gefitinib abrogated this effect, whereas VTKI was ineffective in this respect; this indicates that a radiation therapy–dependent p42/p44 induction in HMME7 cells is initiated through EGFR pathway. Previous studies have reported that cellular responses to ionizing radiation are mediated by signal transduction pathways involving protein kinases and nuclear transcription factors (26–28). Schmidt-Ullrich et al. have shown on MCF-7 and A431 cells that an autophosphorylation of EGFR was a biological response to radiation therapy in the dose range used in radiotherapy (29). The present study shows for the first time that radiation therapy can stimulate EGFR signaling pathway in endothelial cells. Our observation is in line with and provides a mechanistic explanation to the recent data by Sonveaux et al. showing that radiation therapy dose-dependently induced activation of the nitric oxide synthase pathway in endothelial cells (30). Indeed, nitric oxide synthase is regulated in part by cyclooxygenase-2 activity, which can be induced through activation of the EGFR pathway (31). Without ruling out the cytotoxic effects of radiation therapy on endothelial cells as a part of its antitumor effects (32), the present data combined with those of Sonveaux et al. (30)

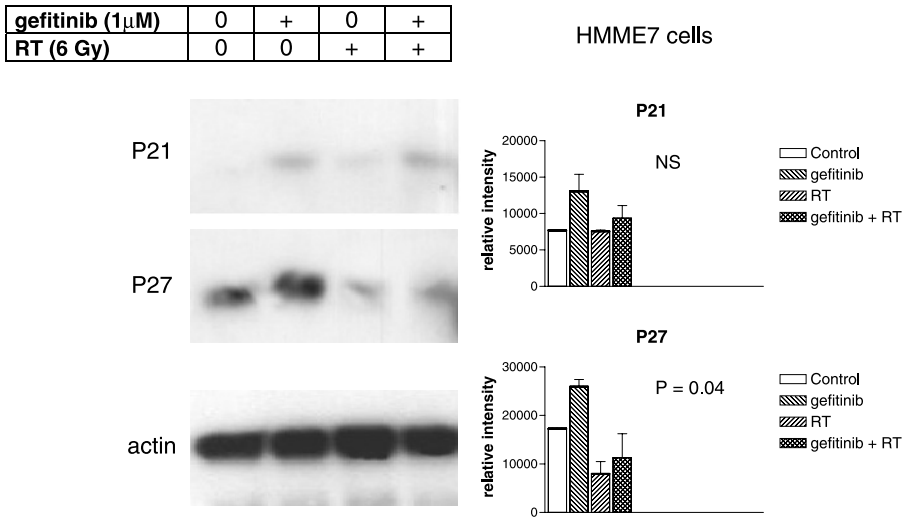

Figure 6. Gefitinib-induced modulation of the proliferation-related proteins p21 and p27 24 h after radiation therapy application on HMME7 cells. Western blots are a representative example of one of three experiments. *Columns,* mean of three experiments; *bars,* SE.

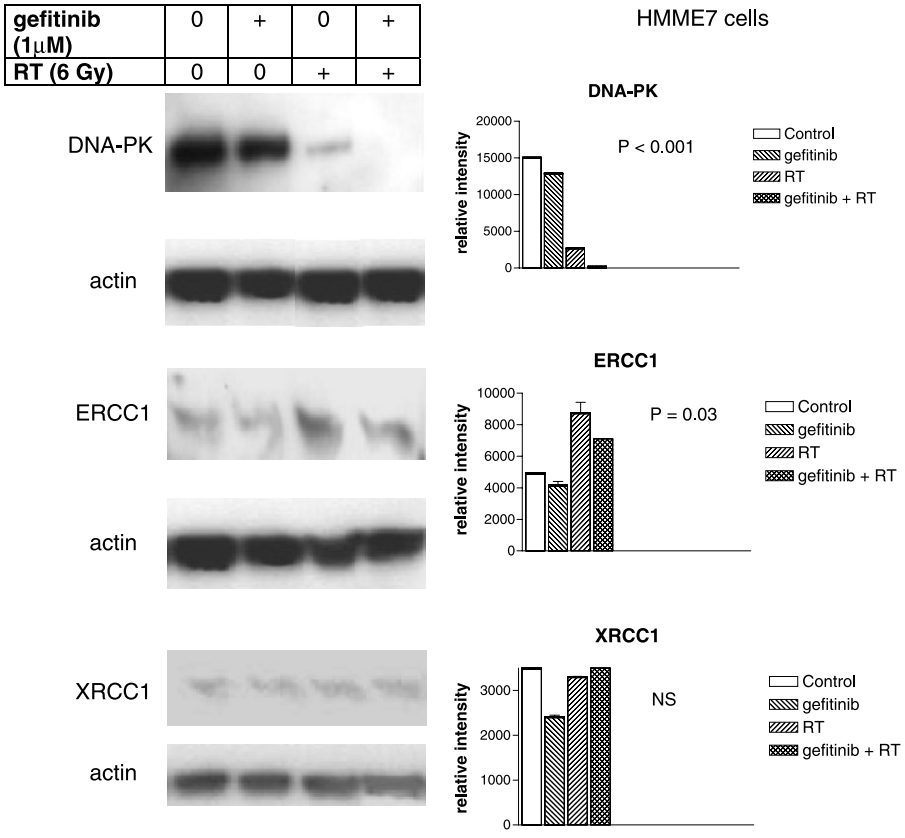

Figure 7. Gefitinib-induced modulation of the DNA repair-related factors DNA-PK (1 h), ERCC1 (2 h), and XRCC1 (2 h) after radiation therapy application on HMME7 cells. Western blots are a representative example of one of three experiments. *Columns,* mean of three experiments; *bars,* SE.

argue in favor of a proangiogenic effect of radiation therapy through the up-regulation of the EGFR pathway. There are clinical data supporting these findings and showing for instance that iris neovascularization is one of the ocular complications following iodine-125 plaque brachytherapy for large ocular melanoma (33). There is also a clinical background supporting that intensified angiogenic growth (angiogenic regeneration) during radiotherapy is associated with failure of radiotherapy (34).

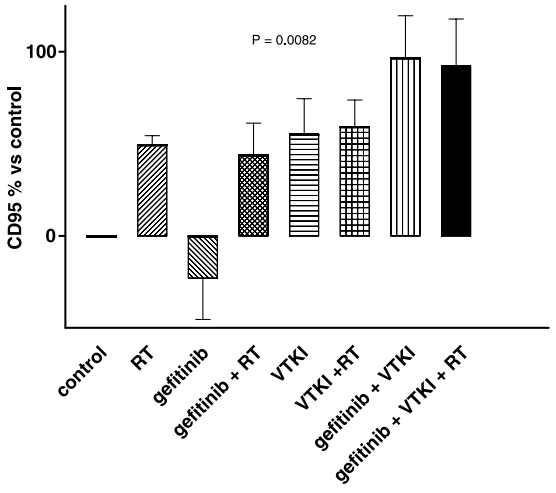

Figure 8. Modulation of CD95 (Apo1-Fas receptor) membrane expression by gefitinib, VTKI, radiation therapy, and their combinations. Western blots are a representative example of one of three experiments. *Columns,* mean of three experiments; *bars,* SE. See Materials and Methods for details.

We found marked cytotoxic synergistic effects on HMME7 cells when combining the application of the two drugs or the three treatments radiation therapy, gefitinib, and VTKI. This observation corroborates the recent data by Williams et al. (35) showing the supra-additivity between ZD6474 and radiation therapy. The fact that VTKI-gefitinib combination totally inhibited the positive effect of VEGF on phospho-Akt may be one of the possible explanations for the synergy observed between the two drugs. The supra-additive antiproliferative effects conferred by the two drugs were also illustrated by a significant change in CD95 (Fas ligand, TNFSFR6) expression. In contrast, the intrinsic apoptosis pathway [Bax, Bcl-2, caspase-3, caspase-9, and cleaved poly(ADP-ribose) polymerase] was not markedly modified by the drug association. Although the combination between radiation therapy and gefitinib was resulting in additivity, there are molecular observations in the present study suggesting more than a simple additivity, such as the radiation therapy–induced EGFR signaling, which is abrogated by the application of gefitinib. In the same way, there was the countereffect of gefitinib on the statistically significant p27 decrease induced by radiation therapy (Fig. 6). Interestingly, the capacity of gefitinib to decrease the expression of DNA repair–related enzymes

was described recently in the tumoral cell (36) and is also reported presently in endothelial cells. Thus, the expression of DNA-PK and ERCC1 were found to be significantly down-regulated by the application of gefitinib on HMME7 cells (Fig. 7; Table 2).

In total, the present data support the notion of the presence of a functional EGFR pathway in endothelial cells and provide a strong rationale for a supra-additive combination of radiation therapy with anti-EGFR and antiangiogenic compounds on endothelial cells and this is complement to the direct antitumor effects that can be conferred by radiation therapy and anti-EGFR drugs.

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