Vascular Endothelial Growth Factor Receptor-1 Contributes to Resistance to Anti–Epidermal Growth Factor Receptor Drugs in Human Cancer Cells

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Abstract

Purpose: The resistance to selective EGFR inhibitors involves the activation of alternative signaling pathways, and Akt activation and VEGF induction have been described in EGFR inhibitor–resistant tumors. Combined inhibition of EGFR and other signaling proteins has become a successful therapeutic approach, stimulating the search for further determinants of resistance as basis for novel therapeutic strategies.

Experimental Design: We established human cancer cell lines with various degrees of EGFR expression and sensitivity to EGFR inhibitors and analyzed signal transducers under the control of EGFR-dependent and EGFR-independent pathways.

Results: Multitargeted inhibitor vandetanib (ZD6474) inhibited the growth and the phosphorylation of Akt and its effector p70S6 kinase in both wild-type and EGFR inhibitor–resistant human colon, prostate, and breast cancer cells. We found that the resistant cell lines exhibit, as common feature, VEGFR-1/Fit-1 overexpression, increased secretion of VEGF and placental growth factor, and augmented migration capabilities and that vandetanib is able to antagonize them. Accordingly, a new kinase assay revealed that in addition to VEGF receptor (VEGFR)-2, RET, and EGFR, vandetanib efficiently inhibits also VEGFR-1. The contribution of VEGFR-1 to the resistant phenotype was further supported by the demonstration that VEGFR-1 silencing in resistant cells restored sensitivity to anti-EGFR drugs and impaired migration capabilities, whereas exogenous VEGFR-1 overexpression in wild-type cells conferred resistance to these agents.

Conclusions: This study shows that VEGFR-1 contributes to anti-EGFR drug resistance in different human cancer cells. Moreover, vandetanib inhibits VEGFR-1 activation, cell proliferation, and migration, suggesting its potential utility in patients resistant to EGFR inhibitors.

Receptor tyrosine kinases (TK), such as epidermal growth factor receptor (EGFR), play a key role in the development and progression of human epithelial cancers (1, 2). Increased expression of EGFR has been widely detected in human carcinomas and is generally associated with poor prognosis as well as resistance to chemotherapy or hormone therapy (3–5). Therefore, inhibition of EGFR signaling has become a valuable anticancer strategy (3–5). Among the various anti-EGFR monoclonal antibodies, cetuximab (Erbitux), a chimeric human-mouse IgG1 monoclonal antibody, has been approved for use in patients with advanced colorectal or head and neck cancers (3). Several compounds have also been developed that inhibit ligand-induced activation of EGFR TK activity; these include gefitinib (Iressa), an orally active anilinoquinazoline under clinical investigation (3–5).

Vascular endothelial growth factor (VEGF), a potent proangiogenic protein (6), binds to three distinct VEGF receptors (VEGFR): VEGFR-1 (Fit-1), VEGFR-2 (KDR or the murine homologue Flk-1), and VEGFR-3 (Flt-4; ref. 6). These receptors are expressed on endothelial cells and regulate cell permeability, proliferation, and differentiation (6) as well as on...
have shown that simultaneous inhibition of EGFR and VEGFR-and increased secretion of VEGF (24, 26, 27). In addition, we EGFR inhibitors cetuximab and gefitinib show overexpression shown that human cancer cells with acquired resistance to downstream signaling effectors (23–25). We and others have cells may develop resistance to EGFR inhibitors via alternative EGFR inhibitors is a relevant issue in cancer patients. Cancer diminishing vascular investment with perivascular cells (22). migration by interfering with the chemotactic response and by tion of VEGFR-1 in primary tumors prevents endothelial cell in the ''angiogenic switch'' and associated with increased cancer cells (8–10). Enhanced expression of VEGF is involved in the “angiogenic switch” and associated with increased neovascularization within the tumor; it is triggered through different mechanisms, most notably hypoxia (11–13). In addition, activation of EGFR signaling can result in the increased expression of VEGF in human cancer cells (14, 15), whereas EGFR inhibition leads to decreased secretion of VEGF and other angiogenic growth factors, including basic fibroblast growth factor, interleukin-8, and transforming growth factor-2 and therapeutic implications. First, using a panel of tumor cells of different types and with different degrees of sensitivity or resistance to epidermal growth factor receptor (EGFR) inhibitors, we show that resistant tumors share the following common features: vascular endothelial growth factor receptor (VEGFR)-1/Flt-1 overexpression and Akt activation, increased secretion of VEGF and placent growth factor, and augmented migration capabilities. We also provide mechanistic evidence of the correlation between VEGFR-1 activity and EGFR drug resistance. These data imply that detection of VEGFR-1 on tumor cells may indicate their increased ability to survive and invade and to escape the inhibition by EGFR inhibitors used in clinical practice, such as cetuximab and gefitinib. Another finding concerns the small-molecule vandetanib/ZD6474, whose mechanisms of action have been documented by us in several previous studies. We here show that vandetanib is able to inhibit VEGFR-1 kinase and EGFR drug-resistant tumors. On these bases, we have reexamined and reported a new kinase inhibition profile for this drug. Because vandetanib is under investigation in several clinical studies, these data may be important for its clinical development.

Translational Relevance

We report several findings that may have relevant clinical and therapeutic implications. First, using a panel of tumor cells of different types and with different degrees of sensitivity or resistance to epidermal growth factor receptor (EGFR) inhibitors, we show that resistant tumors share the following common features: vascular endothelial growth factor receptor (VEGFR)-1/Flt-1 overexpression and Akt activation, increased secretion of VEGF and placental growth factor, and augmented migration capabilities. We also provide mechanistic evidence of the correlation between VEGFR-1 activity and EGFR drug resistance. These data imply that detection of VEGFR-1 on tumor cells may indicate their increased ability to survive and invade and to escape the inhibition by EGFR inhibitors used in clinical practice, such as cetuximab and gefitinib. Another finding concerns the small-molecule vandetanib/ZD6474, whose mechanisms of action have been documented by us in several previous studies. We here show that vandetanib is able to inhibit VEGFR-1 kinase and EGFR drug-resistant tumors. On these bases, we have reexamined and reported a new kinase inhibition profile for this drug. Because vandetanib is under investigation in several clinical studies, these data may be important for its clinical development.

hematopoietic stem cells, osteoblasts, and monocytes. Whereas the expression of VEGFR-2 seems mostly restricted to vascular endothelial cells, VEGFR-1 is present in both vascular endothelial and macrophage-like cells and may promote inflammation, tumor growth, and metastasis (7). Moreover, it has recently been shown that VEGFRs are also expressed in some cancer cells (8–10). Enhanced expression of VEGF is involved in the “angiogenic switch” and associated with increased neovascularization within the tumor; it is triggered through different mechanisms, most notably hypoxia (11–13). In addition, activation of EGFR signaling can result in the increased expression of VEGF in human cancer cells (14, 15), whereas EGFR inhibition leads to decreased secretion of VEGF and other angiogenic growth factors, including basic fibroblast growth factor, interleukin-8, and transforming growth factor-α (16–19). Expression of VEGFR-1 and VEGFR-2 and their ligands may sustain an autocrine loop in some human model cancers. In melanoma (9, 20), mesothelioma (10), and human leukemic cells (21), exogenous VEGF stimulates cell proliferation and migration by activating VEGFR-2. Moreover, inhibition of VEGFR-1 in primary tumors prevents endothelial cell migration by interfering with the chemotactic response and by diminishing vascular investment with perivascular cells (22). The development of constitutive and acquired resistance to EGFR inhibitors is a relevant issue in cancer patients. Cancer cells may develop resistance to EGFR inhibitors via alternative growth signaling pathways or constitutive activation of downstream signaling effectors (23–25). We and others have shown that human cancer cells with acquired resistance to EGFR inhibitors cetuximab and gefitinib show overexpression and increased secretion of VEGF (24, 26, 27). In addition, we have shown that simultaneous inhibition of EGFR and VEGFR-2 causes antitumor effects in several human cancer xenograft models (28, 29), including those with acquired resistance to cetuximab and gefitinib (26). To this aim, we used vandetanib/ZD6474 (Zactima), an orally available TK inhibitor active against VEGFR-2, EGFR, and RET kinases (26, 29–31). The EGFR inhibitor–resistant cancer cells used in our former study were generated by in vivo selection (26); however, their resistant phenotype tended to weaken after several in vitro passages even in the continued presence of cetuximab or gefitinib. Therefore, to investigate the role of certain downstream signal transducers in the resistance to anti-EGFR drugs, in the present study, we generated and used tumor cell lines with different levels of EGFR expression and stable resistance to EGFR inhibitors as well as their sensitive counterparts.

Materials and Methods

Drugs. Vandetanib and gefitinib were kindly provided by Dr. Anderson Ryan (AstraZeneca Pharmaceuticals Ltd.). Cetuximab was supplied by ImClone Systems.

Cell lines. Human GEO (colon carcinoma), PC3 (hormone-refractory prostate adenocarcinoma), MDA-MB-468 (mammary gland carcinoma), and SW480 (colorectal carcinoma) cell lines were obtained from the American Type Culture Collection. GEO-GR (gefitinib resistant), GEO-OR (cetuximab resistant), and PC3-GR (gefitinib resistant) cells were established as previously described (26). In contrast to previous EGFR inhibitor–resistant cancer cells (26), the resulting cell lines that were stably resistant to EGFR inhibitors retained a resistant phenotype even after several passages in the absence of EGFR antagonists. All cell lines were cultured as previously described (26).

Growth in soft agar. Cells (10⁴ per well) were suspended in 0.3% Difco Noble agar (Difco) supplemented with complete medium, layered over 0.8% agar medium base layer, and treated with different concentrations of gefitinib, cetuximab, and vandetanib. After 10 to 14 d, cells were stained with nitro blue tetrazolium (Sigma Chemical Co.) and colonies >0.05 mm were counted (19).

RNA interference. Small interfering RNA (siRNA) kits (Validated Stealth for EGFR and Select Stealth for VEGFR-1/Fli-1 and VEGFR-2/ KDR) were obtained from Invitrogen Life Technologies, Inc. A nonsense sequence was used as a negative control. For siRNA validation, cells were seeded into 60-mm dishes and transfected with 40 nmol/L EGFR siRNA and 120 nmol/L VEGFR-1 or VEGFR-2 siRNA using Lipofectamine 2000 (Invitrogen) in Opti-MEM (Invitrogen). Forty-eight hours after transfection, Western blot analysis for EGFR, VEGFR-1, or VEGFR-2 protein expression was done. The siRNA effects on cell signaling were evaluated through further Western blot analysis.

For the assessment of siRNA effects on cell survival, cells were seeded into 24-well cluster dishes and transfected with EGFRI, VEGFR-1, or VEGFR-2 siRNA. Twenty-four hours after transfection, cells treated with VEGFR-1 or VEGFR-2 siRNA received 140 nmol/L cetuximab or 5 µmol/L gefitinib and cell survival was determined 24 h later.

Transfection. PC3 or SW480 cells were transiently transfected with pcDNA3/hFli-1 or with pcDNA3 as a negative control using the specific Cell Line Nucleofector Kit V for PC3 or SW480 (Amaxa). Briefly, 1 × 10⁶ PC3 or SW480 cells were transfected with 5 µg DNA. To confirm VEGFR-1 expression, cells were plated in 6-well cluster dishes and a Western blot analysis was done 48 h after transfection. For activity experiments, 3 × 10⁴ cells were plated in each well of 24-well cluster dishes; 24 h after transfection, they received 1, 2.5, or 5 µmol/L of gefitinib; and cell survival was determined 48 h later.

Cell survival assay. The culture supernatant was removed and 100 µL 1-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma Chemical) stock solution (5 mg/mL) was added to each well together with 400 µL of medium. After 4 h of incubation, isopropanol
was added and the absorbance was measured at 570 nm. Percentage cell survival was calculated by dividing the mean absorbance of wells containing treated cells by that of untreated control wells.

**Apoptosis detection in cultured cells.** The induction of apoptosis was measured using the Cell Death Detection ELISA Plus kit (Roche Molecular Biochemicals; ref. 32). Briefly, cells (5 \times 10^4 per well) were seeded into 6-multwell cluster dishes and treated on days 1 to 2 with vandetanib (0.5 \mu m/L). Each treatment was done in quadruplicate. Absorbance readings at 405 nm were normalized for cell number and the ratio of absorbance of treated cells to untreated cells was defined as the apoptotic index.

**Kinase assays.** Enzyme inhibition by vandetanib was determined by ProQinase GmbH. IC_{50} values were calculated from 12-point dose-response curves (10^{-4} mol/L to 3 mol/L). All protein kinases were expressed in S9 insect cells as human recombinant glutathione S-transferase fusion proteins or His-tagged proteins by means of the baculovirus expression system. Kinases were purified by affinity chromatography using either reduced glutathione-agarose (Sigma Chemical) or Ni-NTA-agarose (Qiagen). The purity of the protein kinases was examined by SDS-PAGE/silver staining and their identity was checked by Western blot analysis with specific antibodies or by mass spectroscopy. The reaction cocktail was pipetted in five steps in the following order: 20 \mu L of assay buffer, 10 \mu L of substrate (in H_2O), 5 \mu L of vandetanib at 10\% DMSO, 10 \mu L of enzyme solution, and 5 \mu L of ATP solution (in H_2O). The amounts of protein kinase in each assay were as follows: 100 ng VEGFR-2, 40 ng EGFR, 150 ng VEGFR-3, 60 ng VEGFR-1, and 100 ng platelet-derived growth factor receptor \beta. After incubation at 30\°C for 80 min, the reaction was stopped with 2% (v/v) H_2PO_4. All assays were done with a Beckman Coulter Biomek 2000 robotic system. Incorporation of ^32P was determined with a microplate scintillation counter (MicroBeta,Wallac). IC_{50} values were calculated using Prism 4.03 for Windows (GraphPad).

**Immunoprecipitation and Western blot analysis.** Cell protein extracts were prepared from tumor cells cultured for 24 h in the presence or absence of 1 \mu mol/L gefitinib, 1 \mu mol/L vandetanib, or 7 nmol/L cetuximab (19). Protein extracts were resolved by 4\% to 20\% SDS-PAGE and probed with one of the following antibodies: anti-EGFR (Ab-12, NeoMarkers); anti–phospho-(Tyr^{1068} )-EGFR, anti-p70S6K, anti–phospho-p70S6K, and anti-VEGFR-2/KDR (Upstate); Akt and anti–phospho-(Ser^{473} )-Akt (Cell Signaling); anti–extracellular signal-regulated kinase 1/2, anti–phospho-extracellular signal-regulated kinase 1/2, anti–phosphatase and tensin homologue (PTEN), anti–VEGFR-1/Flt-1, anti–phospho-extracellular signal-regulated kinase 1/2, anti–phosphatase and tensin homologue (PTEN), anti–VEGFR-1/Flt-1, anti–phospho-extracellular signal-regulated kinase 1/2, anti–phosphatase and tensin homologue (PTEN), anti–VEGFR-2, and anti–phosphatase and tensin homologue (PTEN). Immunoreactive proteins were visualized by enhanced chemiluminescence (Amersham International).

**Determination of VEGF and placental growth factor concentrations.** The concentrations of VEGF and placental growth factor (PIGF) in conditioned medium from tumor cell lines were determined by ELISA, as previously described (33). The absorbance was measured at 490 nm on a microplate reader (Bio-Rad) and PIGF and VEGF concentrations were determined by interpolation of the standard curve using linear regression analysis.

**Reverse transcription-PCR.** Total RNA was isolated from cells using the Trizol reagent from Invitrogen Life Technologies. Reverse transcription was done using reverse transcriptase (SuperScript RT, Life Technologies). To evaluate VEGFR-1/Flt-1, VEGFR-2/KDR, and VEGF gene expression, aliquots of reverse transcription-RNA were amplified using primers designed based on the coding sequences of the human mRNA (8). PCR products were visualized using ethidium bromide on a 1.8% agarose gel. Human glyceraldehyde-3-phosphate dehydrogenase was used as a control.

**Cell adhesion assay.** To investigate the basement membrane adhesion capabilities of the cancer cell lines, 96-microwell bacterial culture plates were precoated with 50 \mu L/well of serum-free medium containing 0.1\% bovine serum albumin or different dilutions of Matrigel. After 1 h, all coating solutions were removed and 2 \times 10^4 cells per well were plated in serum-free medium. Following incubation for 1 h at 37\°C in 5\% CO_2, cells were fixed and stained with a formalin/ethanol/crystal violet fixing/staining solution, washed extensively, and air dried, and the dye was eluted with ethanol/acetic acid solution. The readings were done at 595 nm and the values were normalized to background adhesion (34).

**Cell migration assay.** Cell migration was investigated using the Boyden chamber chemotaxis assay (35). Polycarbonate filters (8-\mu m pore size, polyvinyl pyrrolidone–free from Costar-Nucleopore) were manually coated with 50 \mu L of a 0.1 mg/mL solution of collagen type IV and dried overnight at room temperature. VEGF was used as chemo-attractant, whereas serum-free medium containing 0.1\% bovine serum albumin was used as a negative control for unstimulated migration. Cells were harvested and placed in the upper compartment of the Boyden chamber. After incubation at 37\°C in 5\% CO_2 for 6 h, cells remaining on the upper surface of the filter were removed, and those that migrated to the lower compartment were fixed with ethanol, stained with toluidine blue (Sigma-Aldrich), and quantified using densitometry.

**Wound-healing assay.** Cancer cell line monolayers grown to confluence on gridded plastic dishes were wounded by scratching with a 10 \mu L pipette tip and then cultured in the presence or absence of doxorubicin (25 ng/mL), vandetanib (2.5 \mu mol/L), gefitinib (5 \mu mol/L), VEGFR-1 or VEGFR-2 siRNA, or with a nonsense RNA sequence (all 120 nmol/L) for 24 h. Under these conditions, all drugs except doxorubicin weakly inhibited cell proliferation. Because doxorubicin did not interfere with cell migration, it was used as a negative control. The wounds were photographed (10\times objective) at 0, 8, or 24 h (36), and healing was quantified by measuring the distance between the edges using Adobe Photoshop (v.8.0.1; Adobe Systems, Inc.). The results are presented as the percentage of the total distance of the original wound enclosed by cells. A survival assay was done to ensure that effect on wound closure reflected inhibition of cell migration and not of cell proliferation.

**Statistical analysis.** The Student’s t test was used to evaluate the statistical significance of the results. All analyses were done with the BMDP New System statistical package (version 1.0) for Microsoft Windows (BMDP Statistical Software).

**Results**

**Human cancer cell lines resistant to EGFR inhibitors are sensitive to vandetanib.** Human cancer cell lines with different levels of EGFR expression were identified/generated to investigate resistance to EGFR inhibitors. MDA-MB-468 human breast cancer cells express high EGFR levels, yet their growth was relatively resistant to gefitinib (37) and resistant to cetuximab at high doses (up to 140 nmol/L; Fig. 1A). This constitutive resistant phenotype is associated with phosphoinositide 3-kinase/Akt hyperactivity, in turn related to mutation of the PTEN gene. GEO human colorectal cancer cells express lower EGFR levels and were sensitive to both cetuximab and gefitinib (IC_{50} <3.5 nmol/L and 0.5 \mu mol/L, respectively). Compared with GEO cells, PC3 cells show similar EGFR expression levels and sensitivity to gefitinib but are resistant to cetuximab (up to 140 nmol/L). Despite similar sensitivity to gefitinib, GEO cells have a functional wild-type PTEN gene, whereas PC3 have a deleted PTEN.

The EGFR inhibitor–resistant cell lines established in this study were insensitive to cetuximab (GEO-GR) and gefitinib (GEO-GR and PC3-GR) at doses up to 560 nmol/L and...
20 μmol/L, respectively (Fig. 1A). They had a morphology, in vitro growth rate, and soft agar cloning efficiency similar to that of parental cells (data not shown). We previously showed that vandetanib inhibits the growth of EGFR inhibitor-resistant GEO xenografts (26). In this study, vandetanib efficiently inhibited soft agar growth of all cell lines (IC₅₀, 0.1-0.5 μmol/L; Fig. 1A), irrespective of their EGFR inhibitor sensitivity, and showed potent proapoptotic activity in the resistant cancer cells even at a dose unable to induce apoptosis in sensitive cancer cells (Fig. 1B).

Resistance to EGFR correlates with activation of downstream signaling pathways via EGFR-independent mechanisms. The investigation of EGFR-dependent signaling pathways revealed interesting differences between the cell lines. Treatment of wild-type GEO and PC3 cells with EGFR inhibitors strongly reduced phosphorylation of EGFR and consequently of the downstream effectors Akt and mitogen-activated protein kinase (MAPK; Fig. 2A). Similarly, vandetanib inhibited EGFR phosphorylation in both cell lines and caused an almost complete down-regulation of phospho-Akt and phospho-MAPK (Fig. 2A).
Fig. 2. Analysis of EGFR-dependent signaling pathways in human cancer cell lines sensitive or resistant to EGFR inhibitors. A, Western blot analysis of protein expression in cell lines treated with 7 nmol/L cetuximab, 1 μmol/L gefitinib, or 1 μmol/L vandetanib for 24 h before protein extraction. B, Western blot analysis of protein expression in PC3-GR and MDA-MB-468 cells 24 h after treatment with 1 μmol/L gefitinib or 1 μmol/L vandetanib and 48 h after transfection with EGFR-specific siRNA or with a nonsense RNA sequence used as a negative control (both 40 nmol/L). C, percent of survival of PC3, PC3-GR, and MDA-MB-468 cells treated with 5 μmol/L gefitinib, 140 nmol/L cetuximab, and EGFR-targeting siRNA or a nonsense RNA sequence (both 40 nmol/L). Results for each treatment are presented relative to untreated control cells.

* Two-sided *P < 0.0001 versus control and versus negative control. Bars, SD.
MDA-MB-468 cells, gefitinib decreased the levels of phospho-EGFR and phospho-MAPK without any change in Akt phosphorylation (37, 38); in contrast, vandetanib inhibited phosphorylation of EGFR, MAPK, and Akt (Fig. 2A). Cetuximab or vandetanib treatment of GEO-CR cells markedly inhibited EGFR phosphorylation; however, vandetanib, but not cetuximab, caused a complete reduction in phospho-Akt and a lesser reduction in phospho-MAPK (Fig. 2A). Similar results were observed in GEO-GR and PC3-GR cells comparing the effects of vandetanib and gefitinib. Although the greatest difference between gefitinib and vandetanib was seen with phospho-Akt, these differential drug effects were also evident for phospho-MAPK (Fig. 2A). We then analyzed downstream effectors of the Akt/mammalian target of rapamycin–dependent pathway. In wild-type GEO and PC3 cells, treatment with gefitinib, cetuximab, and vandetanib reduced p70S6K phosphorylation (Fig. 2A), consistent with the parallel decrease in Akt phosphorylation. Conversely, in resistant cell lines, a reduced p70S6K phosphorylation was observed only following vandetanib treatment (Fig. 2A).

In PC3-GR cells, an EGFR-specific RNA interference, which completely suppressed EGFR expression (Fig. 2B), was unable to reduce phospho-Akt and phospho-MAPK levels, similarly to what we observed after gefitinib treatment. In MDA-MB-468 cells, the EGFR siRNA inhibited MAPK, but not Akt phosphorylation/activation, reproducing also in this case the results obtained with gefitinib (Fig. 2B). Moreover, the EGFR siRNA markedly reduced cell survival (~50%) in wild-type PC3 cells, whereas no effect was seen in PC3-GR and MDA-MB-468 cells (Fig. 2C). This suggests that EGFR inhibition per se is not sufficient to induce growth perturbations in resistant cells and that inhibition of Akt phosphorylation/activation seems to be the most closely associated with significant growth perturbation.

It has been previously shown that the lack of a functional PTEN in MDA-MB-468 cells leads to increased phosphoinositide 3-kinase/Akt activity and resistance to gefitinib (37). However, Western blot analysis of GEO-CR, GEO-GR, and PC3-GR cells did not reveal any differences in PTEN expression (Supplementary Fig. S1A).

Recently, Engelman et al. (39) showed that Met amplification leads to gefitinib resistance in lung cancer by activating ErbB3 signaling. Met evaluation in our models revealed different Met protein levels, higher in GEO cells and lower in PC3 and MDA-MB-468 cells. Nevertheless, no difference in Met expression or activation status was observed between resistant and sensitive cell lines (Supplementary Fig. S1B).

**Human cancer cell lines resistant to EGFR inhibitors express VEGFRs.** The apparent independence of Akt activity from EGFR activation highlights the role that alternative signaling pathways may play in resistance to EGFR antagonists. Vandetanib is able to inhibit, in addition to EGFR, the TK activity of VEGFR-2 (29) and RET (30); we therefore investigated the expression of these receptors in our cancer cell lines.

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cells. No expression of RET was observed in any cell line (data not shown). VEGFR-2 expression was observed with no noticeable differences between parental or resistant cell lines (Fig. 3A).

To examine the potential role of VEGFRs as alternative survival pathways in resistant cell lines, we examined the expression of VEGFR-1 in resistant cells and observed that it was increased compared with parental cells, both at protein and mRNA levels (Fig. 3B). Therefore, we analyzed the effect of vandetanib on VEGFR-1 and VEGFR-2 autophosphorylation, observing a strong inhibition of both receptors in GEO-CR, GEO-GR, and PC3-GR cells (Fig. 3C and D).

To confirm vandetanib capability of inhibiting also VEGFR-1, we did a kinase assay with a new kit to define the IC₅₀ values for VEGFR-1, VEGFR-2, VEGFR-3, EGFR, and platelet-derived growth factor receptor β. As summarized in Table 1, vandetanib had a much broader inhibitory activity than reported before (29) because it efficiently inhibited also VEGFR-1 (IC₅₀, 150 nmol/L). Moreover, the ability to inhibit EGFR resulted almost comparable with VEGFR-2 (IC₅₀, 43 versus 38 nmol/L, respectively). Finally, to a lesser extent, vandetanib inhibited also VEGFR-3 (IC₅₀, 260 nmol/L). These data agree and extend the results formerly reported by a different group (40).

Interestingly, all resistant cell lines synthesized and secreted the VEGF ligands VEGF and PIGF. VEGF mRNA expression was slightly elevated in all resistant cell derivatives compared with parental cell lines (Supplementary Fig. S2A). ELISA assays confirmed that both factors are consistently produced in all cell lines, with VEGF levels considerably higher than PIGF levels, and that their secretion is higher in conditioned medium derived from resistant cells compared with parental cells (Supplementary Fig. S2B and C).

**Table 1. Kinase inhibition by vandetanib**

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<tr>
<th>Kinase</th>
<th>IC₅₀ (nmol/L)</th>
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<tr>
<td>VEGFR-1</td>
<td>150</td>
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<tr>
<td>VEGFR-2</td>
<td>38</td>
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<td>VEGFR-3</td>
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<td>EGFR</td>
<td>43</td>
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<td>PDGFRβ</td>
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Abbreviation: PDGFRβ, platelet-derived growth factor receptor β.

VEGFR-1 siRNA partially restores sensitivity to EGFR antagonists. To further show VEGFR involvement in the resistance to EGFR inhibitors, we investigated whether a reduction of VEGFR-1 or VEGFR-2 expression in resistant cell lines could partially restore sensitivity to cetuximab and gefitinib. Only PC3-GR and MDA-MB-468 were used because of the low transfection efficiency of GEO cells. Transfection with VEGFR-1 or VEGFR-2 siRNA for 48 h partly reduced the respective target protein expression (Fig. 4A and B), and VEGFR-1 protein reduction restored the ability of gefitinib to inhibit Akt and MAPK phosphorylation/activation in PC3-GR cells (Fig. 4C).

Importantly, the reduction of VEGFR-1 expression to levels similar to parental/sensitive cells partially recovered the antiproliferative effect of EGFR inhibitors in PC3-GR and MDA-MB-468 cells, as assessed with a cell survival assay (Fig. 4D and E). The degree of resensitization was ~35% in both cell lines. The reduction of VEGFR-2 expression restored sensitivity to EGFR inhibitors to a lesser extent (~15%; Fig. 4D and E). Therefore, VEGFRs, particularly VEGFR-1, seem to play a role in the resistance to EGFR inhibitors, which, in turn, correlates with Akt kinase activation via EGFR-independent mechanisms.

VEGFR-1 overexpression in wild-type cells reduces sensitivity to gefitinib. To confirm VEGFR-1 contribution to the development of resistance to EGFR inhibitors, we transfected a full-length VEGFR-1 expression vector in gefitinib-sensitive prostate PC3 cells and colon SW480 cells, an other EGFR-expressing cell line (19), and investigated whether VEGFR-1 could confer resistance to gefitinib. In spite of the suboptimal transfection efficiency (~50% of cells), an increase of VEGFR-1 expression was observed 48 h after transfection in both cell lines (Fig. 5A and B), and it was associated with about 30% and 25% reduction of sensitivity to gefitinib, in PC3 and in SW480 cells, respectively, as measured by a survival assay (Fig. 5C and D). Conversely to nontransfected cells, VEGFR-1–overexpressing cells seemed totally insensitive to low doses of gefitinib and their survival was only slightly inhibited by high doses of this drug (Fig. 5C and D).

Human cancer cell lines with acquired resistance to EGFR inhibitors display altered adhesion and migration capabilities. VEGFR expression may influence other tumor cell capabilities, such as migration and adhesion. To compare parental and resistant cell lines for their migration potential, we did a wound-healing assay on PC3 and PC3-GR cells. Eight hours after wound creation, an up to 50% greater migration capability was observed in PC3-GR compared with PC3 cells (Fig. 6A). To confirm these data, we did a Boyden chamber chemotaxis assay on PC3 and PC3-GR cells using VEGF (10 ng/mL) as a chemoattractant. PC3-GR exhibited a 2-fold greater migration capability than the parental cell line (Fig. 6B), and cell migration was not noticeably dependent on the presence of exogenous VEGF. An adhesion assay confirmed that the greater migration capability of PC3-GR cells was not due to a greater adherence to membrane basement components of the Boyden chamber filters (Fig. 6C).

**VEGFRs are involved in migration of human cancer cell lines sensitive and resistant to EGFR inhibitors.** To investigate whether the migration of resistant cells could be affected by VEGFR inhibition, we did a wound-healing assay on PC3 and PC3-GR cells in the presence of vandetanib, gefitinib, or VEGFR-specific siRNAs. Twenty-four hours after wound creation, both PC3 and PC3-GR cells were able to migrate and close the wound to a similar extent. Neither doxorubicin nor gefitinib affected migration, but vandetanib markedly reduced wound closure efficiency in both cell lines, particularly PC3-GR cells (Fig. 6D). Whereas the slight inhibition of wound closure with VEGFR-2–targeted siRNA did not reach statistical significance, VEGFR-1–targeted siRNA inhibited both PC3 (~60%; P < 0.0001) and PC3-GR (~70%; P < 0.0001) cell migration (Fig. 6D).

**Discussion**

The purpose of this study was to examine the signaling mechanisms operating in human tumor cell lines that have acquired resistance to anti-EGFR drugs. Although these agents

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Fig. 4. VEGFR silencing in human cancer cell lines resistant to EGFR inhibitors. Western blot analysis of VEGFR-1/Flt-1 (A) or VEGFR-2/KDR (B) in PC3-GR and MDA-MB-468 cells transfected with 120 nmol/L VEGFR-1 or VEGFR-2 - targeting siRNA, respectively, or with a nonsense RNA sequence (negative control). C, Western blot analysis of protein expression in PC3-GR cells 24 h after treatment with 1 μmol/L vandetanib or 1 μmol/L gefitinib and 48 h after transfection with 120 nmol/L VEGFR-1 - targeting siRNA or with a nonsense RNA sequence (negative control). D and E, cell survival analysis of the VEGFR-1 siRNA-transfected cells in the presence or absence of 5 μmol/L gefitinib or 140 nmol/L cetuximab. Results for each treatment are presented relative to untreated control cells. *, two-sided $P < 0.0001$ versus control and versus negative control. Bars, SD.
have a significant antiproliferative activity, the occurrence of resistance in the clinical setting is an issue. Specific activating mutations within the EGFR TK domain correlate with dramatic responses to gefitinib or erlotinib observed in some subgroups of patients. However, with the exception of a recently shown threonine to methionine (T790M or T766M) point mutations in exon 20 (41), the mechanisms by which some patients become resistant to treatment are still unclear, especially for monoclonal antibodies.

Aberrant activation of phosphoinositide 3-kinase represents one of the most commonly reported mechanisms by which resistance to EGFR inhibitors arises. Inactivating mutations or loss of PTEN could result in constitutive activation of oncogenic signals through Akt and has been associated with resistance to EGFR TK inhibitors (37, 38). Both MDA-MB-468 and PC3 cancer cells lack functional PTEN protein; however, whereas MDA-MB-468 cells are insensitive to both gefitinib and cetuximab, PC3 are gefitinib sensitive, suggesting that the occurrence of the resistant phenotype can arise from signaling pathways other than those regulated by PTEN; no altered expression of PTEN was observed in the resistant lines established in this study. Constitutive activation of the phosphoinositide 3-kinase/Akt pathway is commonly reported in human cancers (42) and seems to correlate with the response to EGFR inhibitors (43). In the present study, treatment with gefitinib and cetuximab, as well as EGFR silencing via siRNA, was able to induce cell growth arrest only if EGFR inhibition was coupled with down-regulation of phospho-Akt. This effect was not detected in resistant cell lines, in which inhibition of EGFR and Akt phosphorylation was only observed following vandetanib administration. Moreover, in cancer cells with elevated activation of Akt, an enhanced mammalian target of rapamycin activity has been detected (44). Because Akt is one of the major positive regulators of mammalian target of rapamycin/p70S6K activity, targeting of these kinases could represent a promising therapeutic approach. The pattern of sensitivity to vandetanib suggests the presence of other TKs that are activated in EGFR inhibitor–resistant cell lines, including the VEGFRs. Alternative signaling pathways that circumvent the inhibition of EGFR are often activated in cancer cells, a key example being insulin-like growth factor-I receptor and Met signaling. The association between insulin-like growth factor-I receptor overactivity and acquired resistance to EGFR blockade has been shown for glioblastoma multiforme and breast and prostate cancer (25, 45), and Met amplification seems to sustain the resistance against small TK inhibitors in non–small cell lung cancer cell lines and patients independently from EGFR mutations (39). However, in our resistant cell lines, no altered
expression of insulin-like growth factor-1 receptor (data not shown) and no altered expression/activation of Met were detected using commercial antibodies. Vandetanib is also a potent inhibitor of RET TK activity (46), but no RET expression was detected in any of our cancer cell lines. Because all the cell lines expressed both VEGFR-1 and VEGFR-2 and VEGFR-1 is significantly increased in cancer cells with acquired resistance to EGFR inhibitors, this receptor may play a potentially important role in determining the EGFR inhibitor–resistant phenotype. Whereas VEGFR-2 has been characterized as one of the major mediators of angiogenesis in human malignancy, through induction of endothelial differentiation, DNA synthesis, and proliferation (47), VEGFR-1 seems to function as a VEGF “sink” during developmental vasculogenesis and may contribute to angiogenesis in ischemic or malignant diseases (48). In addition to their expression on endothelial cells, VEGFRs are expressed in hematopoietic stem cells and also in a variety of tumor types, including breast, prostate, ovarian, melanoma, non–small cell lung, pancreatic, and colon cancers (49). More recently, VEGFR-1 expression has been detected in different human prostate and colorectal cancer cell lines, including GEO cells (8). Although the precise role of VEGFRs in human malignancy is not completely understood, it is possible that the concomitant secretion of proangiogenic growth factors and the expression of VEGFRs support certain biological functions in cancer cells through the activation of autocrine loops (50). All the cell lines used in the present study secreted both VEGF and PlGF, the major growth factors that stimulate and activate VEGFR-1 or VEGFR-2. To further investigate the inhibitory effect observed with vandetanib on our resistant cell lines, we reevaluated the kinase inhibitory profile of this agent using a novel kinase assay. We had previously reported a strong

Fig. 6. Analysis of migration and adhesion capabilities of human cancer cell lines sensitive or resistant to EGFR inhibitors. A, wound-healing assay on PC3 and PC3-GR cells. Cell monolayers were wounded by scratching with a 10 μL pipette tip. The results are presented as the percentage of the total distance of the original wound enclosed by cells. Points, mean at 8 h; bars, SD. *, two-sided \( P < 0.0001 \) versus the other cell line at 8 h. B, migration at 6 h of PC3 and PC3-GR cells ± VEGF, assessed using Boyden chambers. *, two-sided \( P < 0.0001 \) versus the other cell line at 6 h. C, cell adhesion of PC3 and PC3-GR cells ± Matrigel. *, two-sided \( P < 0.0001 \) versus negative control of the same cell line and versus the other cell line. D, wound-healing assay in PC3 and PC3-GR cells done after 24 h of incubation with 25 μg/mL doxorubicin (Dox), 2.5 μmol/L vandetanib, 5 μmol/L gefitinib, and 120 nmol/L VEGFR-1– or VEGFR-2–targeting siRNA. *, two-sided \( P < 0.0001 \) versus control, doxorubicin, and negative control. Bars, SD.
inhibitory activity against VEGFR-2 and RET and, at lesser extent, on EGFR kinases (29, 30). We have now shown that vandetanib efficiently inhibits also VEGFR-1 and that the inhibitory activity on EGFR is higher than formerly reported; moreover, vandetanib at a much lesser extent inhibits also VEGFR-3. These data agree with a previous analysis from a different group (40), enhancing the multitracking profile of vandetanib and providing a clue to its inhibitory activity on resistant cells overexpressing VEGFR-1. In the same fashion, we have shown that siRNA silencing of VEGFRs may result in decreased cancer cell survival. Intriguingly, inhibition of VEGFR-1 activity correlates with a partially restored sensitivity to anti-EGFR drugs in EGFR inhibitor–resistant cancer cells. In fact, VEGFR-1 silencing restores gefitinib ability to inhibit both Akt activation/phosphorylation and cell survival. By contrast, exogenous overexpression of VEGFR-1 in two different tumor cell lines markedly reduces the sensitivity to EGFR inhibitors. VEGFR-1 is implicated in the formation of premalignant niches (51) and may be directly involved in migration of tumor cells, including colorectal carcinoma (52). Therefore, the increased migration efficiency and the reduced adhesion to basement membranes observed in our cancer cells resistant to anti-EGFR drugs and overexpressing VEGFR-1 could result in a greater metastatic potential. In this respect, we have shown that VEGFR-1 inhibition strongly interferes with cell migration, particularly in the anti-EGFR drug-resistant cell lines.

Taken together, the results of our studies suggest that VEGFR-1 may play an important role in determining the development of a resistant phenotype toward EGFR-selective drugs, affecting also adhesion and migration processes. Consequently, the therapeutic use of agents able to inhibit both EGFR and VEGFR-1, including, as reported in this article, vandetanib, may help to efficiently inhibit Akt phosphorylation/activation, antagonizing and overcoming EGFR inhibitor resistance, thus affecting also the microenvironment.

Disclosure of Potential Conflicts of Interest

Dr. Anderson Ryan is an employee of AstraZeneca.

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References


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