Doctorate Program in Molecular Oncology and Endocrinology
Doctorate School in Molecular Medicine

XXI cycle - 2005–2008
Coordinator: Prof. Giancarlo Vecchio

"**Analysis of the mechanisms of resistance to Epidermal growth factor receptor inhibitors and development of multiple targeted strategies"**

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Analysis of the mechanisms of resistance to Epidermal growth factor receptor inhibitors and development of multiple targeted strategies

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Bianco R, **Rosa R**, Damiano V, Daniele G, Gelardi T, Garofalo S, Tarallo V, De Falco S, Melisi D, Benelli R, Albini A, Ryan A, Ciardiello F, Tortora G. Vascular endothelial growth factor receptor-1 contributes to resistance to anti-epidermal growth factor receptor drugs in human cancer cells. Clin Cancer Res 2008; 14:5069-80.

Bianco R, Garofalo S, **Rosa R**, Damiano V, Gelardi T, Daniele G, Marciano R, Ciardiello F, Tortora G. Inhibition of mTOR pathway by everolimus cooperates with EGFR inhibitors in human tumours sensitive and resistant to anti-EGFR drugs. Br J Cancer 2008; 98:923-30.

Damiano V, Caputo R, Garofalo S, Bianco R, **Rosa R**, Merola G, Gelardi T, Racioppi L, Fontanini G, De Placido S, Kandimalla ER, Agrawal S, Ciardiello F, Tortora G. TLR9 agonist acts by different mechanisms synergizing with bevacizumab in sensitive and cetuximab-resistant colon cancer xenografts. Proc Natl Acad Sci U S A 2007; 104:12468-73.

Garofalo S & **Rosa R**, Bianco R, Tortora G. EGFR-targeting agents in oncology. Expert Opin Ther Pat 2008; 18:1-13.

ABSTRACT

Background. Primary and acquired resistance to selective Epidermal growth factor receptor (EGFR) inhibitors remains the most significant obstacle to the success of these targeted agents in cancer therapy. The mechanisms of resistance involve the activation of alternative signaling pathways able to bypass EGFR blockade, and Akt activation and VEGF induction have been described in EGFR inhibitor-resistant tumors. Combined inhibition of EGFR and other signaling proteins has become an effective approach to efficiently inhibit compensatory escape pathways, stimulating the search for further determinants of resistance as basis for novel therapeutic strategies.

Aim of the study. The purpose of this study is to examine the signaling mechanisms operating in human cancers with intrinsic or acquired resistance to EGFR-targeted therapies and responsible for the lack of response to EGFR inhibitors. To this aim, 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 -independent pathways.

Results. Multitargeted inhibitor vandetanib (ZD6474) inhibited human endothelial cells survival thanks to inhibition of both VEGFRs and EGFR signaling pathways. Vandetanib also 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 features, VEGFR-1/Flt-1 overexpression, increased secretion of VEGF and placental growth factor (PlGF), and augmented migration capabilities, and that vandetanib is able to antagonize them. Accordingly, a new kinase assay revealed that in addition to VEGFR-2, RET and EGFR, vandetanib efficiently inhibits also VEGFR-1; this capability plays a key role in determining its activity on EGFR drugs-resistant tumors. 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, while exogenous VEGFR-1 overexpression in wild-type cells conferred resistance to these agents.

Conclusions. This study demonstrates that VEGFR-1 contributes to anti-EGFR drugs resistance in different human cancer models. Moreover, vandetanib inhibits VEGFR-1 activation, cell proliferation and migration, suggesting its potential utility in patients resistant to EGFR inhibitors. Since vandetanib is successfully under investigation in several clinical studies, these data may be important for its clinical development.

1. BACKGROUND

1.1 The Epidermal growth factor receptor (EGFR) as a target for cancer therapy.

In the last decade significant progress has been made in the understanding of the molecular mechanisms which are responsible for human cancer development and progression. The uncontrolled production of specific growth factors and the abnormal, enhanced expression on the cell membranes of growth factor receptors to which growth factors selectively bind play a key role in autonomous and deregulated proliferation of cancer cells, induction of angiogenesis, and metastasis. The majority of human epithelial cancers are marked by functional activation of growth factors and receptors of the erbB/HER family, which are involved in their formation and maintenance. This family consists of four distinct, but structurally similar, trans-membrane tyrosine kinase (TK) receptors, named erbB-1/HER1 (better known as Epidermal growth factor receptor, EGFR), erbB-2/HER2, erbB-3/HER3 and erbB-4/HER4 (Hynes and Lane 2005; Citri and Yarden 2006; Ciardiello and Tortora, 2008).

The EGFR gene encodes a 170kDa trans-membrane glycoprotein containing 1186 amino acids. The receptor consists of an extracellular domain that recognizes and binds to specific ligands, a hydrophobic trans-membrane domain, involved in interactions between receptors within the cell membrane, and an intracellular domain that serves as the site of protein kinase activity. The ligands of the erbB receptors belong to the EGF-family of growth factors: They are characterized by the presence of an EGF-like domain, composed of three disulfide-bonded intra-molecular groups conferring binding specificity, and additional structural motifs such as immunoglobulin-like domains, heparin-binding sites and glycosylation sites. These growth factors have different affinity for the members of erbB family: EGF, transforming growth factor α (TGF-α) and amphiregulin (AR) specifically bind to the EGFR. Ligand binding induces a conformational change of the receptor ectodomain that allows for homo- or hetero-dimerization between EGFR and other members of the erbB-family and autophosphorylation of several tyrosine residues within the COOH-terminal tail of the receptor (Burgess et al. 2003; Hubbard 2005; Bianco et al. 2007; Garofalo and Rosa 2008).

The autophosphorylated receptor initiates the recruitment to the plasma membrane and the activation through phosphorylation of other cytoplasmic substrates, which, in turn, mediate the activation of different signal transduction pathways depending upon type of ligand, levels of receptor expression and partner of EGFR dimerization. The most studied downstream pathways include the phosphatidylinositol-3 kinase (PI3K)/ Akt (protein kinase B, PKB), the Ras/extracellular signal regulated kinase (ERK) and the

phospholipase Cγ (PLCγ)/protein kinase C (PKC) signaling cascades. In the PI3K/Akt pathway, the EGFR c-terminal intracellular domain provides a docking site for the p85 subunit of PI3K either directly or indirectly. Upon activation, PI3K generates phosphatidyl-inositol-3,4,5-tris-phosphate (PIP3) which recruits and activates the serine-threonine kinase Akt/PKB. Phosphatase and tensin homolog protein (PTEN) is a lipid phosphatase that reduces Akt phosphorylation/activation dephosphorylating the D3 position of membrane PIP3. Thus, increased PI3K or reduced PTEN activity result in enhanced Akt function and have been reported in various human tumors. Akt controls some key cellular processes through phosphorylation of several downstream targets, such as apoptotic proteins, transcription factors, and protein kinases (Cantley and Neel 1999; Song et al. 2005; Bianco et al. 2007; Garofalo and Rosa 2008). In the Ras/ERK signaling cascade, the adaptor protein growth factor receptor bound protein 2 (Grb2), pre-associated with the guanine nucleotide exchange son of sevenless (Sos), binds to the activated EGFR, either directly or indirectly. Translocation of the Grb2/Sos complex to the plasma membrane facilitates the activation of membrane-associated small G protein Ras by Sos. Activated Ras induces the activation of the Raf kinase that phosphorylates and activates the mitogen-activated protein kinases (MAPKs). The MAPK superfamily of serine/threonine kinase proteins includes the ERKs, the c-Jun terminal kinases (JNKs) and the p38-MAPKs (Marais and Marshall 1996; Johnson et al. 2005; Bianco et al. 2007; Garofalo and Rosa 2008). The PLCγ/PKC pathway is activated through the interaction of EGFR and phospholipase Cγ (PLCγ), that induces hydrolysis of phosphatidylinositol 4,5-diphosphate (PIP2) to give inositol 1,3,5-triphosphate, an important mediator for intracellular calcium release, and 1,2-diacylglycerol, cofactor in protein kinase C (PKC) activation. PKC can activate both MAPK and JNK, which in turn can translocate into the nucleus and phosphorylate transcription factors leading to inhibition of apoptosis and stimulation of cell proliferation (Patterson et al. 2005; Bianco et al. 2007; Garofalo and Rosa 2008).

Aberrant activity of EGFR signaling pathways is associated with cancer development and growth and is initiated by several events, such as altered ligands production, receptor mutations, deletions or persistent activation. High levels of EGFR expression are a common feature of the malignant phenotype in many solid human tumors, and correlate with advanced tumor stage, poor prognosis, increased risk of metastasis, resistance to chemotherapy or hormone therapy (Baselga and Arteaga 2005; Mendelsohn and Baselga 2006; Ciardiello and Tortora 2008). High expression of EGFR ligands in conjunction with increased expression of EGFR may facilitate the development of an autocrine or paracrine growth pathway, contributing to carcinogenesis (Salomon et al. 1995). EGFR overexpression may result from a variety of mechanisms, including increased gene transcription and gene amplification. EGFR gene amplification is a frequent feature of many human cancers, often accompanied by other structural rearrangements that cause in-frame deletions in the extra-

cellular domain of the receptor. The most frequent deleted form of the human EGFR is the type-III variant (EGFRvIII), characterized by a deletion in the extra-cellular domain that leads to constitutive activation of its TK domain: This EGFR genetic alteration frequently occurs in some cancers, like malignant glioblastoma, breast, lung and ovarian carcinomas (Moscatello et al. 1995, Kuan et al. 2001). Besides perturbations in EGFR expression, mutations and ligands production, downstream intracellular signaling pathways under the control of the receptor are frequently altered in tumor cells, ensuring sustained survival, metastatic spread and resistance to either conventional or targeted therapies. Mutations of Ras, PI3K, PTEN or other downstream signaling transducers are often present in human cancers (Shayesteh et al. 1999; Bianco et al. 2003; She et al. 2003; Friday et al. 2005, Bianco et al. 2006).

The role of EGFR-related signal transduction pathways in cancer progression led pharmaceutical companies to devote efforts to the development of EGFR inhibitors, producing remarkable results in several human malignancies such as colorectal carcinoma (CRC), non-small cells lung cancer (NSCLC) and head and neck squamous cell carcinoma (HNSC). The most promising and well studied EGFR inhibitors are monoclonal antibodies (MAbs) that bind the extra-cellular domain of the receptor and compete with endogenous ligands, and small-molecule tyrosine kinase inhibitors (TKIs) that bind the intracellular portion of the receptor, generally by competing with ATP and inhibiting receptor autophosphorylation. They share the same target but display different mechanisms of action and different specificity for the EGFR: In fact, MAbs are exclusively specific, while TKIs are relatively specific for EGFR. Moreover, MAbs are able to induce EGFR internalization, down-regulation, degradation and activation of host immune response via antibody-dependent cell-mediated cytotoxicity (ADCC). These features may contribute to the observed differences in efficacy and toxicity profiles (Hynes and Lane 2005, Imai and Takaoka 2006)). Two anti-EGFR monoclonal antibodies (cetuximab and panitumumab) and two small-molecule, reversible EGFR TKIs (gefitinib and erlotinib) have been approved in several countries for the treatment of various human cancer types, but more than 10 EGFR-targeting agents are actually in advanced clinical development (Baselga and Arteaga 2005; Mendelsohn and Baselga 2006; Ciardiello and Tortora 2008).

Cetuximab (IMC-225, Erbitux) is a chimeric human:murine immunoglobulin G1 (IgG1) MAb that binds to EGFR with higher affinity compared to TGF-α or EGF and is able to promote EGFR internalization and degradation, such inhibiting EGFR-dependent downstream signaling pathways. Cetuximab has a strong antitumor activity based on direct inhibition of tumor cell growth, induction of cell cycle perturbations with G0/G1 arrest, induction of apoptotic cell death, inhibition of angiogenesis, reduction of invasion capabilities and enhancement of radio- and chemo-sensitivity (Peng et al. 1996; Perrotte et al. 1999; Ciardiello et al. 1999). Moreover, the therapeutic efficacy of cetuximab is supported by ADCC and complement activation (Kimura et al. 2007). Cetuximab is the first MAb approved for clinical use in combination

with either chemotherapy or radiotherapy. It has been approved by several regulatory agencies worldwide, including the Food and Drug Administration (FDA) and the European Medicines Evaluation Agency (EMEA), for the treatment of advanced colorectal cancer refractory to irinotecan-based chemotherapy, alone or in combination with irinotecan in the USA or only in combination with irinotecan in the European Union (Galizia et al. 2007). It has been also approved by the FDA in February 2006 for use in combination with radiotherapy to treat patients with locally advanced, unresectable squamous cell carcinoma of the head and neck or as monotherapy for chemorefractory metastatic disease (Ciardiello and Tortora 2008).

Panitumumab (ABX-EGF, Vectibix) is a fully human, high-affinity anti-EGFR MAb, whose antitumor activity is based on blocking ligand-binding and inducing EGFR internalization but not its degradation, suggesting that the receptor can still be recycled to the cell surface (Yang et al. 2001). It has been approved by several regulatory agencies worldwide, including the FDA, as monotherapy for third-line treatment of colorectal cancer that is refractory to fluoropyrimidines, oxaliplatin, or irinotecan. In December 2007, panitumumab has been approved by the EMEA for use in patients with colorectal cancer who carry a normal, wild type K-Ras gene.

Gefitinib (ZD1839, Iressa) is a small quinazoline derivative, reversible inhibitor of EGFR tyrosine kinase activity. The antitumor effect is based on its capability to bind strongly to EGFR without inducing EGFR internalization or degradation and without reducing EGFR protein levels (Baselga and Averbuch 2000; Sirotnak et al. 2000; Ciardiello et al. 2001). After an accelerated approval process, gefitinib has been approved by the FDA in May 2004 for use as third-line treatment of NSCLC refractory to platinum-based and docetaxel-based chemotherapy regimens, but it has been withheld from the USA market since June 2005. Due to the lack of a survival benefit in the ISEL (Iressa Survival Evaluation in Lung Cancer) study (Thatcher et al. 2005), the FDA restricted the use of gefitinib to patients participating in a clinical trial or continuing to benefit from treatment already initiated. Gefitinib has never been approved in the European Union, but is currently on the market in Japan, Korea, China and several other Asian countries. It is currently an investigational drug in the USA and in the European Union. However, an analysis of tumor samples from NSCLC patients who had a response to gefitinib revealed a correlation between the presence of somatic mutations in the EGFR TK domain and response to gefitinib. The most frequent activating mutations identified were in-frame deletions of amino acids 746-750 in exon 19, amino acid substitution leucine to arginine at codon 858 (L858R) and leucine to glutamate at codon 861 (L861Q) in exon 21, and substitution of glycine to cysteine at codon 719 (G719C) in exon 19 (Lynch et al. 2004). First-line therapy with gefitinib administered in a genotype-directed fashion to chemotherapy-naive patients with advanced NSCLC harboring EGFR mutations resulted in very favorable clinical outcomes with good tolerance (Sequist et al. 2008).

Erlotinib (OSI-774, Tarceva) is a small quinazoline derivative, reversible TKI, able to inhibit EGFR autophosphorylation. It has been approved by several regulatory agencies worldwide, including the FDA and the EMEA, as monotherapy for the treatment of locally advanced or metastatic NSCLC refractory to platinum-based chemotherapy (Gridelli et al. 2007). Recently, erlotinib has been approved for use in combination with gemcitabine as first-line treatment for advanced pancreatic cancer (Heeger 2008).

1.2 Tumor angiogenesis as a therapeutic target.

The role of the EGFR autocrine pathway in human cancers is also related to the regulation of tumor angiogenesis, a process that plays a key role in survival of cancer cells, local tumor growth and development of distant metastasis. In fact, the formation of new blood vessels is essential for providing an adequate oxygen and nutrient supply to the growing tumor mass and for initiating metastatic spread. The EGFR partly controls the production of several proangiogenic growth factors, including vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) and metalloproteases (MMPs) (Goldman et al. 1993; Gille et al. 1997; Ciardiello et al. 2006). Besides the indirect effect of EGFR signaling on angiogenesis, several preclinical studies have provided evidence for direct proangiogenic effects of EGFR pathway. We, and others, demonstrated that stimulation of human endothelial cells with EGF or TGF-α induces tube formation and treatment with gefitinib inhibits endothelial cells proliferation, migration and tube formation (Hirata et al. 2002, Bianco et al. 2008). Recently, it has been demonstrated that phosphorylated EGFR expressed on tumor-associated endothelial cells is a primary target for therapy with EGFR TKIs (Kuwai et al. 2008). Therefore, the antitumor effect of EGFR inhibitors is partially mediated by inhibition of tumor angiogenesis and the combination of anti-EGFR drugs with antiangiogenic drugs produces a synergistic effect in inhibition of VEGF expression and microvessels formation in human cancers (Perrotte et al. 1999; Bruns et al. 2000; Ciardiello et al. 2000; Ciardiello et al. 2001; Ellis 2004; Morelli et al. 2006). Moreover, VEGF overexpression is a major escape pathway used by human cancers to acquire resistance to EGFR antagonists (Ciardiello et al. 2004; Bianco et al. 2005; Ciardiello et al. 2006).

VEGF is a major mediator of tumor angiogenesis in human cancers. Its enhanced expression is involved in the “angiogenic switch” and associated with increased neovascularization within the tumor, and it is triggered through different mechanisms, most notably hypoxia (Fontanini et al. 1997; Ferrara and Kerbel 2005; Folkman 2007; Melillo 2007). In mammals, the VEGF family consists of five members: VEGF-A (usually defined as VEGF), VEGF-B, VEGF-C, VEGF-D, and placental growth factor (PlGF). VEGF binds to three distinct VEGF receptors (VEGFRs), tyrosine kinase receptors with an extracellular ligand-binding domain, a trans-membrane domain, a tyrosine

kinase domain, and a downstream carboxy-terminal region: VEGFR-1 (Flt-1), VEGFR-2 (KDR, or the murine homolog Flk-1) and VEGFR-3 (Flt-4). These receptors are expressed on endothelial cells and regulate cell permeability, proliferation and differentiation, as well as on hematopoietic stem cells, osteoblasts and monocytes. VEGFR-2, whose expression appears mostly restricted to vascular endothelial cells, is the major positive signal transducer for both physiological and pathological angiogenesis. Ligand binding induces receptor dimerization and auto-phosphorylation, activating transduction pathways such as the Ras/MAPK and the PLCγ/PKC signaling cascades (Waltenberger et al. 1994; Ferrara et al. 2003; Ferrara and Kerbel 2005; Kowanetz and Ferrara 2006). VEGFR-1 has an higher affinity for VEGF-A, but its specific ligands are VEGF-B and PlGF. However, its tyrosine kinase activity is relatively weak, therefore it doesn't stimulate significantly the proliferation of endothelial cells. Moreover, an alternative spliced, soluble form of VEGFR-1 is an inhibitor of VEGF activity (Ferrara et al. 2003; Ferrara and Kerbel 2005; Kowanetz and Ferrara 2006). Based on these evidences, it was initially proposed that VEGFR-1 might not be primarily a receptor transmitting a mitogenic signal, but rather a "decoy" receptor, able to negatively regulate the activity of VEGF on vascular endothelium, by preventing the binding of VEGF to VEGFR-2 (Park et al. 1994). Further studies demonstrated a synergism between VEGF and PlGF *in vivo*, especially under pathological conditions: In fact, VEGFR-1 may contribute to angiogenesis in ischemic or malignant diseases. This receptor is expressed on both vascular endothelial and macrophage-like cells and may promote inflammation, tumor growth and metastasis (Hiratsuka et al. 2001). The downstream signaling of VEGFR-1 is not fully understood mainly due to the mild biological activity of this receptor in culture: PI3K is one of the candidates responsible for activation and signaling in certain conditions (Ferrara and Kerbel 2005; Kowanetz and Ferrara 2006; Shibuya 2006). VEGFR-3, whose ligands are VEGF-C and -D, regulates lymphangiogenesis and its expression in the adult seems to be largely restricted to lymphatic endothelial cells (Ferrara et al. 2003; Kowanetz and Ferrara 2006).

It has been recently demonstrated that VEGFRs are also expressed in some cancer cells including breast, prostate, ovarian, melanoma, NSCLC, pancreatic and colon cancers (Liu et al. 1995; Strizzi et al. 2001; Hasan and Jayson 2001; Fan et al. 2005; Sini et al. 2008). 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 in some human model cancers: In melanoma (Liu et al. 1995; Byzova et al. 2000), mesothelioma (Strizzi et al. 2001) and human leukemic cells (Masood et al. 2001; Dias et al. 2002), exogenous VEGF stimulates cancer 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 (Lyden et al. 2001). VEGFR-1-dependent induction of matrix MMP-9 expression in premetastatic lung endothelial cells and macrophages has been reported to promote lung metastasis (Hiratsuka et al. 2002). A recent study has shown that VEGFR-1-positive hematopoietic bone marrow progenitors form cellular clusters at tumor-specific premetastatic sites before the arrival of tumor cells and dictate organ-specific tumor spread (Kaplan et al. 2005). Moreover, it has been shown that VEGFR-1 activates ERK1/2, stress-activated protein kinase/c-Jun NH2-terminal kinase (SAPK/JNK) (Fan et al. 2005) and Src family kinases (Lesslie et al. 2006) to mediate growth and migration of human colorectal carcinoma cells. A recent study has shown that activation of VEGFR-1 in breast cancer cells supports their growth and survival (Wu et al. 2006).

Since 1971, when Folkman proposed the antiangiogenesis as a novel anticancer strategy (Folkman 1971), several pharmacologic approaches to inhibit the VEGF axis have been described, and several pre-clinical and clinical studies demonstrated that combining anti-VEGF treatments with chemotherapy or radiotherapy results in greater antitumor effect than either treatment alone. The first antiangiogenic agent approved by the FDA is bevacizumab (Avastin), a humanized variant of an anti-VEGF neutralizing monoclonal antibody: In 2004, bevacizumab was approved for the treatment of previously untreated metastatic colorectal cancer in combination with 5-fluorouracil-based chemotherapy regimens (Ferrara et al. 2004; Hurwitz et al. 2004). It has been also approved in combination with paclitaxel-carboplatin based regimens for the treatment of patients with unresectable, locally advanced, recurrent, or metastatic non-squamous NSCLC (Sandler et al. 2006). On February 22, 2008, the FDA granted accelerated approval for bevacizumab to be used in combination with paclitaxel for the treatment of patients who have not received chemotherapy for metastatic erbB2-negative breast cancer (Miller et al. 2007) and, more recently, for the treatment of advanced renal cell cancer in combination with IFN. Currently, other anti-VEGF agents are at various stages of clinical development (Ferrara et al. 2003; Kowanetz and Ferrara 2006). These include VEGF Trap, a soluble receptor targeting VEGF, VEGF-B, and PlGF, an antisense oligonucleotide, VEGF-AS, targeting VEGF, VEGF-C, and VEGF-D, and an antibody targeting PlGF.

In addition to strategies aimed at blocking VEGF, a variety of small molecule TKIs targeting VEGF receptors signaling pathway including sorafenib (Bay 43-9006, Nexavar), sunitinib (SU11248, Sutent), and vandetanib (ZD6474, Zactima) have been developed. Sorafenib, initially developed as a Raf kinase inhibitor, was later demonstrated to inhibit several RTKs including VEGFRs and to show efficacy in renal cell cancer (Kowanetz and Ferrara 2006). Sunitinib inhibits VEGFRs, PDGFR, c-kit, and Flt-3 and has efficacy in imatinib-resistant gastrointestinal stromal tumor and renal cell carcinoma (Kowanetz and Ferrara 2006). Vandetanib is a quinazoline-derivative able to bind and inhibit EGFR, VEGFR-2 and rearranged during transfection (RET) tyrosine kinases (Wedge et al. 2002; Carlomagno et al.

2002; Ciardiello et al. 2004; Herbst et al. 2007). Since RET activity is important in some types of thyroid cancer, early data with vandetanib in medullary thyroid cancer has led to orphan-drug designation by the regulatory authorities in the USA and European Union. This investigational agent is currently in Phase III development in NSCLC, with Phase II studies continuing to investigate efficacy in other tumor types, including SCLC and thyroid, breast, glioma and prostate cancers. By simultaneously targeting the VEGFR and EGFR signaling pathways, vandetanib may produce greater clinical benefits than targeting either pathway alone. In fact, EGFR inhibition has a direct effect on tumor proliferation and survival, whereas the inhibition of VEGFR signaling in tumor endothelial cells has indirect antitumor effects through inhibition of tumor angiogenesis. Furthermore, EGFR signaling controls both directly (Hirata et al. 2002, Bianco et al. 2008; Kuwai et al. 2008) and indirectly (Goldman et al. 1993; Gille et al. 1997; Ciardiello et al. 2006) tumor angiogenesis, and VEGFRs expression on cancer cells may sustain autocrine loops in some human model cancers (Liu et al. 1995; Byzova et al. 2000; Strizzi et al. 2001; Dias et al. 2002; Fan et al. 2005; Lesslie et al. 2006; Wu et al. 2006). Preclinical and early clinical studies suggest that dual blockade of these pathways may have greater activity than blockade of either pathway alone (Morelli et al. 2006; Hanrahan et al. 2007; Tortora et al. 2008).

1.3 Mechanisms of resistance to EGFR-targeted therapies.

Although the approval for cancer therapy or the advanced clinical development of several EGFR blocking agents, demonstrating their efficacy in some human metastatic diseases, a relevant issue in cancer patients is the development of primary and secondary resistance to the anti-EGFR drugs. Primary or constitutive resistance refers to patients who either do not achieve stable disease or who progress within 6 month after an initial clinical response, whereas secondary or acquired resistance typically occurs after prolonged treatment (Morgillo et al. 2007). However, despite the differentiation between these two mechanisms, it is not possible to define the molecular basis of each type of resistance. Resistance to targeted agents may occur by mechanisms similar to cytotoxic agent resistance, such as inactivating metabolism, poor absorption, reduced drug availability or defective immune system-mediated functions. Actually, most relevant causes of targeted drug resistance are specific mutations or loss of the target, activation of alternative TK receptors that bypass the pathway targeted by the specific agent, independent or constitutive activation of intracellular molecular effectors downstream to the target protein and activation of tumor-induced angiogenesis (Bianco et al. 2005; Morgillo et al. 2007; Tortora et al. 2007).

EGFR mutations were described in various human malignancies: The most extensively characterized is the EGFR variant III (EGFRvIII), containing an in-frame deletion from exons 2 through 7 in the extracellular domain that

prevents the mutated receptor from binding ligands and results in constitutive EGFR activation and resistance to the anti-EGFR drugs (Kuan et al. 2001; Lorimer 2002; Learn et al. 2004). Glioblastoma cell lines expressing this mutated variant EGFRvIII are relatively resistant to gefitinib; higher doses and longer exposure to this agent are necessary to significantly decrease EGFRvIII phosphorylation (Kuan et al. 2001). The protective activity of EGFRvIII may be due to phosphorylation of Akt, which gefitinib is unable to prevent in cells expressing EGFRvIII (Learn et al. 2004). Conversely, for a wide majority of NSCLC patients with EGFR-sensitizing mutations the mechanisms of acquired resistance to TKIs are represented by secondary mutations in the hydrophobic ATP-binding pocket of the catalytic region (T790M or T766M): In fact, the substitution of a threonine with a bulkier amino acid, such as methionine, could sterically interfere with the binding of gefitinib or erlotinib (Pao et al. 2005).

Cancer cells often simultaneously activate TK growth factor receptors of different families, such as insulin-like growth factor receptor-1 (IGF-1R), VEGFRs, and Met, the hepatocyte growth factor (HGF) receptor, leading to activation of redundant and often overlapping signal transduction pathways that impact multiple cell functions. These receptors can maintain cell survival by replacing EGFR function. Particularly, signaling through the IGF-1R is an important alternative cell survival pathway which leads to EGFR inhibitor resistance. For instance, glioblastoma cells with acquired resistance to the EGFR-TKI AG1478 display enhanced IGF-1R levels and sustained signaling through the PI3K/Akt pathway, and the combined targeting of IGF-1R and EGFR greatly enhances apoptosis and reduces the invasive potential of these resistant cells (Chakravarti et al. 2002). Moreover, recent studies demonstrated a heterodimerization of EGFR and IGF-1R as main determinant of erlotinib and gefitinib resistance in NSCLC cell lines (Morgillo et al. 2006; Morgillo et al. 2007). A role in the development of resistance to anti-EGFR drugs has been demonstrated also for the HGF receptor, Met: in NSCLC cell lines and patients, Met amplification caused gefitinib resistance by driving an erbB3-dependent activation of PI3K pathway (Engelman et al. 2007).

Another relevant mechanism of resistance to the anti-EGFR agents is the independent or constitutive activation of signaling pathways downstream to EGFR. Gene amplification, overexpression of downstream effectors such as PI3K/Akt (Shien et al. 2004; Cully et al. 2006), and/or loss or inactivating mutations of negative regulators such as PTEN (Bianco et al. 2003; She et al. 2003), all lead to a persistent activation of the PI3K/Akt and Ras/MAPK pathways and consequent development and maintenance of an EGFR resistant phenotype. Particularly, a hyperactive PI3K/Akt/mTOR pathway has been found in tumor samples from advanced gastric cancer or colorectal cancer patients failing EGFR-targeted therapy. We, and others, demonstrated that targeting this pathway by using mTOR inhibitors such as everolimus (RAD001, Afinitor) and temsirolimus (CCI-779, Torisel) overcomes resistance to EGFR inhibitors and produces a cooperative effect with EGFR inhibitors, providing a valid therapeutic strategy to be tested in a clinical setting (Wang et

al. 2006; Bianco et al. 2008). Loss or reduction of PTEN expression occurs in some advanced cancers including glioblastoma, melanoma, endometrial, breast, ovarian, renal cell, thyroid, and a small subset of NSCLC, and is related to resistance to anti-EGFR drugs. The reconstitution of PTEN in PTEN-null cells is able to repress Akt and to inhibit tumor growth via induction of apoptosis or inhibition of cell proliferation (Bianco et al. 2003; She et al. 2003). The role of Ras/MAPK signaling pathway in determining resistance to EGFR inhibitors is supported by the demonstration that K-Ras mutations are highly specific negative predictors of response to single-agent EGFR TKIs in advanced NSCLC and to anti-EGFR monoclonal antibodies alone or in combination with chemotherapy in patients with metastatic CRC (Amado et al. 2008; Cappuzzo et al. 2008; Linardou et al. 2008). Moreover, MAPK persistent activation is associated with resistance to EGFR inhibitors in NSCLC and breast cancers (Normanno et al. 2006). Among other signaling transducers downstream to EGFR producing a constitutively activated pathway, high levels of expression of Src, a non-receptor tyrosine kinase, correlate with poor prognosis in solid tumors (Dehm and Bonham 2004).

Finally, the activation of EGFR-independent, tumor-induced angiogenesis can be responsible for the development of resistance to anti-EGFR therapies. We, and others, have shown that human cancer cells with acquired resistance to EGFR inhibitors demonstrate over-expression and increased secretion of VEGF (Viloria-Petit et al. 2001; Ciardiello et al. 2004). Human squamous cell carcinomas xenografted in SCID mice and treated chronically with anti-EGFR MAbs eventually develop resistance to these MAbs by increasing expression and secretion of VEGF (Viloria-Petit et al. 2001). We have provided further evidence of the role played by the VEGF-dependent pathway in the resistance to EGFR inhibitors, generating models of human GEO colon cancer resistant to either cetuximab or gefitinib. Analysis of protein expression in samples from mice xenografted with these resistant tumors revealed a 5-10 fold increase in the expression of cyclooxygenase-2 (COX-2) and VEGF as compared with parental EGFR-inhibitor sensitive xenografts (Ciardiello et al. 2004). This notion was confirmed in colon cancer patients failing treatment with cetuximab, in which it has been demonstrated that gene expression levels of COX-2 and VEGF may be useful markers of clinical outcome in single-agent cetuximab treatment (Valbohmer et al. 2005).

Primary and acquired resistance remains the most significant obstacle to the success of EGFR-targeted agents. Therefore, a major priority is the selection of patients that could benefit from an anti-EGFR therapy through the design of predictive tests that identify specific genetic or epigenetic alterations rendering tumors dependent from EGFR signaling. However, while molecular targeted therapy of individual tumors remains the most ambitious goal, another challenge is the need to identify novel, mechanism-based combinations that have the potential to bypass escape mechanisms and overcome resistance to EGFR inhibitors in relatively unselected patient populations, rendering the EGFR survival signaling pathway unable of recovering.

Combined EGFR and VEGF(Rs) targeting constitutes a good example of a promising combination of targeted agents that has already shown to be feasible in a clinical setting. *In vitro* and *in vivo* studies have demonstrated that simultaneous inhibition of EGFR and VEGF/VEGFRs produces antitumor effects in several human cancer models (Wedge et al. 2002; Jung et al., 2002; Morelli et al. 2006). Particularly, we have first demonstrated that an association of cetuximab with a human VEGF antisense 21-mer phosphorothioate oligonucleotide (VEGF-AS) in mice xenografted with human colon cancer cells results in a selective inhibition of neoangiogenesis and in a synergistic tumor growth inhibition (Ciardiello et al. 2000). Moreover, we have provided the first evidence that a single multi-targeted agent directed against EGFR-dependent and VEGF-dependent signaling, vandetanib, could achieve the same results, demonstrating a strong antitumor activity also in colon cancer xenografts resistant to cetuximab and gefitinib (Ciardiello et al. 2004). In this study, we reported that chronic continuous treatment with selective anti-EGFR drugs of human GEO colon cancer cells propagated as s.c. xenografts in athymic mice results in the development of EGFR inhibitor-resistant tumors. In fact, although chronic administration of optimal doses of cetuximab or gefitinib efficiently blocked GEO tumor growth in the majority of mice, tumors slowly started to grow within 80-90 days, despite continuous treatment. This acquired resistance did not seem to be due to a loss in the expression or to a functional alteration of EGFR. When we established GEO tumors growing during treatment with cetuximab or with gefitinib as cell lines (GEO-C225-RES and GEO-ZD1839-RES, respectively), we found that both cells had only a modest reduction in the expression of cell membrane-associated EGFR compared with parental GEO cells. Moreover, EGFR autophosphorylation could be efficiently inhibited by treatment with either cetuximab or gefitinib in both EGFR inhibitor-resistant GEO cell lines, suggesting that a functional EGFR was expressed in these cells. Western blotting revealed no major change in the expression of the EGFR ligand TGF-α, of bcl-2, bcl-xL, p53, p27, MDM-2, Akt, or MAPK. However, both GEO-C225-RES and GEO-ZD1839-RES cells exhibited a 5-10 fold increase in the expression of COX-2 and of VEGF compared with GEO cells, suggesting that a contributing mechanism to GEO tumor growth escape from chronic EGFR inhibition could be an increased angiogenic potential through enhanced endothelial cell proliferation and permeabilization. In contrast to that reported with EGFR inhibitors, continuous treatment of mice bearing established GEO xenografts with vandetanib resulted in efficient tumor growth inhibition for the entire duration of dosing (up to 150 days). Vandetanib activity was also determined in mice pretreated with gefitinib or cetuximab: After 4 weeks of treatment with EGFR inhibitors, when GEO tumors growth was apparent, mice were re-treated with either EGFR inhibitors or vandetanib. GEO tumor growth was blocked only in mice treated with vandetanib, whereas tumor progression was observed in mice re-treated with cetuximab or gefitinib. Importantly, GEO-C225-RES and GEO-ZD1839-RES cell lines growth as xenografts in nude mice was not significantly affected

by treatment with either cetuximab or gefitinib but was efficiently inhibited by vandetanib. This activity seeemed most probably due to the inhibitory effect of vandetanib on VEGF signaling in endothelial cells (Ciardiello et al. 2004). More recently, we demonstrated that IMO, a TLR9 synthetic agonist impairing also EGFR signaling pathway, synergizes with bevacizumab in sensitive and cetuximab-resistant colon cancer xenografts (Damiano et al. 2006; Damiano et al. 2007). On the basis of these encouraging data several clinical studies were initiated. In breast cancer, a small phase II study performed in patients with advanced disease who were heavily pretreated with chemotherapy showed that the combination of bevacizumab and erlotinib is safe and has some antitumor activity (Dickler et al. 2004; Tortora et al. 2008). The activity and safety of the combination of bevacizumab and erlotinib has been also demonstrated in patients with platinum-refractory advanced NSCLC (Herbst et al. 2005; Sandler and Herbst 2006; Herbst et al. 2007). Two phase I studies in patients with recurrent and/or metastatic HNSCC have evaluated the safety and optimum dosage of bevacizumab plus erlotinib in cases heavily pretreated with chemotherapy and radiation therapy (Mauer et al. 2004; Vokes et al. 2006, Tortora et al. 2008). The Bowel Oncology with Cetuximab Antibody (BOND) 2 study demonstrated the antitumor effect and safety of bevacizumab and cetuximab with or without irinotecan in patients with irinotecan-resistant CRC (Saltz et al. 2007; Tortora et al. 2008). The activity of vandetanib was demonstrated in different phase II studies in NSCLC patients. In the first study, vandetanib induced a significant progression-free survival prolongation when compared with gefinitib (Natale et al. 2006), while in the second study it was evaluated as second-line therapy in combination with docetaxel in patients previously treated with chemotherapy (Heymach et al. 2004; Heymach et al. 2007). Therefore, although the therapeutic approach of combined VEGF/VEGFRs and EGFR inhibition is still investigational, the encouraging results from these phase I-II studies pave the way to finding the optimum strategy aimed at blocking EGFR and VEGF(Rs), in order to maximize therapeutic effects and reduce adverse effects in cancer patients.

2. AIM OF THE STUDY

The purpose of this study is to examine the signaling mechanisms operating in human cancers with resistance to EGFR-targeted therapies and responsible for the lack of response to EGFR inhibitors. In fact, the constitutive resistance in a large number of patients and the development of acquired resistance in the responders represent relevant issues for the clinical utility of this class of targeted agents. Previous studies from my laboratory and from other groups demonstrated that the combined inhibition of EGFR and other signaling proteins could be a successful approach to efficiently inhibit compensatory escape pathways and overcome both intrinsic and acquired resistance to anti-EGFR drugs. This evidence led our group to search for further determinants of resistance as basis for novel therapeutic strategies.

To this aim, we identified human cancer cell lines with different levels of EGFR expression and sensitivity to EGFR inhibitors. We also generated human cancer cell lines with acquired resistance to anti-EGFR drugs through continuous treatment of tumor xenografts with cetuximab or gefitinib for 14 weeks, followed by excision of tumors and establishment of the derived cell lines *in vitro*. In fact, in a previous study we reported that chronic continuous treatment with selective anti-EGFR drugs of human colon cancer xenografts resulted in the development of EGFR inhibitor-resistant tumors, and that growth of the resistant tumors was efficiently inhibited by the multitargeted agent vandetanib. Since vandetanib is a potent inhibitor of the VEGFR-2 tyrosine kinase, we hypothesized that this activity could be due to the inhibitory effect on VEGF signaling in endothelial cells (Ciardiello et al. 2004). The EGFR inhibitor-resistant cancer cells used in our former study were generated by *in vivo* selection; however, their resistant phenotype tended to weaken after several *in vitro* passages, even in the continued presence of cetuximab or gefitinib. Therefore we generated new models of stable resistance to EGFR inhibitors through both *in vivo* selection and *in vitro* establishment of the derived cell lines.

In the present study, we used the multitargeted agent vandetanib as an investigational tool to elucidate the molecular basis of resistance to anti-EGFR drugs. Therefore, we analyzed in the resistant cancer cells signal transducers operating under the control of EGFR-dependent and -independent pathways, and studied their potential involvement in EGFR-inhibition escape mechanisms.

3. MATERIALS AND METHODS

Drugs. Vandetanib and gefitinib were kindly provided by Dr Anderson Ryan (AstraZeneca Pharmaceuticals Ltd, Macclesfield, UK). Cetuximab was supplied by ImClone Systems (New York, NY, USA).

Cell lines. HUVEC (human umbilical vein endothelial cells), human GEO (colon carcinoma), PC3 (hormone-refractory prostate adenocarcinoma), MDA-MB-468 (mammary gland carcinoma) and SW480 (colon carcinoma) cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA). GEO-CR (cetuximab resistant), GEO-GR (gefitinib resistant), and PC3-GR (gefitinib resistant) cells were established as previously described (Ciardiello et al. 2004). In contrast to previous EGFR-inhibitor resistant cancer cells (Ciardiello et al. 2004), the resulting cell lines were stably resistant to EGFR inhibitors retaining a resistant phenotype even after several *in vitro* passages in absence of EGFR antagonists. All cell lines were cultured as previously described (Ciardiello et al. 2004).

Growth in soft agar. Cells (10^4 cells/well) were suspended in 0.3% Difco Noble agar (Difco, Detroit, MI) supplemented with complete medium, layered over 0.8% agar-medium base layer and treated with different concentrations of gefitinib, cetuximab and vandetanib. After 10–14 days, cells were stained with nitro blue tetrazolium (Sigma Chemical Co., Milan, Italy) and colonies >0.05 mm were counted (Ciardiello et al. 2001).

RNA interference. Small interfering RNA (siRNA) Kits (Validated Stealth™ for EGFR and Select Stealth™ for VEGFR-1/Flt-1 and VEGFR-2/KDR) were obtained from Invitrogen Life Technologies (Grand Island, NY, USA). A nonsense sequence was used as a negative control. For siRNA validation, cells were seeded into 60 mm dishes and transfected with 40 nM EGFR siRNA, 120 nM 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 performed. 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-multiwell cluster dishes and transfected with EGFR, VEGFR-1 or VEGFR-2 siRNA. Twenty-four hours after transfection cells treated with VEGFR-1 or VEGFR-2 siRNA received cetuximab 140 nM or gefitinib 5 μM and cell survival was determined 24 hours later.

Transfection. PC3 or SW480 cells were transiently transfected with pcDNA3/hFlt-1 or with pcDNA3 as negative control using the specific Cell

Line Nucleofector Kit V for PC3 or SW480 (Amaxa, Cologne, Germany). Briefly, 1×10^6 cells were transfected with 5 μg of DNA. To confirm VEGFR-1 expression, cells were plated in 6-multiwell cluster dishes and a Western Blot analysis was performed forty-eight hours after transfection; for activity experiments 3×10^4 cells were plated in each well of 24-multiwell cluster dishes, twenty-four hours after transfection they received gefitinib 1, 2.5 or 5 μM and cell survival was determined 48 hours later.

Cell survival assay. The culture supernatant was removed and 100 μl MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma) stock solution (5 $mg \cdot ml^{-1}$) was added to each well together with 400 μl of medium. After 4 hours of incubation, isopropanol was added and the absorbance 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.

Kinase assays. Enzyme inhibition by vandetanib was determined by ProQinase GmbH (Freiburg, Germany). IC_{50} values were calculated from 12 point dose-response curves (10^{-4} M to $3x10^{-9}$ M). All protein kinases were expressed in Sf9 insect cells as human recombinant GST-fusion proteins or His-tagged proteins by means of the baculovirus expression system. Kinases were purified by affinity chromatography using either GSH-agarose (Sigma) or Ni-NTH-agarose (Qiagen, Milan, Italy). 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 5 steps in the following order: 20 μl of assay buffer; 10 μl of substrate (in H_2O); 5 μl of vandetanib in 10% DMSO; 10 μl of enzyme solution; 5 μl 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; 100 ng PDGFRβ. After an incubation at 30° C for 80 minutes, the reaction was stopped with 2 % (v/v) H_3PO_4. All assays were performed with a BeckmanCoulter Biomek 2000 robotic system. Incorporation of ^{33}P was determined with a microplate scintillation counter (Microbeta, Wallac). IC_{50} values were calculated using Prism 4.03 for Windows (Graphpad, San Diego, California, USA).

Immunoprecipitation and Western blot analysis. Cell protein extracts were prepared from tumor or endothelial cells cultured for 24 hours in the presence or absence of 1 μM gefitinib, 1 μM vandetanib, or 7 nM cetuximab (Ciardiello et al. 2001). Protein extracts were resolved by a 4–20% SDS-PAGE and probed with one of the following antibodies: anti-EGFR (Ab-12, NeoMarkers, Fremont, CA); anti-phospho-(Tyr1173)-EGFR, anti-p70S6K, anti-phospho-p70S6K, anti-VEGFR-2/KDR (Upstate, Billerica, MA); anti-Akt, anti-phospho-(Ser473)-Akt (Cell Signaling, Danvers, MA); anti-ERK1-2, anti-phospho-ERK1-2, anti-PTEN, anti-VEGF, anti-VEGFR-1/Flt-1, anti-Met, anti-

phospho-Met (Santa Cruz Biotechnology, Santa Cruz, CA) and anti-actin (Sigma-Aldrich, Milan, Italy). Phosphorylated VEGFR-2 and VEGFR-1 were detected by immunoprecipitation of cell proteins with anti-VEGFR-2 or anti-VEGFR-1 antibody, resolved by a 7.5% SDS-PAGE gel and probed with the PY20 anti-phospho-tyrosine mAb (Upstate, Billerica, MA). Immunoreactive proteins were visualized by enhanced chemiluminescence (Amersham International, London, United Kingdom).

Determination of VEGF and PlGF concentrations. The concentrations of VEGF and PlGF in conditioned medium from tumor cell lines was determined by ELISA, as previously described (Errico et al. 2004). The absorbance was measured at 490 nm on a microplate reader (Bio-Rad, Hercules, CA) and VEGF and PlGF concentrations were determined by interpolation of the standard curve using linear regression analysis.

Reverse transcription (RT)-PCR. Total RNA was isolated from cells using the Trizol reagent from Invitrogen Life Technologies (Grand Island, N.Y., USA). RT was performed using reverse transcriptase (Supertranscript RT, Life Technologies, Inc., Gaithersburg, MD). To evaluate VEGFR-1/Flt-1, VEGFR-2/KDR and VEGF gene expression, aliquots of RT-RNA were amplified using primers designed on the basis of the coding sequences of the human mRNA (Fan et al. 2005). PCR products were visualized using ethidium bromide on a 1.8% agarose gel. Human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) 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 pre-coated with 50 µl/well of serum-free medium containing 0.1%. BSA or different dilutions of matrigel in cell culture medium. After 1 hour, all coating solutions were removed and 2×10^4 cells/well were plated in serum-free medium. Following incubation for 1 hour at 37°C in 5% CO_2, cells were fixed and stained with a formalin/ethanol/crystal-violet fixing/staining solution, washed extensively, air-dried, and the dye was eluted with ethanol/acetic acid solution. The readings were performed at 595 nm and the values were normalized to background adhesion (Benelli et al. 2003).

Cell migration assay. Cell migration was investigated using the Boyden chamber chemotaxis assay (Albini and Benelli 2007). Polycarbonate filters (8-µm pore size, PVP-free from Costar-Nuclepore) were manually coated with 50 µl of a 0.1 mg/ml solution of collagen type IV and dried overnight at room temperature. VEGF was used as chemoattractant, while serum-free medium containing 0.1% BSA was used as 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 hours, 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, Milan, Italy) 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 μl pipette tip and then cultured in the absence or presence of doxorubicin (25 ng/ml), vandetanib (2.5 μM), gefitinib (5 μM), VEGFR-1 or VEGFR-2 siRNA, or with a nonsense RNA sequence (all 120 nM) for 24 hours. Under these conditions, all drugs except doxorubicin weakly inhibited cell proliferation. Since doxorubicin did not interfere with cell migration, it was used as a negative control. The wounds were photographed (10× objective) at 0, 8, 24 or 48 hours (Bennett et al. 2007) and healing was quantified by measuring the distance between the edges using Adobe Photoshop (v. 8.0.1; Adobe System Inc.). The results are presented as the percentage of the total distance of the original wound enclosed by cells. A survival assay was performed 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, Los Angeles, CA)

4. RESULTS AND DISCUSSION

EGFR inhibition contributes to the antiangiogenic effect of vandetanib.

Combined targeting of EGFR and VEGF(Rs) signaling cascades has been identified as a therapeutic approach effective in inhibiting tumor growth and angiogenesis, and already feasible in a clinical setting. In a previous study, we have provided the first evidence that a single multi-targeted agent directed against EGFR-dependent and VEGF-dependent signaling, vandetanib, was able to inhibit tumor growth in mice xenografted with tumors resistant to EGFR inhibitors (Ciardiello et al. 2004). Since the activation of tumor-induced angiogenesis has been described as one of the mechanisms responsible for the development of resistance to anti-EGFR therapies, we first hypothesized that the *in vivo* effect of vandetanib on resistant tumors might depend on inhibition of tumor angiogenesis, probably due to the interference with VEGF signaling in endothelial cells, more than on a direct antitumor effect.

The role of the VEGFRs in the regulation of endothelial cell permeability, proliferation and differentiation in both physiological and pathological conditions has been widely demonstrated (Ferrara et al. 2003; Ferrara and Kerbel 2005; Kowanetz and Ferrara 2006). Moreover, it has been recently reported that EGFR signaling directly controls tumor angiogenesis. In fact, stimulation of human endothelial cells with EGF or TGF-α induces tube formation and treatment with gefitinib inhibits endothelial cells proliferation, migration and tube formation (Hirata et al. 2002, Bianco et al. 2008). Phosphorylated EGFR expressed on tumor-associated endothelial cells has been identified as a primary target for therapy with EGFR TKIs (Kuwai et al. 2008). By simultaneously targeting VEGFR-2 and EGFR signaling pathways, vandetanib may produce a greater antiangiogenic effect than targeting either pathway alone. Based on this evidence, we evaluated the contribution of EGFR signaling to vandetanib activity on human umbilical vein endothelial cells (HUVEC). We first compared the effects of erlotinib and vandetanib on endothelial cells survival. As shown in figure 1A, vandetanib is more effective than erlotinib in affecting HUVEC cells survival, most likely due to inhibition of both VEGFR-2 and EGFR tyrosine kinases. We then analyzed vandetanib effects on EGFR signaling in endothelial cells. We found that vandetanib is able to reduce the EGF-induced phosphorylation/activation of EGFR and of its downstream transducers Akt and MAPK (Fig.1B). Therefore, we demonstrated that inhibition of EGFR signaling in endothelial cells play a key role in the antiangiogenic effect of vandetanib.

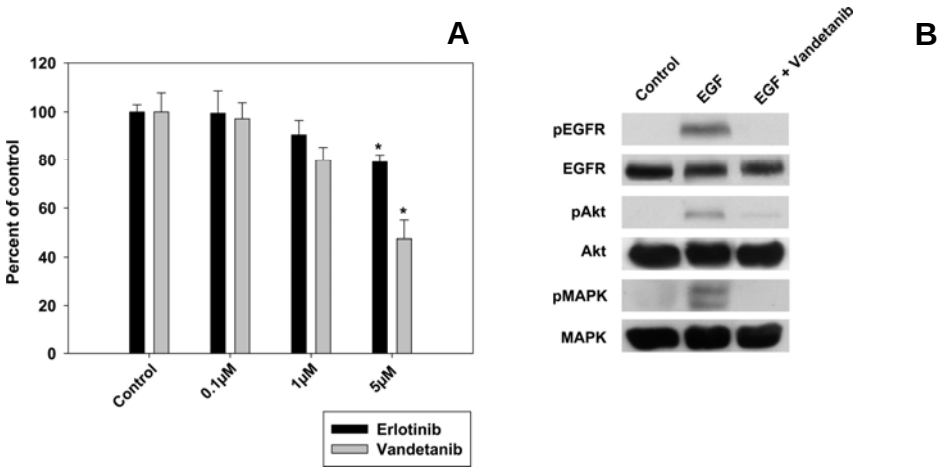

Figure 1. Effects of erlotinib or vandetanib on HUVEC endothelial cells survival and EGFR-dependent signaling. (**A**) Percent of survival of HUVEC cells treated with erlotinib or vandetanib 0.1, 1 or 5 μM. Results for each treatment are presented relative to untreated control cells. *, 2-sided P<0.001 versus control. Bars, SD. (**B**) Western blot analysis of protein expression in HUVEC cells cultured in serum-free medium, treated with vandetanib 1 μM for 24h and stimulated with EGF for 15 minutes before protein extraction.

Human cancer cell lines resistant to EGFR inhibitors are sensitive to vandetanib.

In order to investigate resistance to EGFR inhibitors, we identified human cancer cell lines with different levels of EGFR expression and sensitivity to the anti-EGFR drugs cetuximab and gefitinib. MDA-MB-468 human breast cancer cells express high EGFR levels, yet their growth is relatively resistant to gefitinib (Bianco et al. 2003) and resistant to cetuximab at high doses (up to 140 nM) (Fig.2A-B). This constitutive resistant phenotype is associated with PI3K/Akt hyperactivity, in turn related to mutation of the PTEN gene. GEO human colorectal cancer cells express lower EGFR levels and are sensitive to both cetuximab and gefitinib (IC_{50} <3.5 nM and 0.5 μM, respectively). Compared with GEO cells, PC3 cells demonstrate similar EGFR expression levels and sensitivity to gefitinib but are resistant to cetuximab (up to 140nM). Despite similar sensitivity to gefitinib, GEO cells have a functional wild-type PTEN gene, whereas PC3 have a deleted PTEN.

We also generated human cancer cell lines with acquired resistance to anti-EGFR drugs through continuous treatment of tumor xenografts with cetuximab or gefitinib for 14 weeks, followed by excision of tumors and establishment of the derived cell lines *in vitro*. We demonstrated that the EGFR inhibitor-resistant cell lines established in this study are insensitive to cetuximab (GEO-CR) and gefitinib (GEO-GR, PC3-GR) at doses up to 560 nM and 20 μM, respectively (Fig.2A-B). They exhibited a morphology, *in vitro*

growth rate, and soft agar cloning efficiency similar to that of parental cells (data not shown).

To verify whether the *in vivo* effect of vandetanib on resistant tumors might depend not only on inhibition of tumor angiogenesis, but also on a direct antitumor effect, we tested the *in vitro* sensitivity of resistant cells to vandetanib. As shown in figure 2C, vandetanib efficiently inhibited soft agar growth of all cell lines (IC_{50} 0.1-0.5 μM), irrespective of their EGFR inhibitor sensitivity. Therefore, vandetanib activity on resistant tumors was based not only on indirect antitumor effects through endothelial cells targeting, but also on a direct effect on cancer cells.

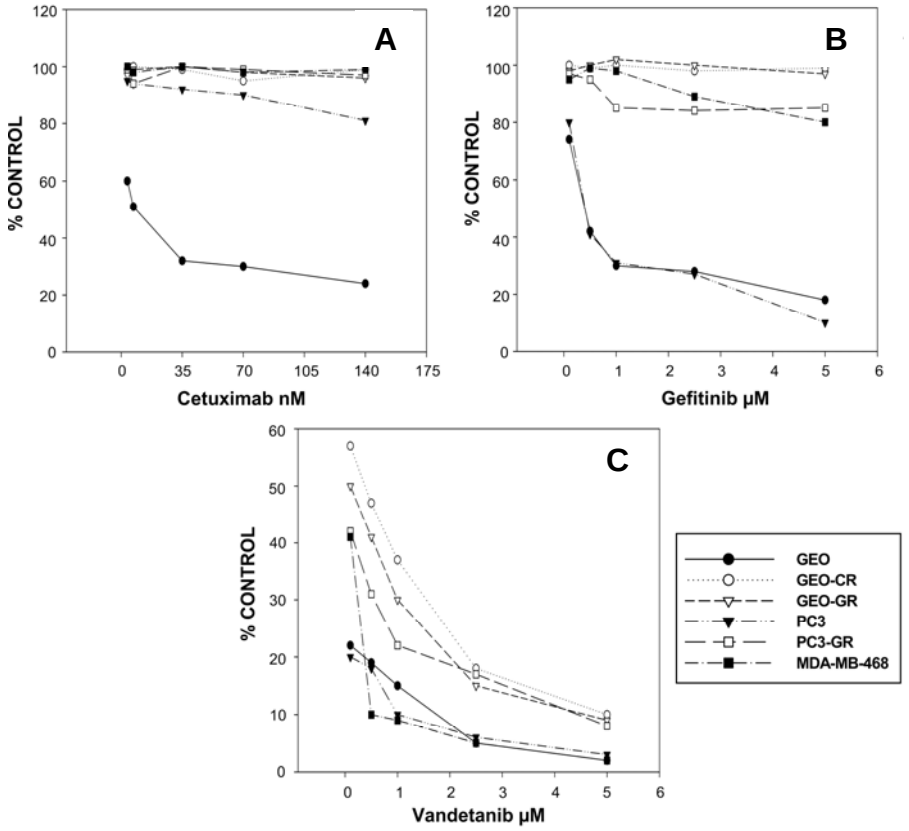

Figure 2. Effects of cetuximab, gefitinib, or vandetanib on growth of human cancer cell lines. GEO, GEO-CR, GEO-GR, PC3, PC3-GR and MDA-MB-468 cells were grown in soft agar and treated with the indicated concentrations of cetuximab (**A**), gefitinib (**B**) or vandetanib (**C**) each day for 3 consecutive days. Colonies were counted after 10-14 days. Data represent the mean (±SD) of three independent experiments, each performed in triplicate, and are presented relative to untreated control cells; while the effects of vandetanib were statistically significant versus control in all cell lines (2-sided $P<0.0001$), the effects of gefitinib and cetuximab treatment were statistically significant versus control in sensitive cell lines (2-sided $P<0.0001$), but not in EGFR-inhibitor resistant cell lines.

Resistance to EGFR inhibitors correlates with activation of downstream signaling pathways via EGFR-independent mechanisms.

Vandetanib is a potent inhibitor of VEGFR-2, EGFR and RET tyrosine kinases. It's a common opinion that the direct effect of vandetanib on tumor cells is due to EGFR and RET inhibition, while VEGFR-2 inhibition accounts for indirect antitumor effect through endothelial cells targeting. In this study, we demonstrated not only that vandetanib activity on endothelial cells is mediated by both VEGFR-2 and EGFR inhibition, but also that the *in vitro* effect of vandetanib on resistant cells is not exclusively related to EGFR inhibition. In fact, in our resistant cells, the EGFR inhibition was achieved also after treatment with the anti-EGFR drugs cetuximab and gefitinib, that are totally ineffective in inhibiting cell growth. Treatment of wild-type GEO and PC3 cells with EGFR inhibitors strongly reduced phosphorylation of EGFR, and consequently of the downstream effectors Akt and MAPK. Similarly, vandetanib inhibited EGFR phosphorylation in both cell lines and caused an almost complete down-regulation of pAkt and pMAPK (Fig.3). In MDA-MB-468 cells, gefitinib decreased the levels of pEGFR and pMAPK without any change in Akt phosphorylation; in contrast, vandetanib inhibited phosphorylation of EGFR, MAPK and Akt. 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. Similar results were observed in GEO-GR and PC3-GR cells comparing the effects of vandetanib and gefitinib (Fig.3). Since Akt is one of the major positive regulators of mTOR/p70S6 kinase (p70S6K) activity, we then analyzed downstream effectors of the Akt/mTOR-dependent pathway. In wild-type GEO and PC3 cells, treatment with gefitinib, cetuximab and vandetanib reduced p70S6K phosphorylation (Fig.3), consistent with the parallel decrease in Akt phosphorylation. Conversely, in resistant cell lines a reduced p70S6K phosphorylation was observed only following vandetanib treatment (Fig.3). In summary, both vandetanib and anti-EGFR drugs inhibited EGFR and MAPK phosphorylation, but only vandetanib inhibited phosphorylation of Akt kinase and of its effector p70S6K in resistant cells.

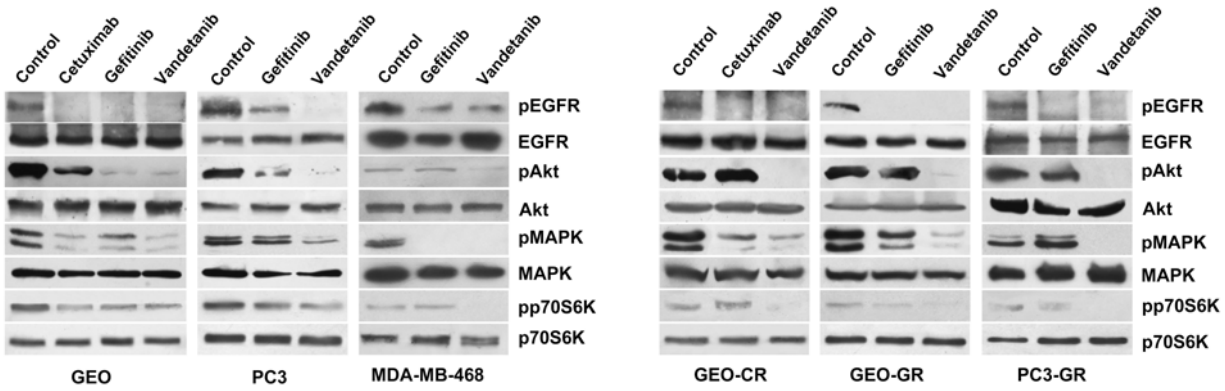

Figure 3. Effects of cetuximab, gefitinib and vandetanib on EGFR-dependent signaling pathways in human cancer cell lines sensitive or resistant to EGFR inhibitors. Western blot analysis of protein expression in cell lines treated with cetuximab 7 nM, gefitinib 1 μM or vandetanib 1 μM for 24 hours prior protein extraction.

To further verify that EGFR inhibition is not sufficient to inhibit growth of resistant cells, we also achieved EGFR gene silencing using an EGFR specific RNA interference which completely suppressed EGFR expression (Fig.4A). In PC3-GR cells, this small interfering RNA (siRNA) was unable to reduce phospho-Akt and phopsho-MAPK levels, similarly to that observed after gefitinib tretament. 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.4B).

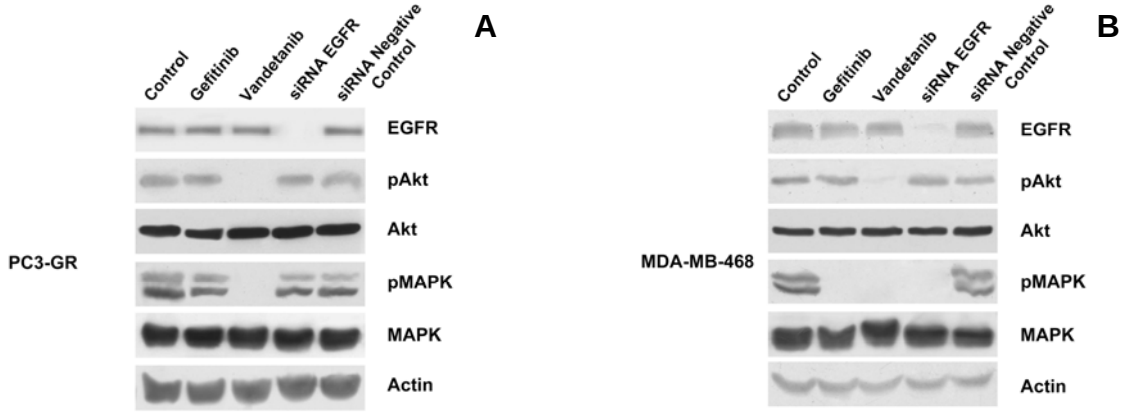

Figure 4. Effects of EGFR silencing via siRNA on EGFR-dependent signaling in human cancer cell lines sensitive or resistant to EGFR inhibitors. Western blot analysis of protein expression in PC3-GR (**A**) and MDA-MB-468 (**B**) cells 24 hours after treatment with 1 μM gefitinib or 1 μM vandetanib, and 48 hours after transfection with EGFR specific siRNA or with a nonsense RNA sequence used as a negative control (both 40 nM).

We also evaluated the effects of EGFR silencing on sensitive and resistant cancer cells survival. Importantly, the EGFR-specific siRNA markedly reduced cell survival (approximately 50%) in wild-type PC3 cells, whereas no effect was seen in PC3-GR and MDA-MB-468 resistant cells (Fig.5).

Therefore, in the present study, treatment with gefitinib and cetuximab, as well as EGFR silencing via siRNA, were able to induce cell growth arrest only if EGFR inhibition was coupled with down-regulation of pAkt. This effect was not detected in resistant cell lines, in which inhibition of EGFR and Akt phosphorylation was only observed following vandetanib administration. This suggests that EGFR inhibition *per se* is not sufficient to induce growth perturbations in resistant cells, and that inhibition of Akt phosphorylation/activation may be the most closely associated with significant growth inhibition.

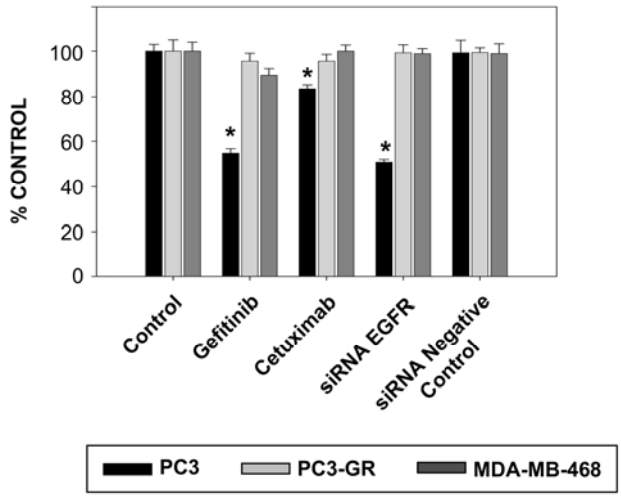

Figure 5. Effects of EGFR silencing via siRNA on survival of human cancer cell lines sensitive or resistant to EGFR inhibitors. Percent of survival of PC3, PC3-GR and MDA-MB-468 cells treated with gefitinib 5 μM, cetuximab 140 nM, EGFR targeting siRNA or a nonsense RNA sequence (both 40 nM). Results for each treatment are presented relative to untreated control cells. *, 2-sided P<0.0001 versus control and versus negative control. Bars, SD.

Resistance to EGFR inhibitors does not strictly depend on PTEN mutational status.

Constitutive activation of the PI3K/Akt pathway is commonly reported in human cancers (Shayesteh et al. 1999; Cully et al. 2006), and appears to correlate with the response to EGFR inhibitors (Shien et al. 2004). It has been previously demonstrated that inactivating mutations or loss of PTEN could result in constitutive activation of oncogenic signals through Akt, and are

associated with resistance to EGFR TKIs (Bianco et al. 2003; She et al. 2003). In fact, the lack of a functional PTEN in MDA-MB-468 cells leads to increased PI3K/Akt activity and resistance to gefitinib (Bianco et al. 2003). Also PC3 cancer cells lack a functional PTEN protein, having a deleted PTEN gene; 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 cell lines established in this study: Western blot analysis of GEO-CR, GEO-GR and PC3-GR cells did not reveal any difference in PTEN expression (Fig.6).

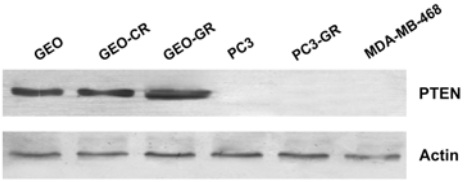

Figure 6. Analysis of PTEN expression in human cancer cell lines sensitive or resistant to EGFR inhibitors. Western blot analysis of PTEN expression in cell lines.

Human cancer cell lines resistant to EGFR inhibitors express VEGF receptors.

The apparent independence of Akt activity from EGFR activation and the pattern of resistant cancer cells sensitivity to vandetanib suggest the activation of other TKRs in the EGFR inhibitor-resistant cells, highlighting the role that alternative signaling pathways may play in resistance to EGFR antagonists.

Alternative signaling pathways that circumvent the inhibition of EGFR are often activated in cancer cells, a key example being IGF-1R and Met signaling. The association between IGF-1R over-activity and acquired resistance to EGFR blockade has been demonstrated for glioblastoma multiforme, breast, prostate and lung cancer (Chakravarti et al. 2002; Morgillo et al. 2006; Morgillo et al. 2007). However, in our resistant cell lines no altered expression of IGF-1R has been detected (data not shown). Recently, Engelman et collaborators (Engelman et al. 2007) 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 (Fig.7).

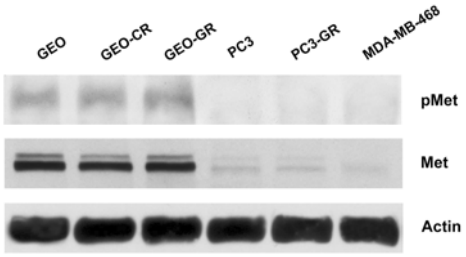

Figure 7. Analysis of pMet and Met expression in human cancer cell lines sensitive or resistant to EGFR inhibitors. Western blot analysis of pMet and Met expression in cell lines.

Since the *in vitro* effect of vandetanib on resistant cells does not depend only on EGFR inhibition, other vandetanib targets may be expressed by tumor cells, activating escape signaling pathways able to circumvent the EGFR inhibition. Vandetanib is able to inhibit, in addition to EGFR, the tyrosine kinase activity of VEGFR-2 (Wedge et al. 2002) and RET (Carlomagno et al. 2002); we therefore investigated the expression of these receptors in our cancer 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.8A).

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 we observed that it was increased compared with parental cells, both at protein and mRNA levels (Fig.8B). Since VEGFR-1 expression 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.

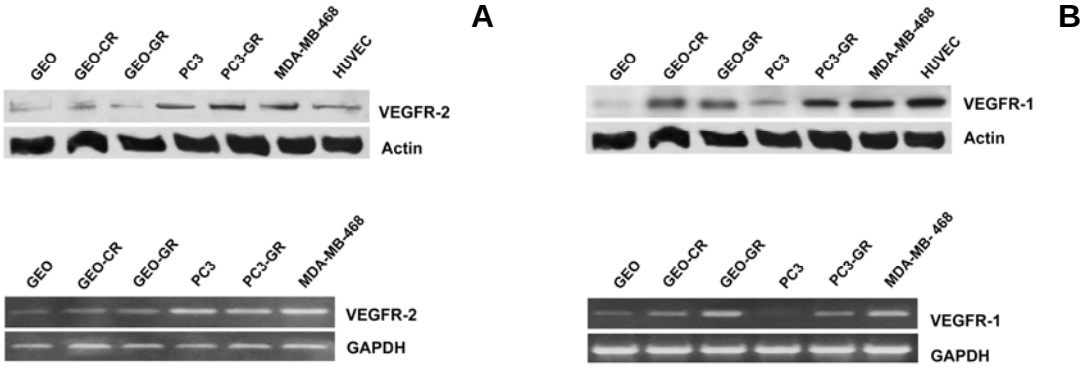

Figure 8. Analysis of VEGFRs expression on human cancer cell lines sensitive or resistant to EGFR inhibitors. (**A**) Analysis of VEGFR-2/KDR protein and mRNA expression in cell lines using Western blot (upper panel) and PCR (lower panel), respectively. (**B**) Analysis of VEGFR-1/Flt-1 protein and mRNA expression in cell lines using Western blot (upper panel) and PCR (lower panel), respectively. HUVEC were used as a positive control.

Therefore we investigated the involvement of VEGFRs in vandetanib activity on resistant cells, evaluating the effect of vandetanib on VEGFR-1 and VEGFR-2 autophosphorylation. We observed a strong inhibition of both receptors in GEO-CR, GEO-GR and PC3-GR cells (Fig.9A-B).

Figure 9. Analysis of VEGFRs activity on human cancer cell lines sensitive or resistant to EGFR inhibitors. (**A**) Inhibition of VEGFR-2/KDR autophosphorylation in GEO-CR, GEO-GR and PC3-GR cells treated for 24 hours with 1 μM vandetanib. (**B**) Inhibition of VEGFR-1/Flt-1 autophosphorylation in GEO-CR, GEO-GR and PC3-GR cells treated for 24 hours with 1 μM vandetanib.

To confirm vandetanib capability of inhibiting also VEGFR-1, we performed a kinase assay with a new kit to define the IC_{50} values for VEGFR-1, -2, -3, EGFR and PDGFRβ. As summarized in Table 1, vandetanib exhibited a much broader inhibitory activity than reported before (Wedge et al. 2002), since it efficiently inhibited also VEGFR-1 (IC_{50} 150 nM). Moreover, the ability to inhibit EGFR resulted almost comparable to VEGFR-2 (IC_{50} 43 nM versus 38 nM, respectively). Finally, to a lesser extent, vandetanib inhibited also VEGFR-3 (IC_{50} 260 nM). These data are in agreement and extend the results formerly reported by a different group (Manley et al. 2002), enhancing the multitargeting profile of vandetanib and providing a clue to its inhibitory activity on resistant cells overexpressing VEGFR-1.

Table 1
Kinase inhibition by vandetanib

Kinase	IC_{50} (nM)
VEGFR-1	150
VEGFR-2	38
VEGFR-3	260
EGFR	43
PDGFRβ	5300

Interestingly, all resistant cell lines synthesized and secreted the VEGFR ligands VEGF and PlGF. VEGF mRNA expression was slightly elevated in all resistant cell derivatives as compared to parental cell lines (Fig.10A). ELISA assays confirmed that both factors are consistently produced in all cell lines, with VEGF levels considerably higher than PlGF levels, and that their secretion is higher in conditioned medium derived from resistant cells compared to parental cells (Fig.10B-C).

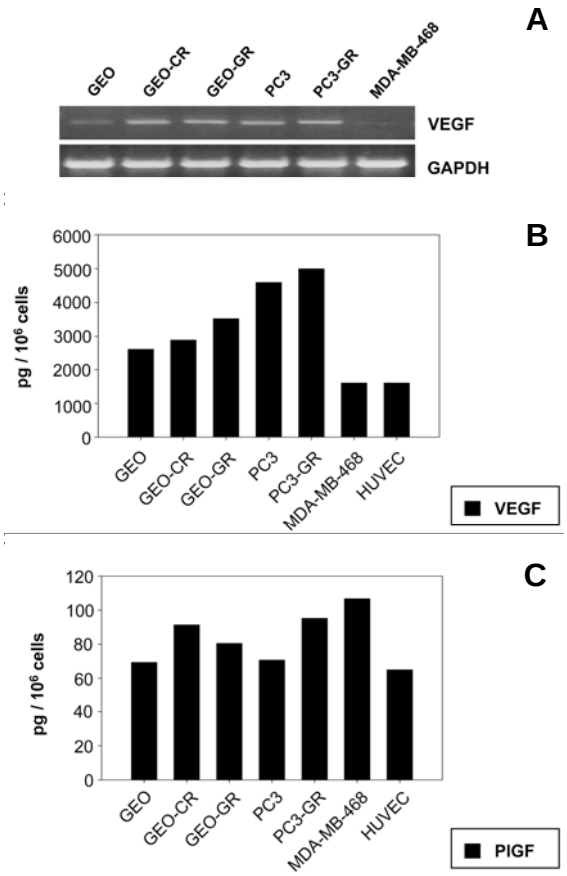

Figure 10. Analysis of VEGF and PlGF production by human cancer cell lines sensitive or resistant to EGFR inhibitors. (**A**) VEGF mRNA levels measured by PCR analysis in cell lines. Concentrations of secreted VEGF (**B**) and PlGF (**C**) in conditioned medium from each cell line were determined by ELISA. HUVEC were used as a positive control.

VEGFR-1 silencing partially restores sensitivity to EGFR antagonists.

To further demonstrate VEGFRs 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. We verified that transfection with VEGFR-1 or VEGFR-2 siRNA for 48 hours partly reduced the respective target protein expression (Fig.11A-B).

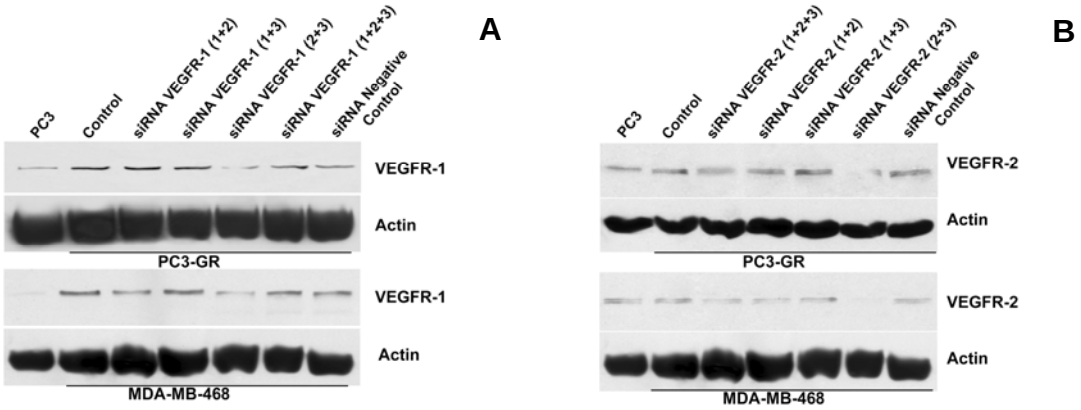

Figure 11. VEGFRs silencing in human cancer cell lines resistant to EGFR inhibitors. Western blot analysis of VEGFR-1/Flt-1 (**A**) or VEGFR-2/KDR (**B**) in PC3-GR and MDA-MB-468 cells transfected with 120 nM VEGFR-1- or VEGFR-2- targeting siRNA, respectively, or with a nonsense RNA sequence (negative control).

Therefore, we verified the effects of VEGFR-1 silencing on signal transducers activation. Intriguingly, in PC3-GR cells VEGFR-1 protein reduction through siRNA restored the ability of gefitinib to inhibit Akt and MAPK phosphorylation/activation (Fig.12).

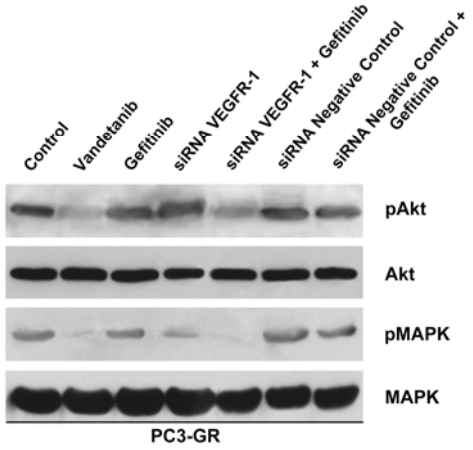

Figure 12. Effects of VEGFRs silencing on EGFR-dependent signaling in human cancer cell lines resistant to EGFR inhibitors. Western blot analysis of protein expression in PC3-GR cells 24 hours after treatment with 1 μM vandetanib or 1 μM gefitinib, and 48 hours after transfection with 120 nM VEGFR-1 targeting siRNA or with a nonsense RNA sequence (negative control).

Moreover, 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.13A-B). The degree of re-sensitization was approximately 35% in both cell lines. The reduction of VEGFR-2 expression restored sensitivity to EGFR inhibitors to a lesser extent (about 15%) (Fig.13A-B). Therefore, VEGFRs, particularly VEGFR-1, appear to play a role in the resistance to EGFR inhibitors, which, in turn, correlates with Akt kinase activation via EGFR-independent mechanisms.

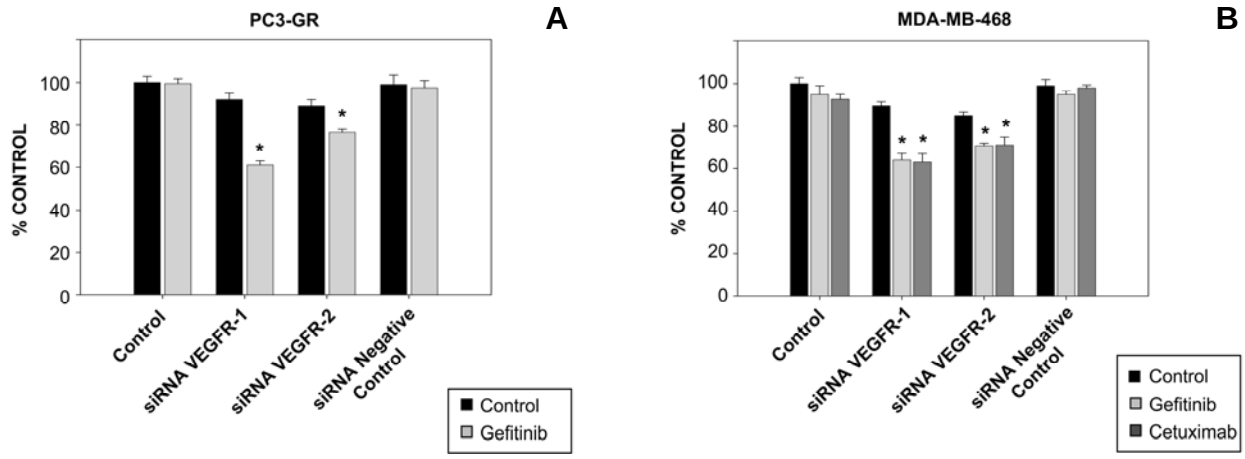

Figure 13. Effects of VEGFRs silencing on survival of human cancer cell lines resistant to EGFR inhibitors. Cell survival analysis of the VEGFR-1 siRNA transfected PC3-GR (**A**) or MDA-MB-468 (**B)** cells in the presence or absence of gefitinib 5 μM or cetuximab 140 nM. Results for each treatment are presented relative to untreated control cells. *, 2-sided P<0.0001 versus control and versus negative control. Bars, SD.

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, another EGFR-expressing cell line (Ciardiello et al. 2001), and investigated whether VEGFR-1 could confer resistance to gefitinib. In spite of the suboptimal transfection efficiency (about 50% of cells), an increase of VEGFR-1 expression was observed 48 hours after transfection in both cell lines (Fig.14A-B).

Figure 14. VEGFR-1 overexpression in human cancer cell lines sensitive to EGFR inhibitors. Western blot analysis of VEGFR-1/Flt-1 in PC3 cells (**A)** and in SW480 cells (**B**) transfected with pcDNA3/hFlt-1 or pcDNA3 as negative control. HUVEC were used as a positive control.

Importanlty, VEGFR-1 overexpression 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.15A-B). Conversely to non-transfected cells, VEGFR-1 overexpressing cells appeared totally insensitive to low doses of gefitinib and their survival was only slightly inhibited by high doses of this drug (Fig.15A-B).

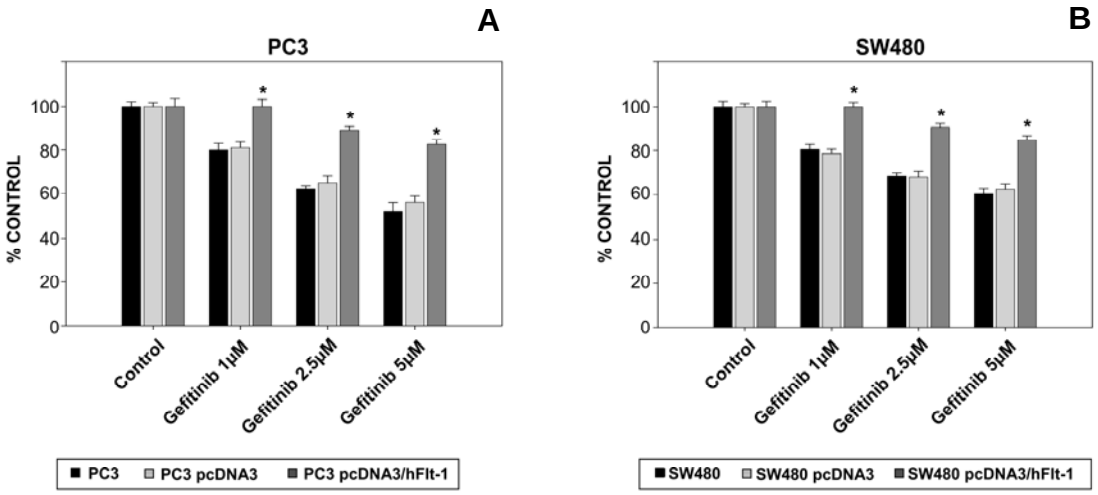

Figure 15. Effects of VEGFR-1 overexpression on survival of human cancer cell lines sensitive to EGFR inhibitors. Cell survival analysis of PC3 (**A**) and SW480 (**B**) transfected cells in the presence or absence of gefitinib 1, 2.5 or 5 μM. Results for each treatment are presented relative to untreated control cells. *, 2-sided P<0.0001 versus control and pCDNA3 cells treated at the same dose of gefitinib. Bars, SD.

Human cancer cell lines with acquired resistance to EGFR inhibitors display altered adhesion and migration capabilities.

VEGFRs expression may influence other tumor cell capabilities, such as migration and adhesion. Particularly, VEGFR-1 is implicated in the formation of pre-metastatic niches (Kaplan et al. 2005), and may be directly involved in migration of tumor cells, including colorectal carcinoma (Lesslie et al. 2006).

Therefore, we compare parental and VEGFR-1 overexpressing resistant cell lines for their migration potential. We performed wound-healing assays on GEO, PC3 cells and their counterparts resistant to cetuximab and gefitinib, respectively. GEO and GEO-CR cells migrated slowly, however GEO-CR exhibited a greater migration capability compared to GEO cells, reaching a statistically significant difference at forty-eight hours from the beginning of the experiment (Fig.16A). Eight hours after wound creation, an up to 50% greater migration capability was observed in PC3-GR compared to PC3 cells (Fig.16B).

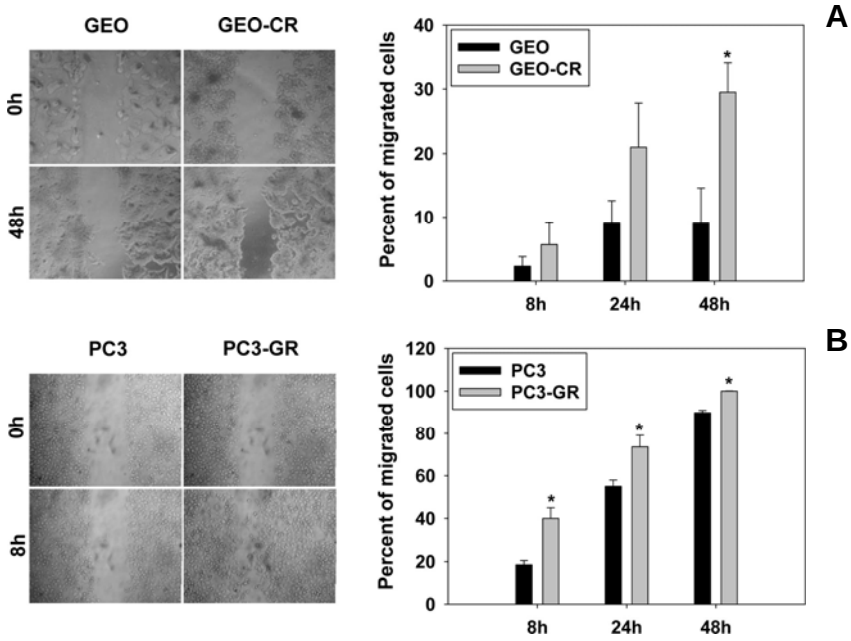

Figure 16. Analysis of migration capabilities of human cancer cell lines sensitive or resistant to EGFR inhibitors. Wound healing assay on GEO versus GEO-CR (**A**) and PC3 versus PC3-GR (**B**) cells. Cell monolayers were wounded by scratching with a 10 µl pipette tip. The results are presented as the percentage of the total distance of the original wound enclosed by cells and represent the mean ± SD at 8, 24 or 48 hours. *, 2-sided $P<0.001$ versus the other cell line.

To confirm these data, we performed a Boyden chamber chemotaxis assay on PC3 and PC3-GR cells using VEGF (10 ng/ml) as a chemoattractant. PC3-GR exhibited a two-fold greater migration capability than the parental cell

line (Fig.17A), 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 chambers filters (Fig.17B).

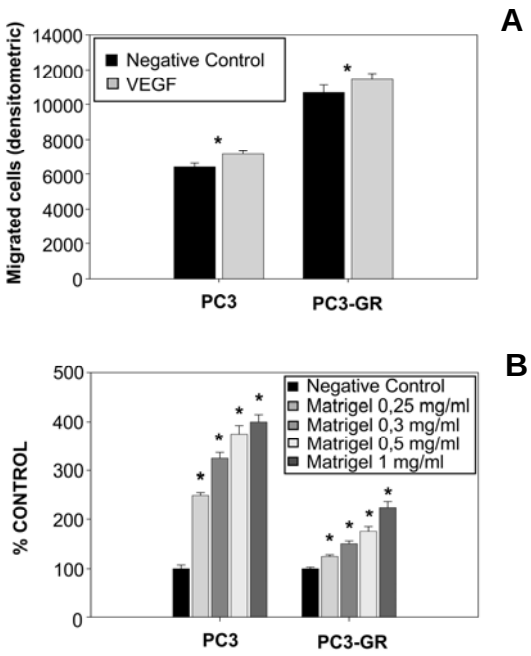

Figure 17. Analysis of migration and adhesion capabilities of human cancer cell lines sensitive or resistant to EGFR inhibitors. (**A**) Migration at 6 hours of PC3 and PC3-GR cells ± VEGF, assessed using Boyden chambers. *, 2-sided P<0.0001 versus the other cell line at 6 hours. Bars, SD. (**B**) Cell adhesion of PC3 and PC3-GR cells ± matrigel dilutions. *, 2-sided P<0.0001 versus negative control of the same cell line and versus the other cell line. Bars, SD.

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 VEGFRs inhibition, we performed a wound healing assay on PC3 and PC3-GR cells, in the presence of vandetanib, gefitinib or VEGFRs-specific siRNAs. Treatments with doxorubicin or with a nonsense RNA sequence were used as negative controls. 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.18A-B). Whereas the slight inhibition of wound closure with VEGFR-2-targeted siRNA did not reach statistical significance, VEGFR-1-targeted siRNA inhibited both PC3 (about 60%, p<0.0001) and PC3-GR (about 70% p<0.0001) cell migration (Fig.18A-B).

Therefore we have demonstrated that VEGFR-1 inhibition strongly interferes with cell migration, particularly in the anti-EGFR drugs resistant cell lines. Further studies will evaluate whether 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.

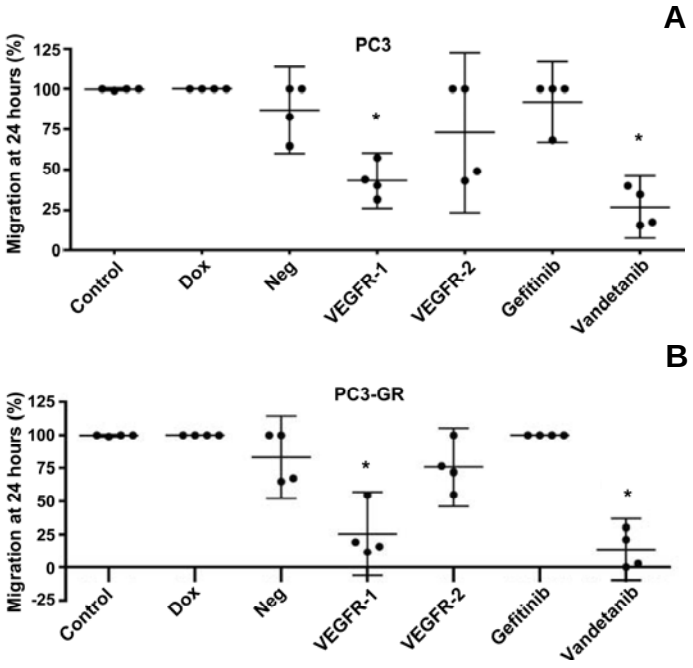

Figure 18. Role of EGFR and VEGFRs in migration capabilities of human cancer cell lines sensitive or resistant to EGFR inhibitors. Wound healing assay on PC3 (**A**) and PC3-GR (**B**) cells performed after 24 hours incubation with doxorubicin (dox) 25 ng/ml, vandetanib 2.5 μM, gefitinib 5 μM, 120 nM VEGFR-1 or VEGFR-2 targeting siRNA. *2-sided $P<0.0001$ versus control, doxorubicin and negative control. Bars, SD.

5. CONCLUSIONS

The studies described in this thesis report several findings that may have clinical and therapeutic implications.

First, using a panel of tumor cells of different types and with different degrees of sensitivity or resistance to EGFR inhibitors, we demonstrated that resistant tumors share the following common features: VEGFR-1/Flt-1 overexpression and Akt activation, increased secretion of VEGF and PlGF, and augmented migration capabilities. We also provided mechanistic evidence of the correlation between VEGFR-1 activity and anti-EGFR drugs resistance. These data imply that detection of VEGFR-1 on tumor cells may dictate an increased ability to survive and to escape the inhibition by anti-EGFR drugs used in clinical practice, such as cetuximab and gefitinib.

Another finding concerns the small molecule TKI vandetanib, whose main mechanisms of action were reported by our laboratory in several previous studies. We demonstrated that vandetanib-induced inhibition of EGFR kinase in endothelial cells contributes to its antiangiogenic effect. We also demonstrated that vandetanib is able to inhibit VEGFR-1 kinase and this capability plays a key role in determining its activity on EGFR drugs-resistant tumors. On these bases we have measured and reported a new kinase inhibition profile for this drug. Since vandetanib is successfully under investigation in several clinical studies, these data may be important for its clinical development.

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, which, in turn, correlates with Akt kinase activation via EGFR-independent mechanisms. Therefore vandetanib, inhibiting not only EGFR, but also VEGFRs in tumor cells, is able to turn off Akt activation and to inhibit survival and growth also in resistant cells. Our results further validate the clinical utility of the combined inhibition of EGFR and VEGFRs pathways, clarifying the molecular mechanisms which contribute to the efficacy of this strategy in overcoming resistance to EGFR inhibitors.

Moreover, we demonstrated the involvement of VEGFR-1 in regulating other important cells function, such as adhesion and migration capabilities. Consequently, the therapeutic use of agents able to inhibit both EGFR and VEGFR-1, including, as reported in this manuscript, vandetanib, may help to efficiently counteracts these process, potentially interfering with metastatic process.

6. ACKNOWLEDGEMENTS

This study was supported by grants from the Associazione Italiana per la Ricerca sul Cancro (AIRC), the Ministry of Health Strategic Project and the Regione Campania.

I acknowledge Prof. Giampaolo Tortora, for his continuous tutorship and encouragement during these doctorate years. I also thank all colleagues in the laboratory of Molecular Cancer Therapy with whom I have worked: Dr. Roberto Bianco, Dr. Vincenzo Damiano, Dr. Gennaro Daniele, Dr. Rosa Caputo, Dr. Teresa Gelardi, Dr. Sonia Garofalo. Especially I thank Sonia for sharing with me this experience.

I acknowledge Prof. Paul J. Chiao who received me in his laboratory of the Department of Surgical Oncology at the MD Anderson Cancer Center. My thanks to Dr. Davide Melisi who assisted me during my work at MD Anderson Cancer Center.

I acknowledge Prof. Giancarlo Vecchio, as the Coordinator of the PhD Program in Molecular Oncology and Endocrinology, who gave me the chance to enrich my knowledge and achieve a major professional training.

I sincerely thank my family, Domenico and my friends for their precious support. I thank Ruggero who always inspires my work.

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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/Flt-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.

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Received 11/21/07; revised 5/21/08; accepted 5/21/08.

Grant support: Associazione Italiana per la Ricerca sul Cancro, Ministry of Health Strategic Project, and Regione Campania. V. Damiano is supported by a fellowship from Associazione Italiana per la Ricerca sul Cancro.

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doi:10.1158/1078-0432.CCR-07-4905

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 (Flt-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

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 remeasured 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 (colon carcinoma) cell lines were obtained from the American Type Culture Collection. GEO-GR (gefitinib resistant), GEO-CR (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 (10^4 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/Flt-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-multiwell cluster dishes and transfected with EGFR, 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/hFlt-1 or with pcDNA3 as a negative control using the specific Cell Line Nucleofector Kit V for PC3 or SW480 (Amaxa). Briefly, 1×10^6 PC3 or SW480 cells were transfected with 5 μg DNA. To confirm VEGFR-1 expression, cells were plated in 6-multiwell cluster dishes and a Western blot analysis was done 48 h after transfection. For activity experiments, 3×10^4 cells were plated in each well of 24-multiwell 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 3-(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 (5×10^4 per well) were seeded into 6-multiwell cluster dishes and treated on days 1 to 2 with vandetanib (0.5 μmol/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 3×10^{-9} mol/L). All protein kinases were expressed in Sf9 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 μL of assay buffer, 10 μL of substrate (in H_2O), 5 μL of vandetanib in 10% DMSO, 10 μL of enzyme solution, and 5 μL 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 β. After an incubation at 30°C for 80 min, the reaction was stopped with 2% (v/v) H_3PO_4. All assays were done with a Beckman Coulter Biomek 2000 robotic system. Incorporation of ^{33}P 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 μmol/L gefitinib, 1 μmol/L vandetanib, or 7 nmol/L cetuximab (19). Protein extracts were resolved by a 4% to 20% SDS-PAGE and probed with one of the following antibodies: anti-EGFR (Ab-12, NeoMarkers); anti–phospho-(Tyr^{1173})-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-VEGF, anti-VEGFR-1/Flt-1, anti-Met, and anti–phospho-Met (Santa Cruz Biotechnology); and anti-actin (Sigma-Aldrich). Phosphorylated VEGFR-2 and VEGFR-1 were detected by immunoprecipitation of cell proteins with anti-VEGFR-2 or anti-VEGFR-1 antibody, resolved by a 7.5% SDS-PAGE gel, and probed with the PY20 anti-phosphotyrosine monoclonal antibody (Upstate). 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 (PlGF) 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 PlGF 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 (Supertranscript 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 μL/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 2×10^4 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-μm pore size, polyvinyl pyrrolidone–free from Costar-Nuclepore) were manually coated with 50 μL of a 0.1 mg/mL solution of collagen type IV and dried overnight at room temperature. VEGF was used as chemoattractant, 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 μL pipette tip and then cultured in the presence or absence of doxorubicin (25 ng/mL), vandetanib (2.5 μmol/L), gefitinib (5 μmol/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× 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 μmol/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-CR) 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 (IC_{50}, 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). In

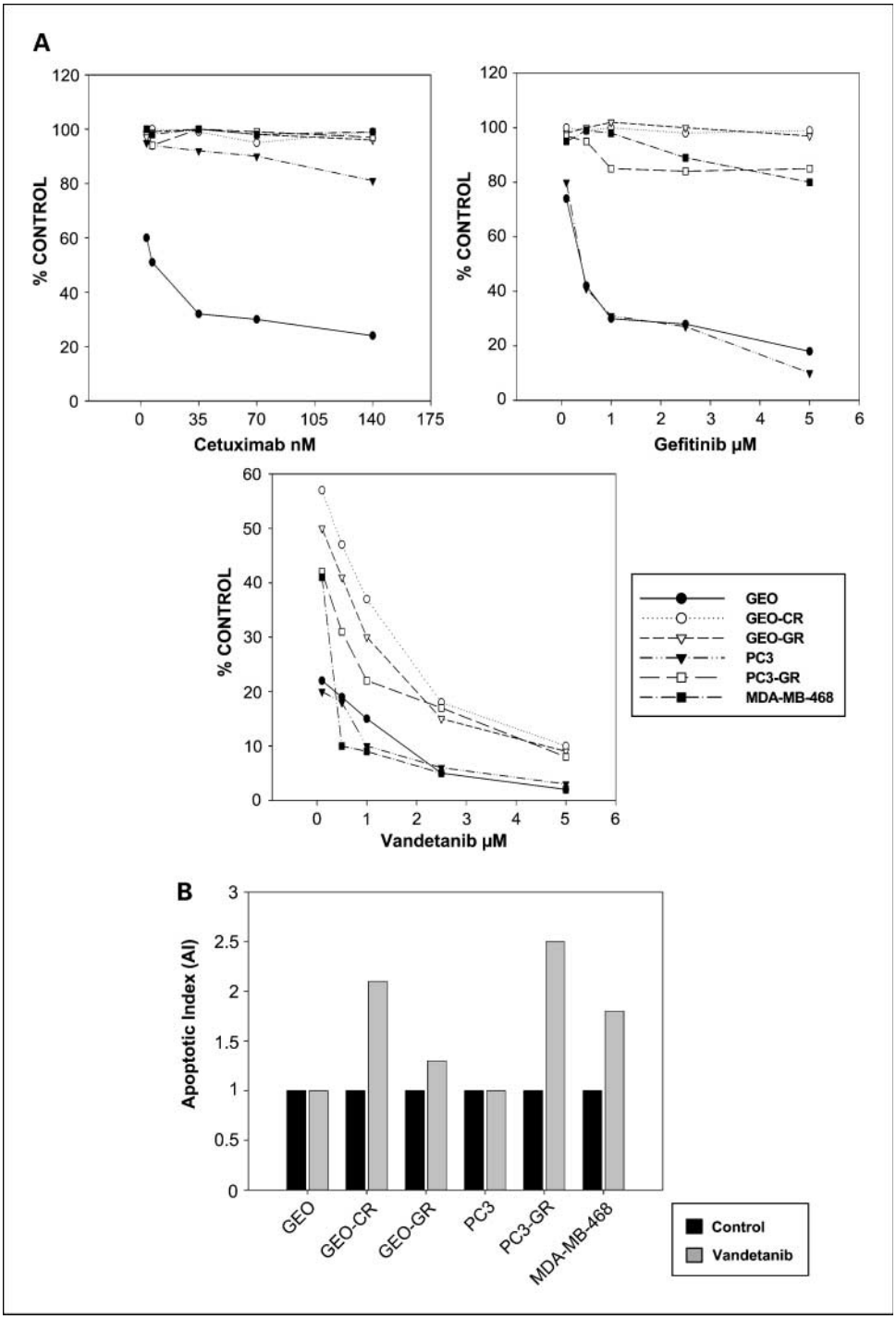

Fig. 1. Effects of cetuximab, gefitinib, or vandetanib on growth and induction of apoptosis of human cancer cell lines. *A,* effects of cetuximab, gefitinib, or vandetanib on the soft agar growth of GEO, GEO-CR, GEO-GR, PC3, PC3-GR, and MDA-MB-468 cells. Cells were treated with the indicated concentrations of drug each day for 3 consecutive days. Colonies were counted after 10 to 14 d. Points, mean of three independent experiments, each done in triplicate; bars, SD. Data are presented relative to untreated control cells. Whereas the effects of vandetanib were statistically significant versus control in all cell lines (two-sided $P < 0.0001$), the effects of gefitinib and cetuximab treatment were statistically significant versus control in sensitive cell lines (two-sided $P < 0.0001$) but not in EGFR inhibitor – resistant cell lines. *B,* effect of 0.5 μmol/L vandetanib on the induction of apoptosis. Data are expressed as apoptotic index (absorbance ratio at 405 nm of treated cells/untreated cells, normalized for the same number of cells).

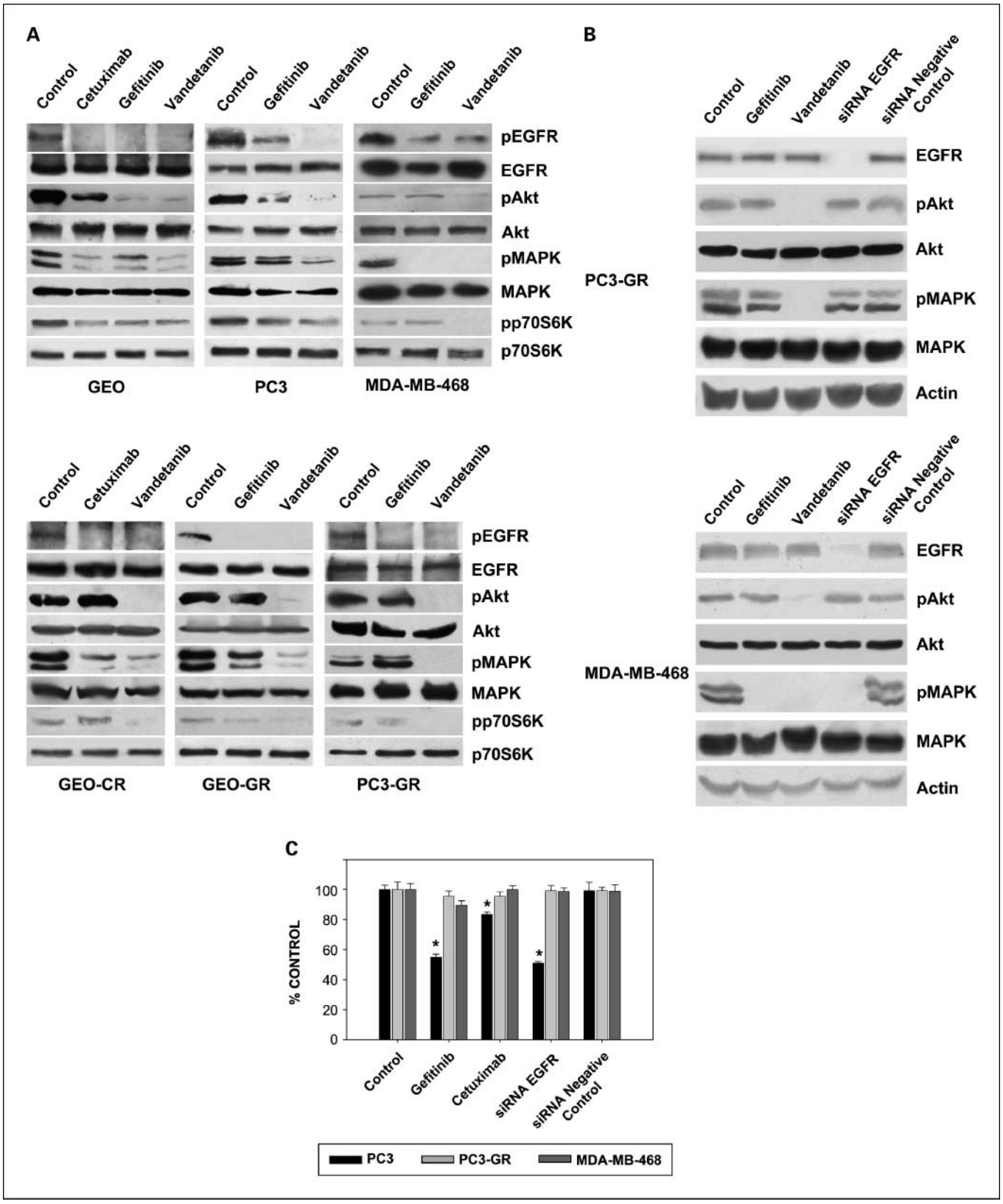

Fig. 2. Analysis of EGFR-dependent signaling pathways in human cancer cell lines sensitive or resistant to EGFR inhibitors. *A,* Western blot analysis of protein expression in cell lines treated with 7 nmol/L cetuximab, 1 μmol/L gefitinib, or 1 μmol/L vandetanib for 24 h before protein extraction. *B,* 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

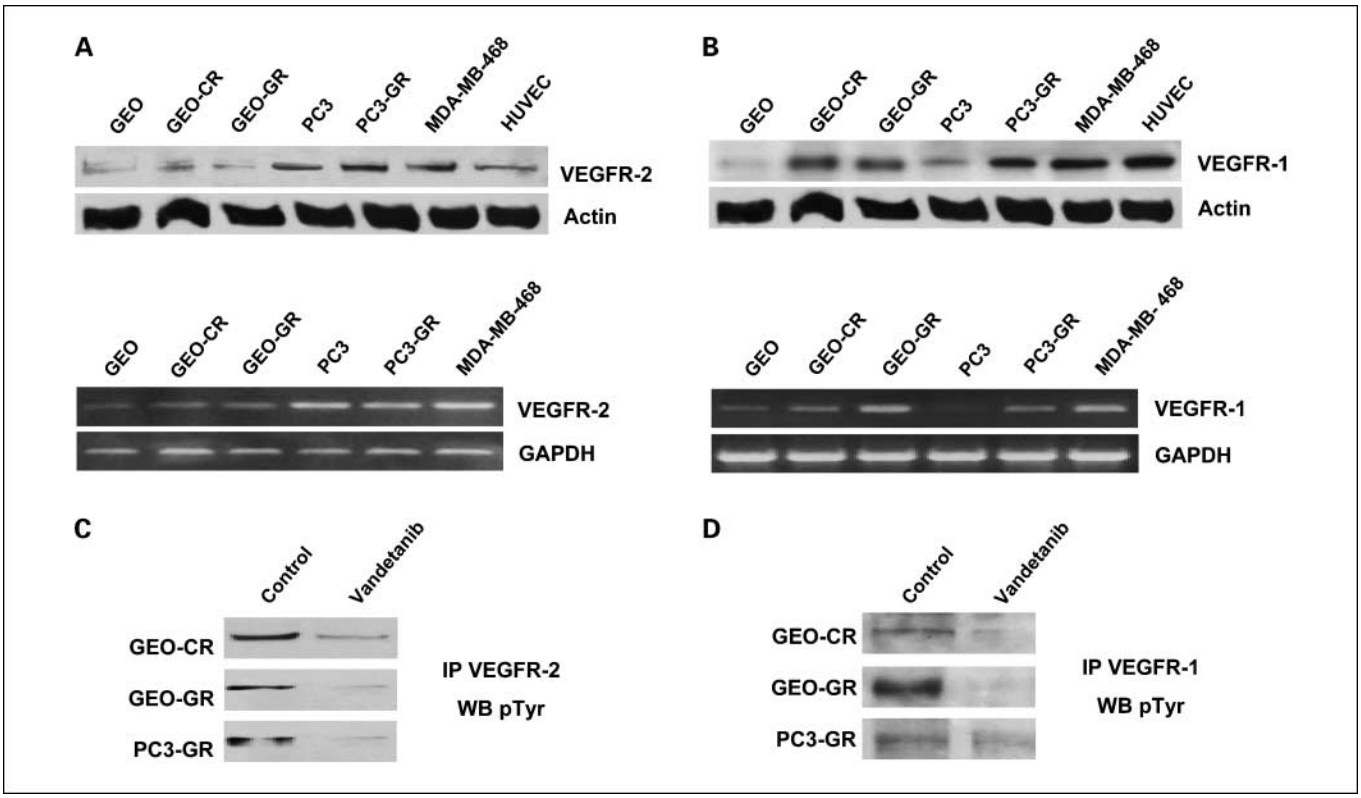

Fig. 3. Analysis of VEGFR expression and activity on human cancer cell lines sensitive or resistant to EGFR inhibitors. *A,* analysis of VEGFR-2/KDR protein and mRNA expression in cell lines using Western blot (*top*) and PCR (*bottom*), respectively. *B,* analysis of VEGFR-1/Flt-1 protein and mRNA expression in cell lines using Western blot (*top*) and PCR (*bottom*), respectively. *C,* inhibition of VEGFR-2/KDR autophosphorylation in GEO-CR, GEO-GR, and PC3-GR cells treated for 24 h with 1 μmol/L vandetanib. *D,* inhibition of VEGFR-1/Flt-1 autophosphorylation in GEO-CR, GEO-GR, and PC3-GR cells treated for 24 h with 1 μmol/L vandetanib. HUVEC, human umbilical vein endothelial cells; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Table 1. Kinase inhibition by vandetanib

Kinase	IC_{50} (nmol/L)
VEGFR-1	150
VEGFR-2	38
VEGFR-3	260
EGFR	43
PDGFRβ	5,300

Abbreviation: PDGFRβ, platelet-derived growth factor receptor β.

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 IC_{50} 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 (IC_{50}, 150 nmol/L). Moreover, the ability to inhibit EGFR resulted almost comparable with VEGFR-2 (IC_{50}, 43 versus 38 nmol/L, respectively). Finally, to a lesser extent, vandetanib inhibited also VEGFR-3 (IC_{50}, 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 VEGFR ligands VEGF and PlGF. 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 PlGF levels, and that their secretion is higher in conditioned medium derived from resistant cells compared with parental cells (Supplementary Fig. S2B and C).

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

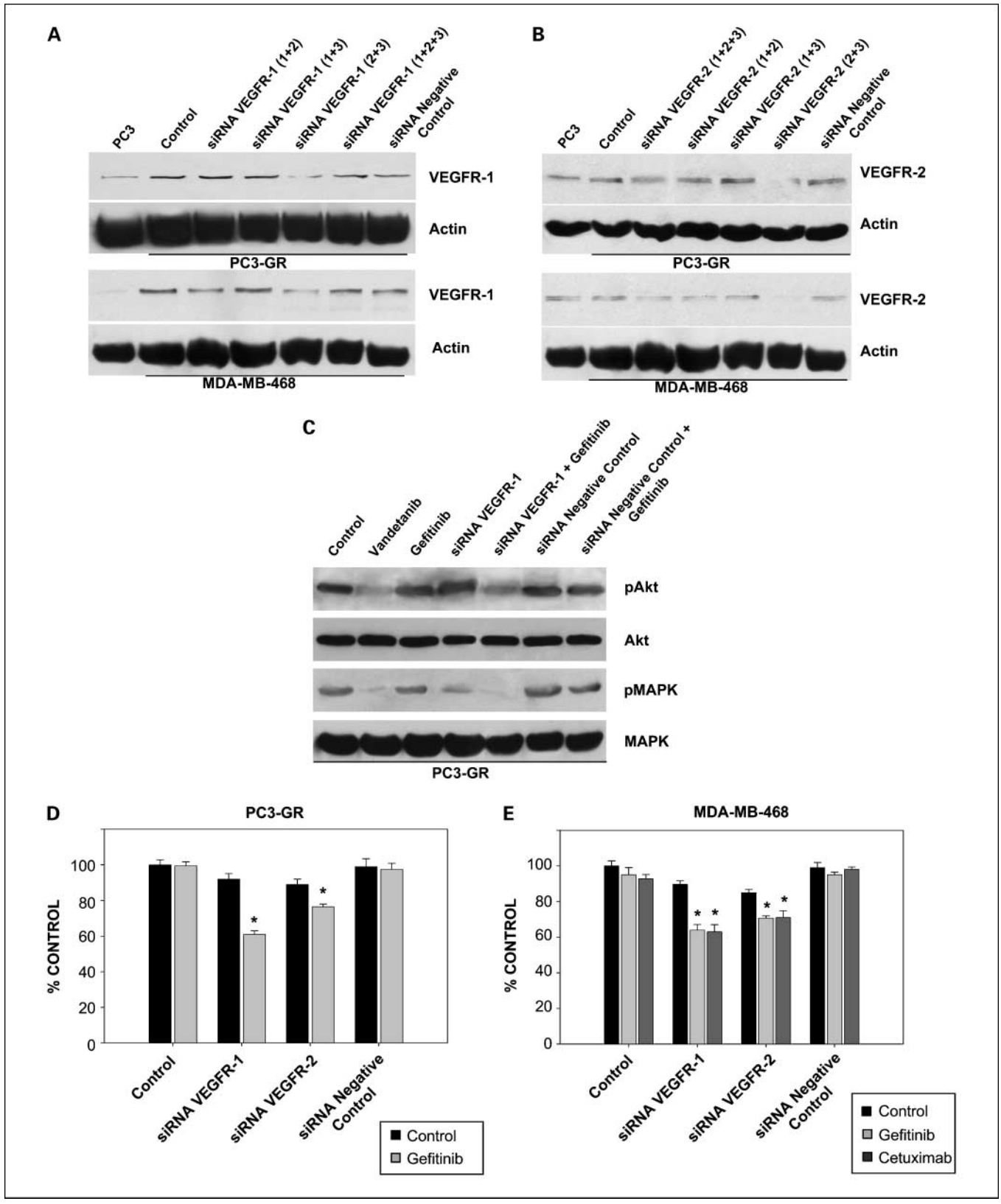

Fig. 4. VEGFR silencing in human cancer cell lines resistant to EGFR inhibitors. Western blot analysis of VEGFR-1/Flt-1 (*A*) or VEGFR-2/KDR (*B*) in PC3-GR and MDA-MB-468 cells transfected with 120 nmol/L VEGFR-1 – or VEGFR-2 – targeting siRNA, respectively, or with a nonsense RNA sequence (negative 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.

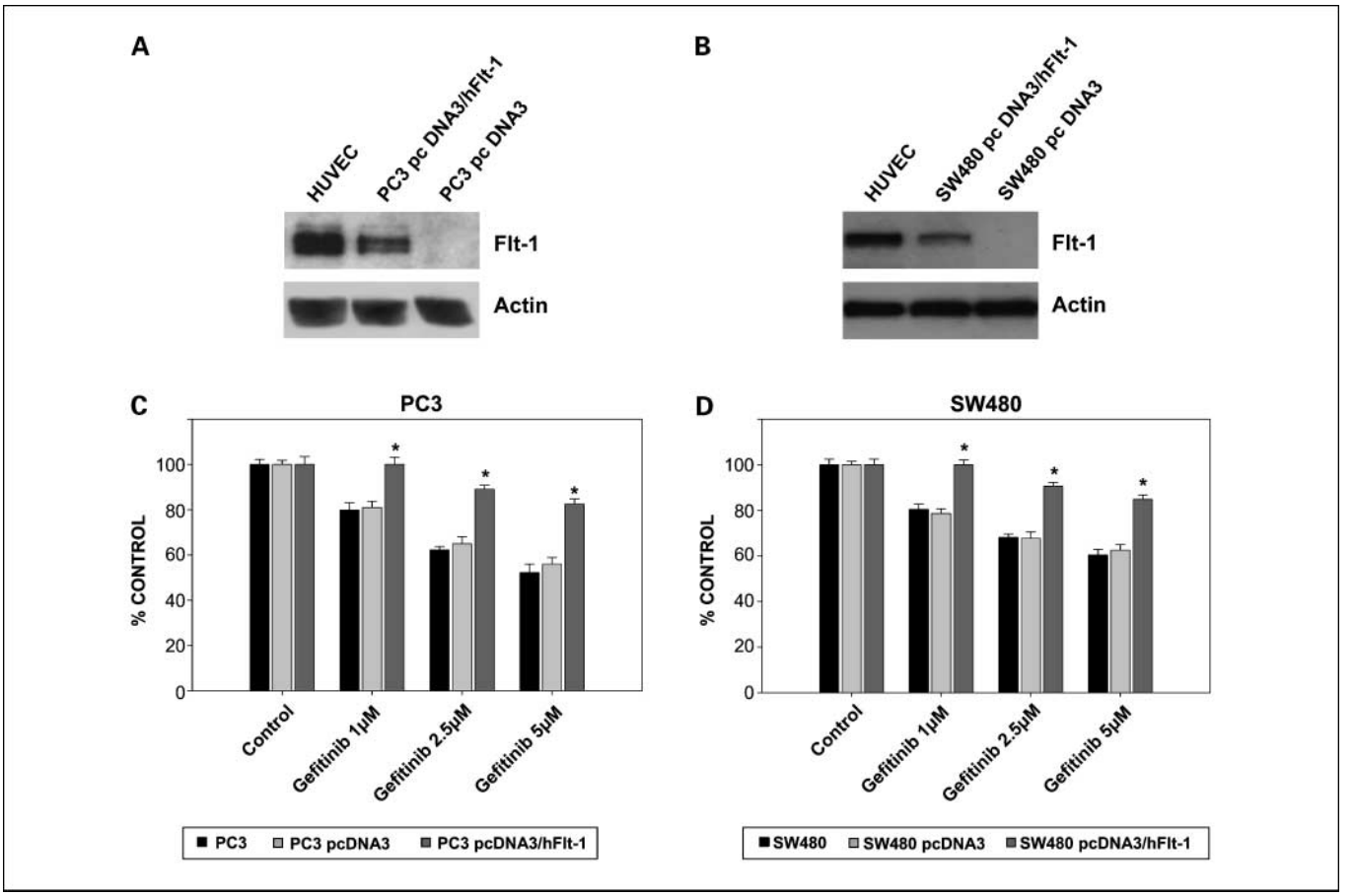

Fig. 5. VEGFR-1 overexpression in human cancer cell lines sensitive to EGFR inhibitors. Western blot analysis of VEGFR-1/Flt-1 in PC3 cells (*A*) and in SW480 cells (*B*) transfected with pcDNA3/hFlt-1 or pcDNA3 as negative control. Cell survival analysis of PC3-transfected (*C*) and SW480-transfected (*D*) cells in the presence or absence of 1, 2.5, or 5 μmol/L of gefitinib. Results for each treatment are presented relative to untreated control cells. *, two-sided $P < 0.0001$ versus PC3 and PC3pCDNA3 treated at the same dose of gefitinib. 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-I 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

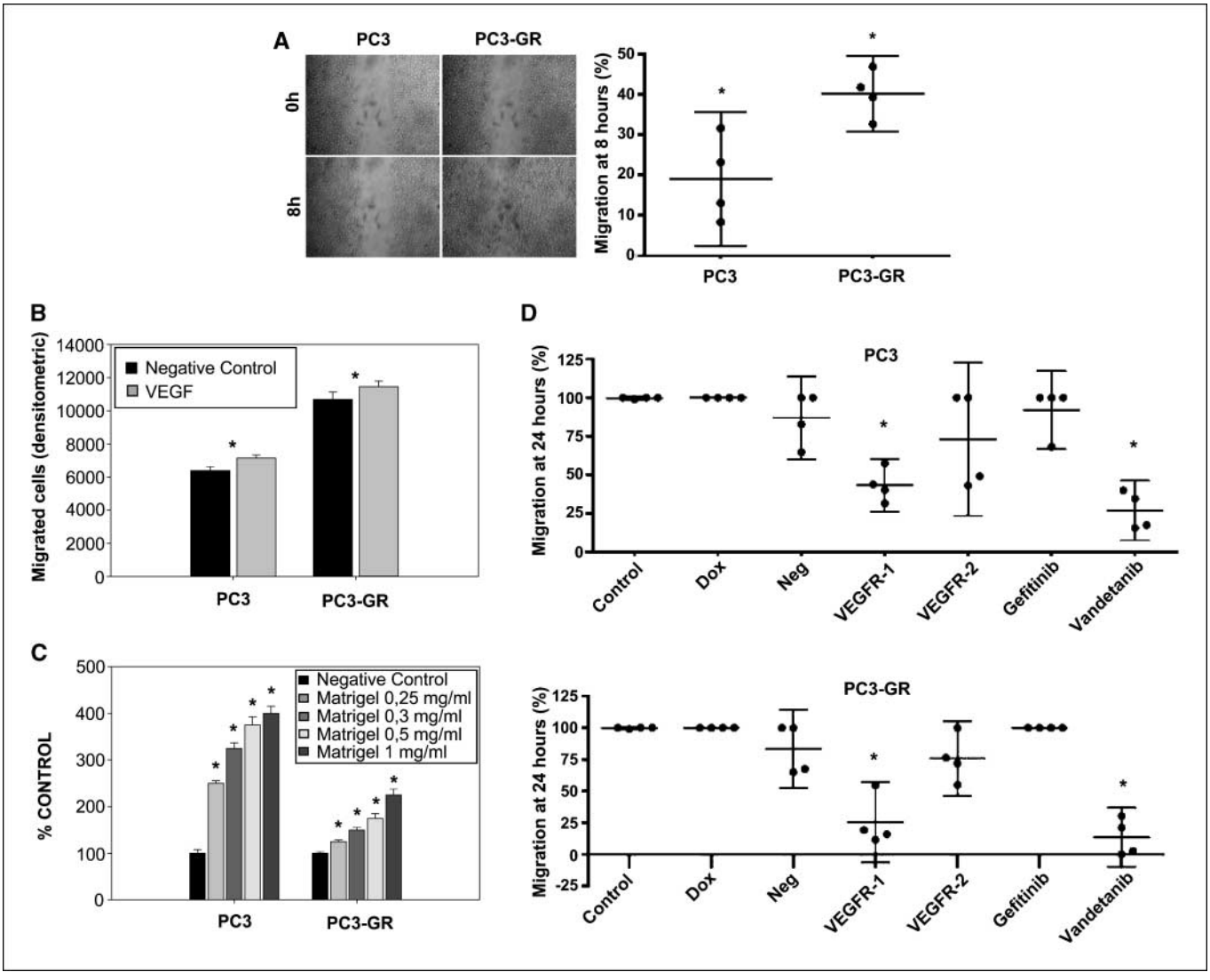

Fig. 6. Analysis of migration and adhesion capabilities of human cancer cell lines sensitive or resistant to EGFR inhibitors. *A,* wound-healing assay on PC3 and PC3-GR cells. Cell monolayers were wounded by scratching with a 10 μL pipette tip. The results are presented as the percentage of the total 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 ng/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 multitargeting 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 premetastatic 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.

Acknowledgments

We thank Gaetano Borriello for his excellent technical assistance.

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