Anticancer effects of ZD6474, a VEGF receptor tyrosine kinase inhibitor, in gefitinib ("Iressa")-sensitive and resistant xenograft models

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ZD6474 is a novel, orally available inhibitor of vascular endothelial growth factor (VEGF) receptor-2 (KDR) tyrosine kinase, with additional activity against epidermal growth factor receptor (EGFR) tyrosine kinase. ZD6474 has been shown to inhibit angiogenesis and tumor growth in a range of tumor models. Gefitinib ("Iressa") is an selective EGFR tyrosine kinase inhibitor (TKI) that blocks signal transduction pathways. We examined the antitumor activity of ZD6474 in the gefitinib-sensitive lung adenocarcinoma cell line, PC-9, and a gefitinib-resistant variant (PC-9/ZD). PC-9/ZD cells showed cross-resistance to ZD6474 in an *in vitro* dye formation assay. In addition, ZD6474 showed dose-dependent inhibition of EGFR phosphorylation in PC-9 cells, but inhibition was only partial in PC-9/ZD cells. ZD6474-mediated inhibition of tyrosine residue phosphorylation (Tyr992 and Tyr1045) on EGFR was greater in PC-9 cells than in PC-9/ZD cells. These findings suggest that the inhibition of EGFR phosphorylation by ZD6474 can contribute a significant, direct growth-inhibitory effect in tumor cell lines dependent on EGFR signaling for growth and/or survival. The effect of ZD6474 (12.5–50 mg/kg/day p.o. for 21 days) on the growth of PC-9 and PC-9/ZD tumor xenografts in athymic mice was also investigated. The greatest effect was seen in gefitinib-sensitive PC-9 tumors, where ZD6474 treatment (>12.5 mg/kg/day) resulted in tumor regression. Dose-dependent growth inhibition, but not tumor regression, was seen in ZD6474-treated PC-9/ZD tumors. These studies demonstrate that the additional EGFR TKI activity may contribute significantly to the antitumor efficacy of ZD6474, in particular in those tumors that are dependent on continued EGFR-signaling for proliferation or survival. In addition, these results provide a preclinical rationale for further investigation of ZD6474 as a potential treatment option for both EGFR-TKI-sensitive and EGFR-TKI-resistant tumors. (Cancer Sci 2004; 95: 984–989)

ZD6474 is a novel, orally available inhibitor of VEGF receptor-2 (KDR) tyrosine kinase, with additional activity against EGFR tyrosine kinase, and it inhibits angiogenesis and tumor growth in a diverse range of tumor models.[1, 2] Phase I clinical evaluation has shown ZD6474 to be generally well tolerated, and tumor responses in patients with non-small cell lung cancer (NSCLC) have been documented.[3, 4] Thus, ZD6474 is considered to be a multi-target tyrosine kinase inhibitor active against solid tumors. The purpose of this study is to clarify the mode of antitumor action of ZD6474 as compared with that of gefitinib ("Iressa," ZD1839). Gefitinib is an orally active, selective EGFR tyrosine kinase inhibitor (EGFR-TKI) that blocks signal transduction pathways implicated in the proliferation and survival of cancer cells and other host-dependent processes promoting tumor growth.[5–7] Gefitinib is now available clinically for non-small cell lung cancer patients. In order to elucidate the mode of action of ZD6474, the antitumor activity and pharmacodynamics were investigated in an established human lung cancer cell line resistant to gefitinib (PC-9/ZD cells).[8] This approach allowed us to clarify the common and differential modes of actions of gefitinib and ZD6474 in lung cancer, and this will be important for deciding how to use ZD6474 in non-small cell lung cancer patients in combination with gefitinib.

Materials and Methods

Reagents and cell culture. ZD6474 and gefitinib ("Iressa," ZD1839) were provided by AstraZeneca (Macclesfield, UK). Human NSCLC cell lines PC-9 and PC-14 were used.[9, 10] In addition, a gefitinib-resistant subline, PC-9/ZD, was derived from PC-9 cells by short-term exposure to the mutagen *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine, continuous exposure to 0.2–0.5 μ*M* gefitinib for 28 days, and subcloning. The resistant phenotype has been stable for at least 6 months under drug-free conditions.[8] The PC-9/ZD cell line shows no cross-resistance to conventional anticancer drugs.[8] Cells were maintained in RPMI-1640 (Sigma Chemical Co., St. Louis, MO) supplemented with 10% heat-inactivated fetal bovine serum (Gibco BRL, Grand Island, NY).

Antibodies. Anti-vonWillebrand Factor (vWF) antibody was purchased from Chemicon, Temecula, CA. Affinity-purified antibody to EGFR was purchased from Santa Cruz, CA and affinity-purified antibodies to phospho-EGFR specific for Tyr845, Tyr992, Tyr1045, and Tyr1068 were purchased from Cell Signaling Technology, Beverly, MA.

Growth inhibition assay. Cell sensitivity to ZD6474 and gefitinib was estimated by means of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described previously.[11] Briefly, PC-9, PC-9/ZD, or PC-14 cells were exposed to 0–10 μ*M* ZD6474 or gefitinib for 72 h before measuring absorbance. Optical density was assessed at 562–630 nm using an EL340 96-well microtiter plate reader (Bio-Tek, Winooski, VT).

Xenograft studies in athymic mice. Suspensions of PC-9 cells (5×10^6) or PC-9/ZD cells (3×10^6) were injected subcutaneously into the backs of 5-week-old female athymic mice (Japan Charles River Co., Atsugi, Japan). After 1 week (tumors >95 mm^3), mice were randomly allocated into groups of six animals to receive ZD6474 (12.5, 25, or 50 mg/kg/day), gefitinib (12.5, 25, or 50 mg/kg/day) or vehicle only by oral gavage. Tumor diameter and body weight were measured twice weekly. The tumor volume was calculated ($width^2\times length/2$) and is presented as a percentage of the pretreatment value. A tumor volume below 100% of the pretreatment volume was defined as "tumor reduction." Experiments were performed in accordance with the UK Coordinating Committee on Cancer Research Guidelines for the welfare of animals in experimental neoplasia (second edition). After 3 weeks of treatment, tumors were removed.

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Abbreviations: VEGF, vascular endothelial growth factor; EGFR, epidermal growth factor receptor; TKI, tyrosine kinase inhibitor; NSCLC, non-small cell lung cancer; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

Two tumor specimens per group were processed for immunohistochemical analysis.

Immunohistochemical analysis. Immunohistochemistry was performed on formalin-fixed, paraffin-embedded tissue sections as reported previously.[1,5] An anti-Ki67 monoclonal antibody (clone MIB1; DBA, Milan, Italy) was used and the proportion of positive (proliferating) cells was assessed. At least 1000 cancer cells were counted and scored per slide. Both the percentage of specifically stained cells and the intensity of immunostaining were recorded. Blood vessels were detected with an anti-von Willebrand Factor (vWF) antibody (Chemicon). Microvessel density was determined by calculating the proportion of vWF-positive cells.

Evaluation of apoptosis (TUNEL). Sections were stained with an *in situ* Death Detection POD Kit (Roche Diagnostic GmbH, Mannheim, Germany) according to the manufacturer's instructions. At least 1000 tumor cell nuclei from the most evenly and distinctly labeled areas were examined. The TUNEL-positive tumor cell nuclei were counted, and the apoptotic index was calculated as the proportion of cells with apoptotic nuclei.

Immunoprecipitation and immunoblotting. Cells were maintained in medium without serum for 12 h. Then serum-starved cells were exposed to ZD6474 or gefitinib, incubated for 1 h and stimulated in medium including 10% fetal bovine serum for 30 min. The cells were subsequently washed twice with ice-cold PBS, scraped in lysis buffer (50 m*M* Tris-HCl [pH 8.0], 120 m*M* NaCl, 0.5% Nonidet P-40, 100 m*M* NaF, 200 μ*M* Na_3VO_4, and 10 μg/ml each of aprotinin, leupeptin, and PMSF), and incubated on ice for 60 min. The lysates were centrifuged at 8000*g* for 20 min, and total protein was obtained from the supernatants. Protein concentration was measured with the bicinchoninic acid protein assay (Pierce, Rockford, IL). Cell lysates for immunoprecipitates contained 2 mg of total protein. Anti-EGFR antibody (3 μg) was incubated overnight with the lysates at 4°C, and the precipitates were collected with 40 μliters of Protein G Sepharose beads over a 1 h period. Antibody-complexed proteins were washed with lysis buffer, analyzed by SDS-PAGE and visualized using an enhanced chemiluminescence solution (ECL; Amersham Pharmacia Biotech UK, Buckinghamshire, UK). Quantative analysis was performed using Kodak software. Quantified values of phospho-EGFR bands were standardized according to those of EGFR bands.

Results

***In vitro* evaluation of ZD6474 and gefitinib inhibition of tumor cell growth.** The IC_{50} values of gefitinib for growth inhibition of PC-9 and PC-9/ZD cells were 0.038 μ*M* and 6.8 μ*M*, respectively. The IC_{50} values of ZD6474 were 0.14 μ*M* and 5.92 μ*M*, respectively (Fig. 1A). PC-9 cells were 180-fold more sensitive to gefitinib than PC-9/ZD cells, and PC-9/ZD cells were cross-resistant to ZD6474. Experiments with another VEGFR-TKI, SU5416, and PDGFR-TKI, Tyrphostin 9, revealed no cross-resistance (data not shown).

In a separate experiment, the IC_{50} values of gefitinib were 0.006 μ*M* and 20.5 μ*M*, in PC-9 and PC-14 (another human NSCLC cell line), respectively (Fig. 1B). PC-9 cells were therefore approximately 3400-fold more sensitive to gefitinib than PC-14 cells. Corresponding IC_{50} values for ZD6474 were 0.11 μ*M* and 9.81 μ*M*, demonstrating cross-resistance to ZD6474.

Other workers have examined the ability of gefitinib or ZD6474 to inhibit serum-dependent tumor cell growth *in vitro*, and have demonstrated IC_{50} values of gefitinib[12] and ZD6474[13] of >1 μ*M* for tumor cell lines. Therefore, PC-9 is particularly sensitive to *in vitro* growth inhibition by both gefitinib and ZD6474, whereas the sensitivities of both gefitinib-resistant PC-9/ZD and PC-14 fall within the normal range reported for other tumor cell lines.

***In vivo* antitumor effects.** ZD6474 treatment (12.5–50 mg/kg/day) resulted in inhibition of PC-9 tumor growth, with robust tumor regression seen even at the lowest dose tested. ZD6474 treatment also resulted in dose-dependent inhibition of PC-9/ZD tumor xenograft growth, although in this case, regression was not seen (Fig. 2, A and B). This antitumor effect of ZD6474 was very similarly to that of gefitinib we previously reported (Fig. 2, C and D).[8]

Effect of treatment on cell proliferation, apoptosis, and vascularization. ZD6474 treatment resulted in a dose-dependent decrease in the proportion of proliferating cells in the PC-9 tumors, but not in PC-9/ZD xenografts (Fig. 3). No significant

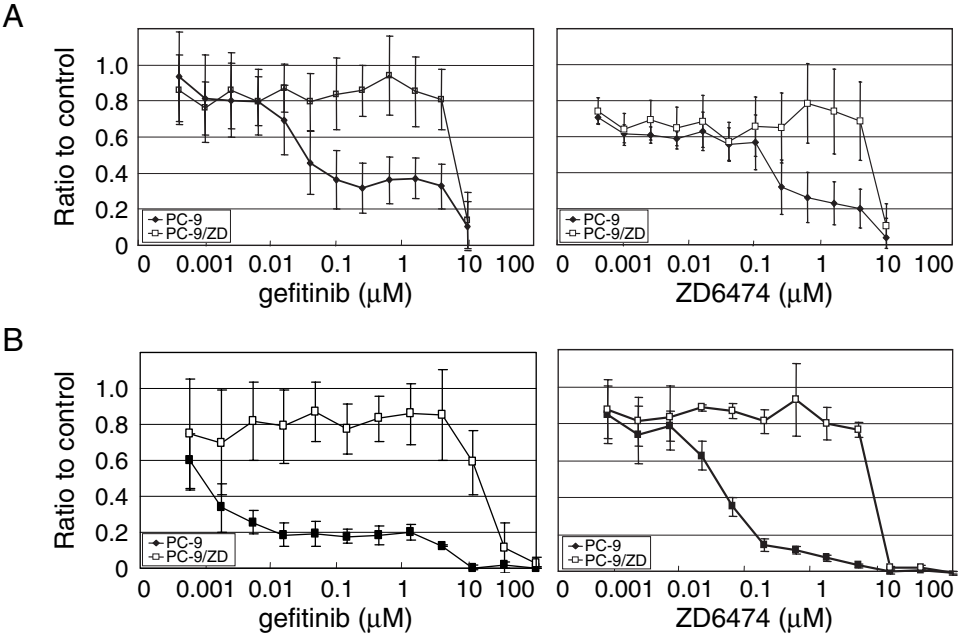

Fig. 1. Growth inhibitory effect of gefitinib (ZD1839) and ZD6474. A: PC-9 and PC-9/ZD, B: PC-9 and PC-14 cells. Data shown are mean values from three experiments (±SD).

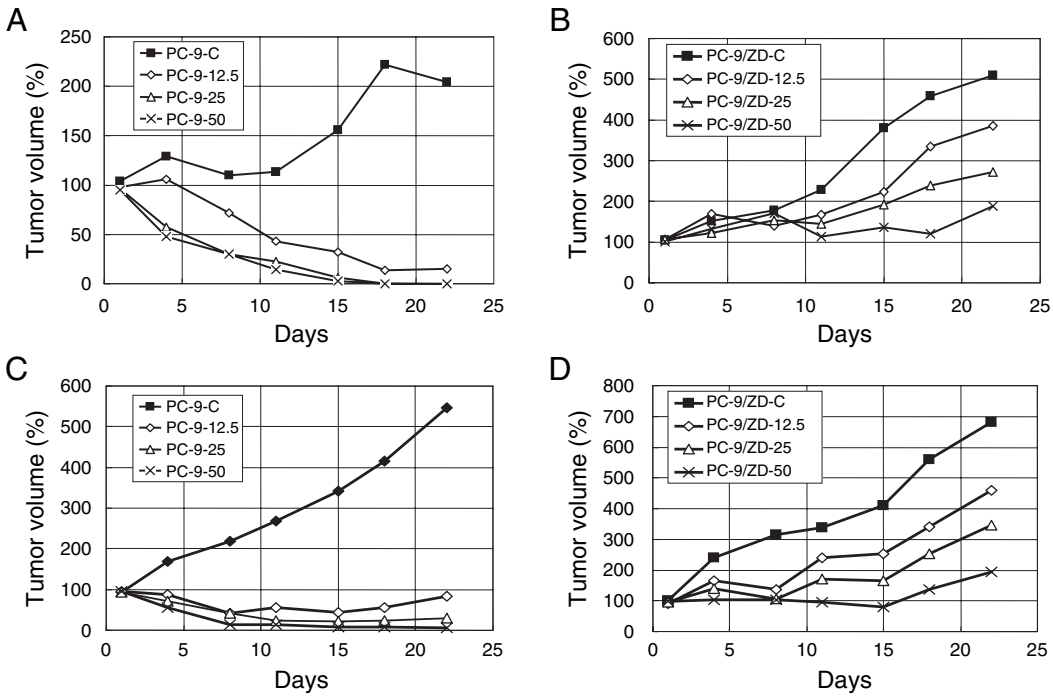

Fig. 2. Antitumor activity of ZD6474 (A, B) and gefitinib (C, D) on established PC-9 (A, C) and PC-9/ZD (B, D) human lung cancer xenografts.

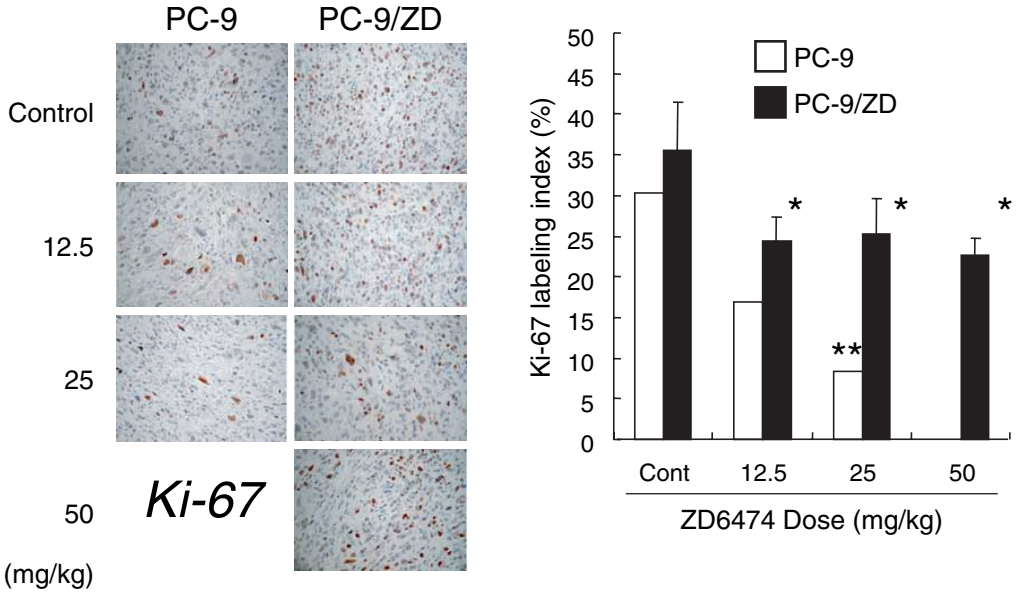

Fig. 3. Effect of ZD6474 on the Ki67 labeling index of PC-9 and PC-9/ZD tumors *in vivo*. Data represent mean values (±SD). Significant difference from control shown by the Dunnett test (* $P<0.05$, ** $P<0.01$).

increase in apoptosis was observed in either tumor type (Fig. 4).

Assessment of tumor vascularization showed a significant reduction in vascular density following ZD6474 treatment of PC-9 tumor xenografts, although no effect was seen in PC-9/ZD tumors (Fig. 5). Differences in the action of ZD6474 on PC-9 and PC-9/ZD tumors are summarized in Table 1.

Inhibition of EGFR activity. It is possible that the antitumor activity of ZD6474 is partly attributable to EGFR inhibition based on the evidence of cross-resistance to gefitinib (Figs. 1–3). Therefore, site-specific anti-phosphorylated-EGFR antibodies were used to investigate inhibition of EGFR phosphorylation by ZD6474 in PC-9 and PC-9/ZD cells at four different tyrosine phosphorylation sites (Tyr845, Tyr992, Tyr1045, and Tyr1068; Fig. 6). ZD6474 dose-dependently inhibited phosphorylation of the four EGFR tyrosine residues in PC-9 cells (Fig. 6). In PC-9/ZD cells, drug-related inhibition of phosphorylation at the Tyr992 site was highly resistant to ZD6474 treatment (Fig. 6), and the Tyr845 and Tyr1045 sites were moderately resistant, while the effect of phosphorylation at the Tyr1068 site did not differ significantly between the sensitive and resistant cell lines (Table 1). The spectrum of activity of ZD6474 on the

four EGFR tyrosine residues examined in PC-9/ZD cells differed from that of gefitinib. ZD6474 displayed a variety of actions on each tyrosine residue, which may be responsible for the wide range of biological activities.

Discussion

In the NSCLC xenograft model reported here, ZD6474 treatment significantly inhibited PC-9 tumor growth, inducing tumor regression. In addition, ZD6474 caused dose-dependent PC-9/ZD tumor growth inhibition. These data indicate that ZD6474 exerts potent antitumor activity against gefitinib-sensitive and resistant lung cancers *in vivo*. Although PC-9/ZD cells are less sensitive to gefitinib than PC-9 cells, the *in vitro* sensitivity of these cells falls within the normal range for other tumor cell lines. Accordingly, gefitinib has significant *in vivo* activity against PC-9/ZD, producing a dose-dependent inhibition of xenograft growth, rather than the tumor regression seen with PC-9 xenografts. Therefore, the antitumor activity of ZD6474 appeared to parallel that of gefitinib in PC-9 and PC-9/ZD tumor cells, both *in vitro* and *in vivo*. Since gefitinib is a TKI with a high degree of selectivity for EGFR,[1, 2, 4] inhibition of EGFR autophosphorylation is likely to contribute to the antitumor activity of ZD6474, particularly in tumor cells which are dependent on EGFR signaling for continued growth and survival. This was shown *in vitro*, as ZD6474 inhibited EGFR

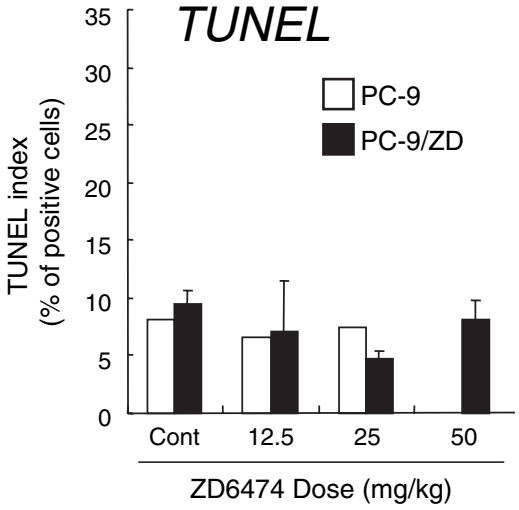

Fig. 4. Effect of ZD6474 on the TUNEL index of PC-9 or PC-9/ZD tumors *in vivo*. Data represent mean values (±SD).

Table 1. Site-specific effect of ZD6474 on EGFR tyrosine residues in PC-9 and PC-9/ZD cells

Tyr residue of EGFR	Inhibition of phosphorylation			
	ZD6474		Gefitinib	
	PC-9	PC-9/ZD	PC-9	PC-9/ZD
845	++	+	++	+
992	++	–	++	++
1045	++	+	–	–
1068	++	++	++	+

++ strong; + moderate; – not significant.

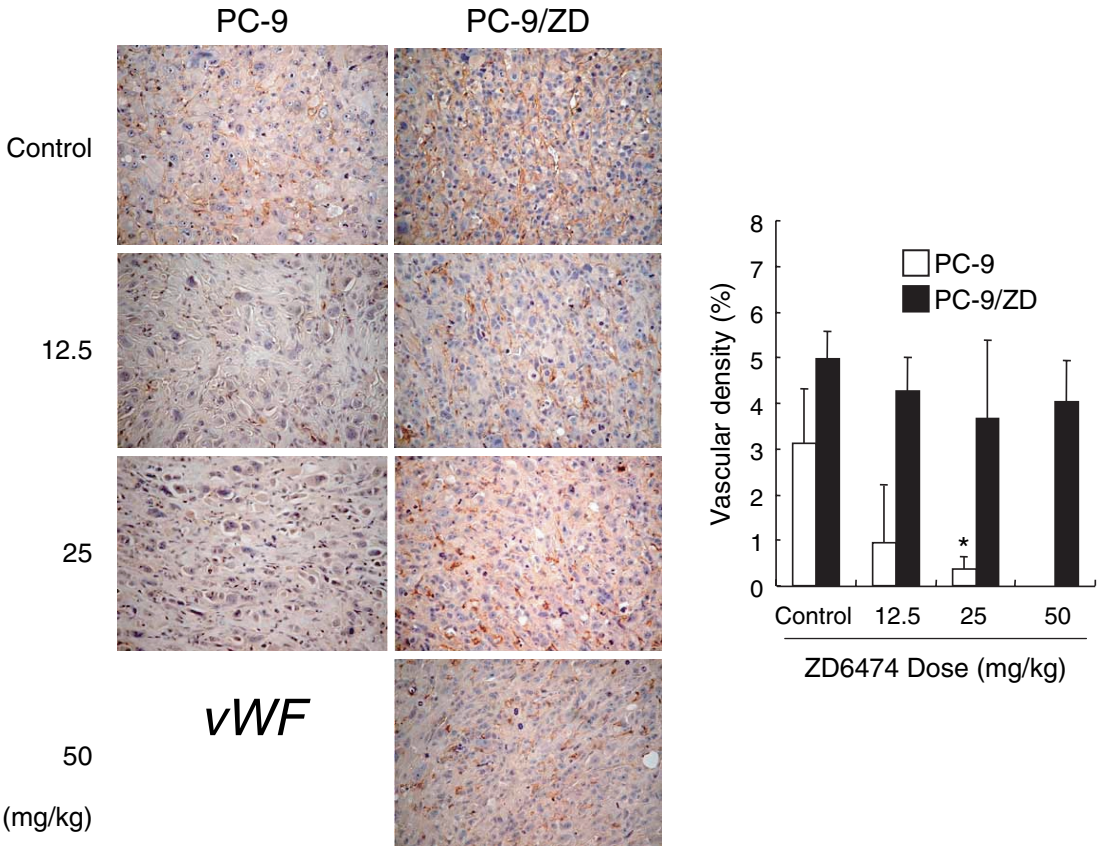

Fig. 5. Effect of ZD6474 on the vascular density of PC-9 and PC-9/ZD tumors stained *in vivo* with anti-vWF. Values are means±SD. Significant difference from the control by the Dunnett test (* $P<0.05$).

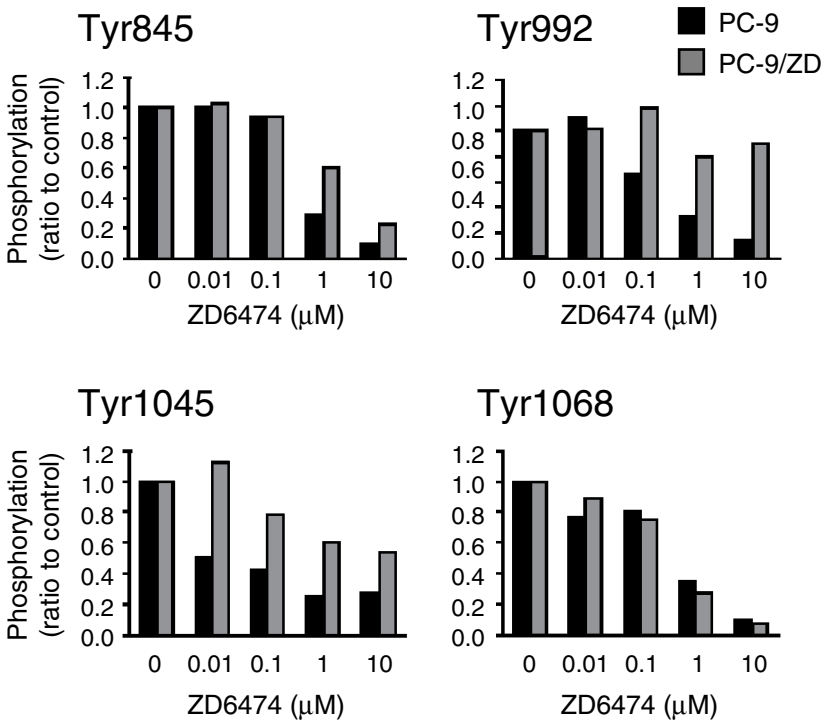

Fig. 6. Phosphorylation of EGFR tyrosine residues in PC-9 and PC-9/ZD cells after exposure to ZD6474.

phosphorylation in a dose-dependent manner. These results are consistent with previous reports[1] and indicate that ZD6474 is a potent EGFR TKI. *In vivo*, ZD6474 decreased vascular density in PC-9 tumors but not in PC-9/ZD cells, suggesting that ZD6474 may affect the angiogenic process via EGFR blockade. This could be mediated by inhibition of EGFR-induced paracrine production of angiogenic growth factors, such as VEGF, bFGF, and TGF from cancer cells, but the exact mechanism of action is unclear. This activity is, however, likely to be of less significance than the VEGFR-2-mediated antiangiogenic effect, since ZD6474 has been shown to have consistent *in vivo* antitumor activity in a range of histologically diverse human tumor xenografts, including activity in tumor models which do not respond to treatment with an EGFR TKI.[13] In addition, any change, or lack of change in microvessel density needs to be interpreted with caution as a either positive or negative indication of antiangiogenic activity, since the efficacy of antiangiogenic agents may not be related to microvessel density measurements.[14] ZD6474 was expected to induce increased apoptosis in tumor cells; although no induction of apoptosis was in fact observed, this may have been due to experimental factors.

Phosphorylations of Tyr845 and Tyr1045 of PC-9 and PC-9/ZD cells are similarly inhibited by ZD6474. On the other hand, while the inhibition pattern of Tyr845 phosphorylation by ZD6474 is coincident with that by gefitinib, the patterns at Tyr1045 are different. Therefore, we considered that the Tyr1045 is more important than Tyr845 for assessing the distinctive mode of action of ZD6474. In searching for a common mode of action of ZD6474 and gefitinib, Tyr845 seems to be the most promising site.

Phosphorylation of Tyr992 has been reported to transduce the signal to phospholipase C and protein kinase C.[15–17] In contrast, no inhibition of pan-phospho-PKC (the downstream signal of Tyr992) by gefitinib or ZD6474 was observed (data not shown). Tyr1045 has been reported to be linked to the Cbl-ubiquitin signaling pathway.[18] We have previously reported that Tyr1068 is a possible target site of EGFR for gefitinib[7], and gefitinib inhibited phosphorylation of Tyr1068 to varying degrees in PC-9 and PC-9/ZD cells, whereas ZD6474 inhibited Tyr1068 in both cell lines. These results suggest that the mode of inhibition of phosphorylation of EGFR by ZD6474 is subtly different to that of gefitinib. Therefore, although ZD6474 shows cross-resistance to gefitinib in these PC-9/ZD tumor cells, it has the potential for activity against gefitinib-resistant tumors through at least two mechanisms: (i) inhibition of EGFR-dependent downstream signaling pathways through differential effects on the phosphorylation status of tyrosine residues in the intracellular domain of EGFR, and (ii) inhibition of tumor angiogenesis through inhibition of VEGFR2 tyrosine kinase activity, which has not been examined in the present study. Site-directed mutagenesis studies are now under way to elucidate the biological significance of these sites.

1. Ciardiello F, Caputo R, Damiano V, Troiani T, Vitagliano D, Carlomagno F, Veneziani BM, Fontanini G, Bianco AR, Tortora G. Antitumor effects of ZD6474, a small molecule vascular endothelial growth factor receptor tyrosine kinase inhibitor, with additional activity against epidermal growth factor receptor tyrosine kinase. *Clin Cancer Res* 2003; **9**: 1546–56.
2. Ciardiello F, Bianco R, Caputo R, Damiano V, Troiani T, Melisi D, De Vita F, De Placido S, Bianco AR, Tortora G. Antitumor activity of ZD6474, a vascular endothelial growth factor receptor tyrosine kinase inhibitor, in human cancer cells with acquired resistance to antiepidermal growth factor receptor therapy. *Clin Cancer Res* 2004; **10**: 784–93.
3. Minami H, Ebi H, Tahara M, Sasaki Y, Yamamoto N, Yamada Y, Tamura T, Saijo N. A phase I study of an oral VEGF receptor tyrosine kinase inhibitor ZD6474, in Japanese patients with solid tumors. *Proc Am Soc Clin Oncol* 2003; **22**: abstr 778.
4. Hurwitz H, Holden SN, Eckhardt SG, Rosenthal M, de Boer R, Rischin D, Green M, Basser R. Clinical evaluation of ZD6474, an orally active inhibitor of VEGF signaling, in patients with solid tumors. *Proc Am Soc Clin Oncol* 2002; **22**: abstr 325.
5. Ciardiello F, Caputo R, Bianco R, Damiano V, Pomatico G, De Placido S, Bianco AR, Tortora G. Antitumor effect and potentiation of cytotoxic drugs activity in human cancer cells by ZD-1839 (Iressa), an epidermal growth factor receptor-selective tyrosine kinase inhibitor. *Clin Cancer Res* 2000; **6**: 2053–63.
6. Moasser MM, Basso A, Averbuch SD, Rosen N. The tyrosine kinase inhibi-

tor ZD1839 ("Iressa") inhibits HER2-driven signaling and suppresses the growth of HER2-overexpressing tumor cells. *Cancer Res* 2001; **61**: 7184–8.
7. Koizumi F, Kanzawa F, Ueda Y, Koh Y, Tsukiyama S, Taguchi F, Tamura T, Saijo N, Nishio K. Synergistic interaction between the EGFR tyrosine kinase inhibitor gefitinib ("Iressa") and the DNA topoisomerase I inhibitor CPT-11 (irinotecan) in human colorectal cancer cells. *Int J Cancer* 2004; **108**: 464–72.
8. Koizumi F, Taguchi F, Shimoyama T, Saijo N, Nishio K. Mechanism of resistance to epidermal growth factor receptor inhibitor ZD1839: a role for inhibiting phosphorylation of EGFR at Tyr1068. *Am Assoc Cancer Res* 2003: abstr 1001.
9. Kawamura-Akiyama Y, Kusaba H, Kanzawa F, Tamura T, Saijo N, Nishio K, Arioka H, Ishida T, Fukumoto H, Kurokawa H, Sata M, Ohata M, Morikage T, Ohmori T, Fujiwara Y, Takeda Y. Non-cross resistance of ZD0473 in acquired cisplatin-resistant lung cancer cell lines. *Lung Cancer* 2002; **38**: 43–50.
10. Nishio K, Arioka H, Ishida T, Fukumoto H, Kurokawa H, Sata M, Ohata M, Saijo N, Morikage T, Ohmori T, Fujiwara Y, Takeda Y. Enhanced interaction between tubulin and microtubule-associated protein 2 via inhibition of MAP kinase and CDC2 kinase by paclitaxel. *Int J Cancer* 1995; **63**: 688–93.
11. Naruse I, Ohmori T, Ao Y, Fukumoto H, Kuroki T, Mori M, Saijo N, Nishio K. Antitumor activity of the selective epidermal growth factor receptor-tyrosine kinase inhibitor (EGFR-TKI) Iressa (ZD1839) in an EGFR-expressing multidrug-resistant cell line *in vitro* and *in vivo*. *Int J Cancer* 2002; **98**: 310–5.
12. Ono M, Hirata A, Kometani T, Miyagawa M, Ueda S, Kinoshita H, Fujii T, Kuwano M. Sensitivity to gefitinib (Iressa, ZD1839) in non-small cell lung cancer cell lines correlates with dependence on the epidermal growth factor (EGF) receptor/extracellular signal-regulated kinase 1/2 and EGF receptor/Akt pathway for proliferation. *Mol Cancer Ther* 2004; **3**: 465–72.
13. Wedge SR, Ogilvie DJ, Dukes M, Kendrew J, Chester R, Jackson JA, Boffey SJ, Valentine PJ, Curwen JO, Musgrove HL, Graham GA, Hughes GD, Thomas AP, Stokes ES, Curry B, Richmond GH, Wadsworth PF, Bigley AL, Hennequin LF. ZD6474 inhibits vascular endothelial growth factor signaling, angiogenesis, and tumor growth following oral administration. *Cancer Res* 2002; **62**: 4645–55.
14. Hlatky L, Hahnfeldt P, Folkman J. Clinical application of antiangiogenic therapy: microvessel density, what it does and doesn't tell us. *J Natl Cancer Inst* 2002; **94**: 883–93.
15. Holbrook MR, O'Donnell JB Jr, Slakey LL, Gross DJ, Bishayee A, Beguinot L, Bishayee S. Epidermal growth factor receptor internalization rate is regulated by negative charges near the SH2 binding site Tyr992. *Biochemistry* 1999; **38**: 9348–56.
16. Bishayee A, Beguinot L, Bishayee S. Phosphorylation of tyrosine 992, 1068, and 1086 is required for conformational change of the human epidermal growth factor receptor c-terminal tail. *Mol Biol Cell* 1999; **10**: 525–36.
17. Nogami M, Yamazaki M, Watanabe H, Okabayashi Y, Kido Y, Kasuga M, Sasaki T, Maehama T, Kanaho Y, Holbrook MR, O'Donnell JB Jr, Slakey LL, Gross DJ, Bishayee A, Beguinot L, Bishayee S. Requirement of autophosphorylated tyrosine 992 of EGF receptor and its docking protein phospholipase C gamma 1 for membrane ruffle formation. *FEBS Lett* 2003; **536**: 71–6.
18. Ravid T, Sweeney C, Gee P, Carraway KL 3rd, Goldkorn T. Epidermal growth factor receptor activation under oxidative stress fails to promote c-Cbl mediated down-regulation. *J Biol Chem* 2002; **277**: 31214–9.