Effects of Vandetanib on Lung Adenocarcinoma Cells Harboring Epidermal Growth Factor Receptor T790M Mutation In vivo

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Abstract
Vandetanib is a novel multitarget tyrosine kinase inhibitor (TKI) that inhibits vascular endothelial growth factor receptor-2 (VEGFR-2), with additional inhibition of epidermal growth factor receptor (EGFR) and rearranged during transfection receptor signaling, which has shown promising results in clinical trials for advanced non–small cell lung cancer. However, the mechanisms of acquired resistance to vandetanib remain unclear. Therefore, we established in vitro vandetanib-resistant PC-9/VanR cells from PC-9, a vandetanib-sensitive lung adenocarcinoma cell line, by chronic exposure to this agent. PC-9/VanR cells were 50-fold more resistant to vandetanib than PC-9 cells in vivo. Compared with PC-9 cells, PC-9/VanR cells showed emergence of an EGFR T790M mutation, moderately elevated MET amplification, and similar VEGFR-2 inhibition by vandetanib. Note that phospho-MET in PC-9/VanR was suppressed following EGFR inhibition by an irreversible EGFR-TKI, indicating that MET signaling of PC-9/VanR was dependent on EGFR signaling and that MET amplification was not the primary mechanism of resistance to vandetanib. In contrast to the in vitro experiment, vandetanib effectively inhibited the growth of PC-9/VanR tumors in an in vivo xenograft model through the antiangiogenesis effects of VEGFR-2 inhibition. In conclusion, the multitarget TKI vandetanib induced or selected for the EGFR T790M mutation as observed previously with highly selective EGFR-TKIs. However, vandetanib retained significant efficacy in vivo against xenografts harboring the T790M mutation, providing a strong scientific rationale for investigating vandetanib in clinical settings where acquired resistance through emergence of EGFR T790M mutations limits the effectiveness of highly selective EGFR-TKIs. [Cancer Res 2009;69(12):5091–8]

Introduction
Dramatic clinical responses to the selective epidermal growth factor receptor (EGFR)-tyrosine kinase inhibitors (TKI) gefitinib and erlotinib have been observed in patients with advanced non–small cell lung cancer (NSCLC), especially in those with tumors harboring activating EGFR mutations (1–3). However, selective EGFR-TKIs have shown only limited survival benefits in NSCLC in some clinical trials (4, 5) and the majority of NSCLCs initially sensitive to gefitinib or erlotinib become resistant to these agents within 1 year of therapy (6). In patients with NSCLC who acquire resistance to gefitinib or erlotinib, ~50% have tumors with a secondary T790M mutation in exon 20 of EGFR (7–10) and ~20% have tumors with amplification of MET gene (11–13).

Recently, multitarget treatment strategies have been developed for various solid cancers, and a variety of multitarget TKIs [e.g., laptatinib (14), sunitinib (15), and sorafenib (16)] have been introduced for clinical use. In particular, vascular endothelial growth factor receptor (VEGFR) and EGFR seem to be key molecular targets in the treatment of NSCLC (5, 17). VEGFR-2 signaling promotes angiogenesis of the tumor microenvironment and is indirectly related to tumor growth. Bevacizumab, an anti-VEGF monoclonal antibody, has received Food and Drug Administration approval for treatment of nonsquamous NSCLC as well as for colorectal cancer and breast cancer (17, 18). A combination of bevacizumab with erlotinib seemed to have clinical benefit in the treatment of nonsquamous NSCLC (19). Vandetanib (ZACTIMA) is an orally available multitarget TKI inhibiting VEGFR-2, with additional inhibition of EGFR and rearranged during transfection (RET) receptor signaling (20–22). Vandetanib has shown promising results in phase II clinical trials in patients with pretreated NSCLC (23–25). Currently, phase III trials of vandetanib versus erlotinib (ZEST) and vandetanib in combination with docetaxel versus docetaxel alone (ZODIAC) are under way in patients with advanced NSCLC who have received prior therapy. However, it remains unknown whether prolonged treatment with multitarget TKIs, such as vandetanib, results in the emergence of the T790M EGFR mutation or MET gene amplification as reported previously for acquired resistance to the highly selective EGFR-TKIs gefitinib and erlotinib (7–13).

In this study, we established an in vitro vandetanib-resistant cell line from vandetanib-sensitive cells by chronic exposure to vandetanib to elucidate the mechanism of acquired resistance to this agent. We also examined the sensitivity of the resistant cells to vandetanib in an in vivo xenograft model.

Materials and Methods
Establishment of a vandetanib-resistant cell line. PC-9 cells were cultured at 37°C with 5% CO2 in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum. To establish a vandetanib-resistant subline, the cells were treated with gradually increasing concentrations of vandetanib, starting at 0.01 μM/L (lower than the IC50 of PC-9 cells). After 3 mo, the cells grew rapidly in the presence of 4 μM/L vandetanib, and then we performed a single-cell cloning by soft agar and established the vandetanib-resistant cell line (PC-9/VanR).

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Growth inhibition was determined using a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay using Cell Counting Kit-8 (Dojindo; ref. 26). Briefly, the cells were placed on
A, c cells continued to grow under this condition (Fig. 1A, a). 

IC50 ± SD values of vandetanib in PC-9 and PC-9/VanR cells were determined periodically. After the completion of the treatment, two mice per group were sacrificed and the tumor specimens were obtained for analysis. All experiments involving animals were performed under the auspices of the Institutional Animal Care and Research Advisory Committee at the Department of Animal Resources, Okayama University Advanced Science Research.

Immunofluorescence staining. Frozen tissues were used for immunofluorescence analyses to assess microvessel density in xenograft tissues. The samples were cut into 8-μm-thick sections and mounted on MAS-coated glass slides. After fixation with 4% paraformaldehyde, they were washed with PBS, incubated with 10% bovine serum albumin in PBS for 15 min, and then incubated with a 1:100 dilution of rabbit polyethylene-conjugated anti-CD31 polyclonal antibody (BD Biosciences) overnight at 4°C in the dark. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI; Roche Applied Sciences GmbH) for 2 h in the dark. These slides were examined under a fluorescence microscope (LSM510, Carl Zeiss, Inc.).

Results

Vandetanib-resistant PC-9/VanR cell line established by continuous exposure to vandetanib in vitro. We established a stable vandetanib-resistant PC-9/VanR cell line from vandetanib-sensitive cells by continuous exposure to increasing concentrations of this agent. The human NSCLC cell line PC-9 was chosen as the parent cell line because it is highly sensitive to vandetanib (27). PC-9 was derived from an untreated Japanese patient with pulmonary adenocarcinoma carrying an in-frame deletion in EGFR exon 19 (deletion of exons 18-22). On microscopic observation, PC-9/VanR cells showed the same morphology as PC-9 cells (Fig. 1A, a and b). PC-9/VanR cells proliferated more rapidly than PC-9 cells with doubling times of 20.5 and 27.1 hours, respectively (Fig. 1B). The parent PC-9 cells were very sensitive to vandetanib and could not proliferate in the presence of 1 μmol/L of this agent, whereas PC-9/VanR cells continued to grow under this condition (Fig. 1A, c and d). The IC50 ± SD values of vandetanib in PC-9 and PC-9/VanR cells determined by MTT assay were 0.091 ± 0.027 and 4.6 ± 0.28 μmol/L, respectively (Fig. 1C; Table 1). PC-9/VanR cells were 50-fold more resistant to vandetanib than the parental PC-9 cells. The resistance was stable for at least 2 years without exposure to vandetanib. PC-9/VanR cells showed cross-resistance to the selective reversible EGFR-TKI, gefitinib (IC50 ± SD: 8.2 ± 2.0 μmol/L; Table 1), and collateral sensitivity to irreversible EGFR-TKI, ZACTIMA (CL387,785). These results indicate that the PC-9/VanR cell line is a useful model for vandetanib-resistant NSCLC cells,

Phosphorylation levels of EGFR and its downstream signal in PC-9/VanR cells were not suppressed by vandetanib treatment. Immunoblotting assay revealed that phosphorylation of EGFR (pEGFR), phospho-HER2, and phospho-HER3 levels in PC-9/VanR cells were slightly higher than those in the parental PC-9 cells (Fig. 2a). RET receptor was not detected by immunoblotting in PC-9 or PC-9/VanR cells (data not shown); RET is mainly expressed in thyroid cancer (29).

The effects of vandetanib on pEGFR, phospho-Akt (pAkt) and pMAPK were also examined by immunoblotting (Fig. 2B). Vandetanib markedly suppressed pEGFR, pAkt, and pMAPK levels in PC-9 cells but not in the vandetanib-resistant PC-9/VanR cells.

EGFR T790M mutation and moderately increased MET amplification were detected in PC-9/VanR cells. The secondary T790M mutation is the major cause of acquired resistance to gefitinib and erlotinib in vivo and in vitro (7–10). To examine the genetic alterations, including the T790M mutation, we conducted direct sequencing assays of EGFR at exons 18 to 22, which revealed the T790M mutation at exon 20 as a minor peak in PC-9/VanR cells.
cDNA of PC-9/VanR cells was amplified by a PCR method using a primer specific to a wild-type EGFR or EGFR with exon 19 in-frame deletion and was sequenced. T790M mutation was detected only in cDNA amplified by the exon 19 in-frame deletion-specific primer, indicating that the T790M mutation of PC-9/VanR cells was in cis (data not shown). Recently, D761Y or L747S mutations at exon 19 have also been reported to be responsible for acquired EGFR-TKI resistance (30, 31); neither of these mutations was detected in the PC-9 and PC-9/VanR cells. No other genetic differences in EGFR DNA sequences between PC-9 and PC-9/VanR cells were detected (32).

The second major cause of the acquired resistance of NSCLC to EGFR-TKIs in vitro and in vivo involves amplification and overexpression of MET (11–13). We examined differences in MET amplification between PC-9 and PC-9/VanR cells using a quantitative PCR method. MET amplification was moderately but significantly higher in PC-9/VanR cells (Fig. 3B). MET phosphorylation and expression levels were also moderately higher in PC-9/VanR cells based on an immunoblotting analysis (Fig. 3C). Quantitative PCR of the cDNA revealed elevated mRNA levels of HGF, a ligand of MET, in PC-9/VanR cells (Fig. 3D).

**Table 1. IC<sub>50</sub> values of each TKI in PC-9 and PC-9/VanR cells determined by MTT assay**

<table>
<thead>
<tr>
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<th>Vandetanib</th>
<th>Gefitinib</th>
<th>CL387,785</th>
<th>SU11274</th>
</tr>
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<tbody>
<tr>
<td>PC-9</td>
<td>0.091 ± 0.027*</td>
<td>0.059 ± 0.045*</td>
<td>1.6 ± 0.79*</td>
<td>&gt;10</td>
</tr>
<tr>
<td>PC-9/VanR</td>
<td>4.6 ± 0.28*</td>
<td>8.2 ± 2.0*</td>
<td>0.72 ± 0.51*</td>
<td>&gt;10</td>
</tr>
<tr>
<td>RR</td>
<td>50</td>
<td>138</td>
<td>0.45</td>
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**Abbreviation: RR, relative resistance.**

*P < 0.05 (t test).
(pVEGFR-2) was inhibited to a similar extent in PC-9 and PC-9/VanR cells.

Taken together, these observations indicated that the T790M secondary mutation was the major mechanism of in vitro resistance to vandetanib in PC-9/VanR cells.

Effects of vandetanib on T790M harboring tumor in a xenograft model. We examined xenograft tumors to determine the degree of vandetanib resistance caused by the T790M mutation in vivo. In the vehicle-treated animals, PC-9 and PC-9/VanR xenograft tumors grew at almost the same rate (Fig. 5A, left, top and bottom). As anticipated, both vandetanib and gefitinib strongly inhibited the growth of PC-9 xenografts, producing marked tumor regression (Fig. 5A, left, top). In contrast to the in vitro experiment, vandetanib effectively inhibited the growth of PC-9/VanR tumors in the xenograft model, albeit to a lesser extent than in the PC-9 tumor xenografts, producing sustained inhibition of tumor growth rather than tumor regression (Fig. 5A, left, bottom). In contrast, gefitinib only modestly inhibited the growth of PC-9/VanR tumors. To know whether the superiority of vandetanib over gefitinib is confirmed in other cancer cells harboring T790M mutation,
two xenografts were examined using two lung adenocarcinoma cell lines, RPC-9 (exon 19 in-frame deletion/exon 20 T790M) and H1975 (exon 21 L858R/exon 20 T790M), both of which were highly resistant to gefitinib but were more sensitive to vandetanib than gefitinib in vitro (7, 9). The IC$_{50}$ ± SD values of gefitinib and vandetanib in the H1975 cells were 14.3 ± 0.7 and 6.9 ± 0.3 μmol/L, respectively. The RPC-9 and H1975 cells are more sensitive to vandetanib than gefitinib in vitro. Vandetanib inhibited the growth of RPC-9 xenografts to a significantly greater extent than gefitinib (P < 0.05), resulting in tumor regression (Fig. 5A, right, top). In the H1975 xenografts, vandetanib inhibited tumor growth to a greater extent than gefitinib (P < 0.05) but was unable to inhibit the growth of H1975 tumors completely (Fig. 5A, right, bottom).

We next examined the cause of the different inhibitory effects of vandetanib and gefitinib on PC-9/VanR xenografts via direct sequencing assay, an immunoblotting analysis, and immunofluorescence staining of PC-9/VanR tumor specimens obtained after completion of 4 weeks of treatment. T790M mutation was preserved in xenografts of every group (data not shown). As anticipated, vandetanib suppressed pVEGFR-2, whereas gefitinib did not (Fig. 5B, top). In addition, vandetanib treatment reduced pEGFR levels more effectively than gefitinib in PC-9/VanR tumors (Fig. 5B, top), although vandetanib and gefitinib inhibited pEGFR to a similar degree in the in vitro experiment (Fig. 5B, middle). Moreover, the total amount of EGFR was also decreased in vandetanib-treated PC-9/VanR tumors compared with vehicle-treated controls but remained unchanged in gefitinib-treated PC-9/VanR tumors.

To exclude the possibility that the difference of drug distribution between vandetanib and gefitinib caused the different tumor inhibition, we assessed pEGFR levels in xenograft tumors 2 hours after administering a single dose of vehicle, vandetanib (25 mg/kg), or gefitinib (25 mg/kg). Vandetanib and gefitinib incompletely inhibited pEGFR to a similar extent and did not alter the total amount of EGFR (Fig. 5B, bottom). From this result, it was estimated that vandetanib and gefitinib achieved similar distribution to PC-9/VanR xenografts.

Because vandetanib can inhibit VEGF signaling, we next stained by immunofluorescence with anti-CD31 antibody detecting microvessels to evaluate the state of angiogenesis in PC-9/VanR tumors. Compared with vehicle- or gefitinib-treated tumors, vandetanib-treated tumors showed markedly fewer CD31-positive cells (Fig. 5C). To confirm whether the superiority of vandetanib over gefitinib in the xenograft models was due to the antiangiogenic effects of vandetanib mediated by inhibition of VEGF signaling, PC-9/VanR xenografts were treated with bevacizumab (a VEGF inhibitor) alone or gefitinib in combination with bevacizumab (Fig. 5D). Bevacizumab alone inhibited tumor growth and combined gefitinib with bevacizumab inhibited tumor growth more efficiently than gefitinib alone.

**Discussion**

This is the first report of a secondary EGFR T790M mutation detected during treatment with a reversible multitarget TKI in vitro. Note that xenograft tumors bearing the T790M mutation remained sensitive to vandetanib, but not gefitinib, although these cell lines were resistant to both agents in vitro.

The T790M mutation at EGFR exon 20 (7–9) and MET gene amplification (11–13) were reported to be major causes of resistance to the selective EGFR-TKIs gefitinib and erlotinib. IGF-IR activation...
PTEN loss (37–39), and D761Y and L747S mutations at EGFR exon 20 (30, 31) have also been reported as other causes of resistance. PC-9/VanR cells acquired the secondary T790M mutation and moderately elevated MET amplification. Although it is possible that both mechanisms could be the causes of vandetanib resistance in PC-9/VanR cells, the collateral sensitivity to an irreversible EGFR-TKI, CL387,785 (Table 1), like other gefitinib-resistant cell lines bearing the T790M mutations (8, 9), and resistance to the MET inhibitor, SU11274, indicated ongoing oncogene addiction to EGFR signaling in PC-9/VanR cells. The microvessel density in PC-9/VanR xenograft tumors was evaluated by immunofluorescence staining. Frozen samples were taken from xenograft tumors treated with vehicle, vandetanib, or gefitinib after 4 wk of treatment. Vessel endothelial cells and nuclei of tumor cells were stained with anti-CD31 antibody and DAPI, respectively. PC-9/VanR xenograft tumor volume after 3 wk of treatment with each drug. Tumor volume relative to vehicle control group was measured. Bars, SE. Differences in tumor volume were compared using Student’s t test.

The mechanism associated with emergence of the T790M mutation during treatment with gefitinib or erlotinib is not fully understood. Vandetanib shares some common structural features with gefitinib and erlotinib (42–44), and these may contribute to the emergence of the T790M mutation during prolonged exposure to vandetanib.
We next examined whether vandetanib still had some advantages over selective EGFR-TKIs even in the presence of an EGFR T790M mutation due to its ability to inhibit other RTKs, including VEGFR-2. First, we compared the sensitivity to vandetanib with that to gefitinib in PC-9/VanR cells in vitro. The IC_{50} value of vandetanib in PC-9/VanR cells was lower than that of gefitinib, although in the parent PC-9 cells, the IC_{50} value of vandetanib was higher than that of gefitinib (Table 1). A similar tendency was also observed in RPC-9 cells (exon 19 in-frame deletion/exon 20 T790M), a subline that we had previously established from PC-9 cells by continuous exposure to gefitinib (IC_{50} values of vandetanib and gefitinib in RPC-9 cells were around 5 and 8 μmol/L, respectively; ref. 9) and H1975 (exon 21 L858R/exon 20 T790M; IC_{50} values of gefitinib and vandetanib were around 7 and 14 μmol/L). VEGFR-2 signaling not only may act to promote angiogenesis of tumor vessels but may also act as a signal in tumor cells (45), and the effect of VEGFR-2 inhibition by vandetanib could become more prominent following the emergence of the EGFR T790M mutation. However, compared with PC-9 cells, PC-9/VanR cells were still relatively resistant to vandetanib, suggesting that VEGFR-2 signaling did not play a major role in PC-9/VanR cell proliferation, although the IC_{50} value of vandetanib was lower than that of gefitinib.

Therefore, we next investigated vandetanib in a xenograft model with the expectation that the antiangiogenic nature of the microenvironment may show additional inhibitory effects on PC-9/VanR tumors. Vandetanib inhibited the growth of PC-9/VanR tumors in vivo more effectively than gefitinib (P < 0.05; Fig. 5A, bottom). This advantage for vandetanib over gefitinib was not particular to the PC-9/VanR xenograft but was also seen in T790M-harboring RPC-9 and H1975 xenografts. Vandetanib prevented angiogenesis of the tumor microenvironment through the inhibition of VEGFR-2 (Fig. 5B) and C, resulting in an indirect antitumor effect. Note that in the PC-9/VanR xenograft model, the total amount of EGFR decreased in the vandetanib-treated group but remained unchanged in the gefitinib-treated group (Fig. 5B, left). Thus, the vandetanib treatment resulted in lower pEGFR levels than the gefitinib treatment in PC-9/VanR xenograft tumors (Fig. 5B, left), although vandetanib was reported to have less potent EGFR inhibitory activity than gefitinib in vitro (43). These observations raise the question of what may be responsible for the difference in EGFR expression between the vandetanib- and gefitinib-treated xenograft groups. In both groups, levels of EGFR mRNA determined by quantitative PCR were the same as in the vehicle control group (data not shown). Thus, the decreased EGFR expression was considered due to a postranslational process. The precise cause of this postranslational difference in EGFR expression is not known. One possible explanation is that vandetanib may have had a structural advantage over gefitinib with regard to the EGFR exon 19 in-frame deletion plus the exon 20 T790M mutation. An alternative explanation is that vandetanib improved drug delivery through VEGFR-2 inhibition resulting in normalization of microenvironmental vessels (46). Although it is not yet possible to determine which, if either, of these explanations is correct, VEGFR-2 inhibition and greater EGFR inhibition in vivo by vandetanib would have worked together and thus inhibited the growth of PC-9/VanR xenografts more potently than gefitinib.

With regard to how best to overcome the T790M resistance mutation, irreversible EGFR-TKIs such as HKI-272 and EKB-569 are attracting attention because of their promising effectiveness in ex vivo experiments (47). However, to date, no reports have described their effectiveness against the T790M mutation in a clinical setting. In the phase É trial of HKI-272 and EKB-569, dose-limiting toxicity occurred with these drugs at plasma concentrations of 0.04 to 0.2 μmol/L (48, 49), whereas the in vivo studies were performed using concentrations of >1 μmol/L (41, 47). In the clinical setting, irreversible EGFR-TKIs may reach the maximum tolerated dose (MTD) before exerting the antitumor effect, possibly due to their strong inhibition of EGFR. In contrast to these irreversible EGFR-TKIs, the plasma concentration of vandetanib at MTD was reported to be >2 μmol/L, although this is still below the IC_{50} value of vandetanib in PC-9/VanR cells. In the case of vandetanib, it may be possible that VEGFR-2 inhibition together with the ability to achieve higher plasma drug concentrations would overcome the T790M mutation in a clinical setting.

In conclusion, prolonged exposure of PC-9 cells to the multitarget TK1 vandetanib selected for or induced the EGFR T790M mutation, as reported for other highly selective EGFR-TKIs, gefitinib and erlotinib, and produced a cell line (PC-9/VanR) that was resistant to the antitumor effects of vandetanib in vitro. However, in vivo, vandetanib remained effective against PC-9/VanR tumor xenografts. This in vivo efficacy also extended T790M-harboring RPC-9 and H1975 tumor cell xenografts. These data suggest that vandetanib may produce more durable effects than highly selective EGFR-TKIs in a clinical setting and further investigation of vandetanib in patients harboring T790M mutations is warranted.

Disclosure of Potential Conflicts of Interest

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