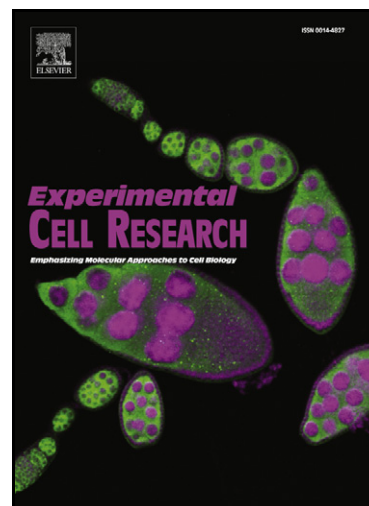


## Author's Accepted Manuscript

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# Vandetanib is effective in EGFR-mutant lung cancer cells with PTEN deficiency

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**Abstract** (195 ≤ 200 words)

The effectiveness of vandetanib, an agent that targets RET, VEGFR and EGFR signaling, against EGFR-mutant lung cancer cells with PTEN loss was investigated. Two EGFR mutant

non-small cell lung cancer (NSCLC) cell lines, PC-9 (PTEN wild type) and NCI-H1650 (PTEN null), were used. We transfected an intact PTEN gene into H1650 cells and knocked down PTEN expression in PC-9 cells using shRNA. The effectiveness of gefitinib and vandetanib was assessed using a xenograft model. While PC-9 cells were more resistant to vandetanib than to gefitinib, H1650 cells were more sensitive to vandetanib than to gefitinib. Both gefitinib and vandetanib suppressed the activation of EGFR and MAPK in H1650 cells, although phosphorylated AKT levels were not affected. In an H1650 cell xenograft model, vandetanib was also more effective than gefitinib. Although PTEN-transfected H1650 cells did not show restoration of sensitivity to gefitinib *in vitro*, the xenograft tumors responded to gefitinib and vandetanib. Knockdown of PTEN in PC-9 cells caused resistance to gefitinib. In conclusion, vandetanib might be effective in NSCLC with EGFR mutations and that lack PTEN expression. The contribution of PTEN absence to vandetanib activity in NSCLC cells harboring EGFR mutations should be further examined.

**Highlights** (3 to 5 bullet points)

- Vandetanib is effective against EGFR mutant lung cancer cell lines without PTEN.
- PTEN restoration causes sensitization to gefitinib *in vivo*, but not *in vitro*.
- PTEN ablation leads to resistance to gefitinib *in vitro*.

**Keywords** ( $\leq 6$  words)

Lung cancer, vandetanib, gefitinib, EGFR, VEGFR, PTEN.

**Abbreviations**

Epidermal growth factor receptor (EGFR), vascular endothelial growth factor receptor (VEGFR), phosphatase and tensin homolog (PTEN), non-small cell lung cancer (NSCLC), tyrosine kinase inhibitor (TKI).

**Introduction**

Epidermal growth factor receptor (EGFR) is a receptor tyrosine kinase that acts as a mediator of cell proliferation and survival signaling. The EGFR gene is frequently mutated in non-small cell lung cancer (NSCLC) with an adenocarcinoma histology in never smokers, particularly in Asians [1]. EGFR-activating mutations lead to so-called “oncogene addiction”, a state in which a cancer cell is dependent on continued activation of a specific gene to retain a malignant phenotype [2]. Mutations often predict a dramatic response to EGFR tyrosine kinase inhibitors (TKIs) such as gefitinib and erlotinib [3-5]. The majority of EGFR mutant lung cancers that are initially sensitive to EGFR-TKIs become resistant to these agents within 1 year [6]. Possible mechanisms of the acquired resistance have been identified, the most common of which is development of an EGFR T790M gatekeeper mutation, which occurs in

~50% of cases [7-10]. Other reported mechanisms of acquired resistance include MET amplification [11], hepatocyte growth factor expression [12], small cell transition [13], and epithelial-mesenchymal transition [14].

*Phosphatase and tensin homolog (PTEN)* is a tumor suppressor gene located on human chromosome 10q23 that deactivates PI3K, which signals downstream of EGFR [15]. Although genetic alterations of the PTEN gene in NSCLC are rare, PTEN loss caused by promoter methylation is not uncommon [16]. Approximately 2–9% of NSCLC tumors lack PTEN [17]. In one study, PTEN mutations were found in eight (4.5%) of 176 NSCLC tumors, one which had a concurrent EGFR mutation [18]. In another study, PTEN loss and EGFR mutations co-occurred in one of 24 EGFR mutant patients with lung adenocarcinoma [19]. PTEN loss is considered indicative of primary or acquired resistance to EGFR-TKIs [20-23]. Additionally, co-occurrence of EGFR mutation and loss of PTEN was correlated with EGFR-TKI potency in glioblastoma, a common primary malignant brain tumor [24]. Therefore, a new strategy to combat EGFR-TKI resistance is needed.

Vandetanib is a multi-targeted TKI that inhibits EGFR, VEGFR and rearranged during transfection (RET) receptor [25, 26]. This agent demonstrated efficacy in NSCLC cell lines harboring EGFR-activating mutations, including the T790M mutation [10, 27]. Four phase III trials of vandetanib in a broad population of NSCLC patients have been reported: as monotherapy, *versus* placebo, in patients previously treated with anti-EGFR therapy

(ZEPHYR) [28]; *versus* erlotinib (ZEST) [29]; and in combination with docetaxel (ZODIAC) [30] or pemetrexed (ZEAL) [31] in global trials. Only the ZODIAC trial met its primary endpoint (progression-free survival). While no study reported an advantage in overall survival with vandetanib, erlotinib and vandetanib showed equivalent progression-free survival and overall survival in the ZEST trial [29]. In four all-comers trials, the efficacy of vandetanib could not be demonstrated. Meanwhile, the BATTLE trial, a prospective, biopsy-mandated, biomarker-based, adaptively randomized phase II study, demonstrated that the individual markers that predicted better 8-week disease control by treatment [*versus* the opposite status (absence or presence)] were EGFR mutations for erlotinib ( $P = 0.04$ ) and high VEGFR2 expression for vandetanib ( $P = 0.05$ ) [32]. If the patients were selected according to target molecules, efficacy of vandetanib was presumed, even in a small sample.

In this study, we focused on PTEN status in EGFR-mutated NSCLC and hypothesized that vandetanib might overcome gefitinib resistance in tumors lacking PTEN.

## **Materials and methods**

### **Cell lines**

The human NSCLC cell lines PC-9 and NCI-H1650 were derived from patients with pulmonary adenocarcinomas that carried in-frame deletions in *EGFR* exon 19 (del E746-A750). PC-9 cells are highly sensitive to EGFR-TKI [10]. NCI-H1650 cells also harbor

homozygous deletion in *PTEN*, with the 3' part of exon 8 and all of exon 9 being deleted [19].

PC-9 cells and H1650 cells were purchased from Immuno-Biological Laboratories (Gunma, Japan) and from ATCC (American Type Culture Collection, Rockville, MD), respectively.

The cells were cultured at 37°C in 5% CO<sub>2</sub> in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum.

### **3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay**

Growth inhibition was measured by a modified MTT assay. Briefly, the cells were plated on 96-well plates at a density of 2,000 cells per well and exposed to each gefitinib or vandetanib for 72 h. Each assay was performed in triplicate. The 50% inhibitory concentration (IC<sub>50</sub>) of each drug was determined as the mean ± standard deviation (SD).

### **Protein extraction and Western blot analysis**

Vandetanib and gefitinib were kindly provided by AstraZeneca. H1650 cells were exposed to gefitinib and vandetanib for 6 h. Cells were lysed in RIPA buffer [1% Triton X-100, 0.1% SDS, 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10 mM β-glycerol-phosphate, 10 mM NaF, 1 mM Na-orthovanadate] containing a protease inhibitor tablet (Roche). Rabbit antibodies against EGFR, phospho (p)EGFR (Y1068), pHER2 (Y1248), pHER3 (Y1289), mitogen-activated protein kinase (MAPK), pMAPK (T202/pY204), pAKT (Ser473), PTEN and β-actin were purchased from Cell Signaling Technology. Polyclonal antibodies against pVEGFR2 (Y1054) and VEGFR2 were purchased

from BioSource. Each sample was incubated with the appropriate primary antibody, and signals were detected by HRP-mediated chemiluminescence (ECL Plus).

### ***PTEN* transfection**

The *PTEN* gene was cloned from PC-9 cells. PC-9 mRNA samples were prepared for reverse transcription-polymerase chain reaction (RT-PCR) using an RNeasy Mini Kit (Qiagen), according to the manufacturer's protocol. cDNA was synthesized using SuperScript II Reverse Transcriptase (Invitrogen). The *PTEN* gene was cloned from cDNA from PC-9 cells by PCR using the following primers: forward,

5'-TGTTGAATTCTTCAGCCACAAGCTCCCAGACATGACAGCCATCATCAAAGAGA

TCG-3'; and reverse,

5'-TTGCTCTAGATTATCAGACTTTTGTAATTTGTGTATGCTGATCTTCATC-3'. The

product of this PCR reaction was digested using *EcoRI* and *XbaI*, and then inserted into the pcDNA3.1(+) plasmid vector (Invitrogen). The *PTEN* expression vector was introduced into H1650 cells using Fugene6 (Roche). Stable *PTEN*-expressing clones were isolated by limiting dilution.

### ***PTEN* knockdown**

The pBasi-hU6 Neo vector (Takara bio) for expression of shRNA was employed to suppress the *PTEN* gene. The target sequence for *PTEN*-suppression was 5'-

AUAGCUACCUGUAAAGAA -3'. This vector was introduced into PC-9 cells using



Fugene6, and the clone with the greatest PTEN suppression was isolated by limiting dilution.

#### **Animal husbandry and drug administration**

Five-week-old female athymic mice were purchased from Japan Charles River Co. All mice were provided with sterilized food and water and housed in a barrier facility under a 12-h light/dark cycle. All animals were kept under conditions that complied with the guidelines of the Department of Animal Resources, Okayama University Advanced Science Research Center. Gefitinib and vandetanib were administered once per day, 5 days per week, by gavage as a 15 mg/kg suspension. The suspension was prepared in 1% polysorbate 80 by homogenization and ball-milled with glass beads. All procedures were performed in accordance with institutional guidelines for the protection of animals.

#### **Xenograft model**

One million H1650 cells or H1650/PTEN cells (H1650 cells with a transfected *PTEN* gene) were injected subcutaneously into the backs of each mouse. At 10 days after injection, mice were randomly assigned to three groups, which received either vehicle, vandetanib (15 mg/kg/day), or gefitinib (15 mg/kg/day). Vehicle, vandetanib, and gefitinib were administered once per day p.o., five times per week. Tumor volume (width × width × length / 2) and body weight were determined periodically. Tumor volumes were expressed as means ± SD. Differences in tumor volume were evaluated using Student's *t*-test.

## Results

The dose-response curve of H1650 cells is shown in Fig. 1A. The  $IC_{50}$  values of gefitinib and vandetanib were  $34.3 \pm 3.3 \mu\text{M}$  and  $3.5 \pm 1.2 \mu\text{M}$ , respectively. The sensitivity to vandetanib was significantly (3 to 10 times) higher than that to gefitinib in H1650 cells *in vitro*. Next, the efficacies of vandetanib and gefitinib against H1650 cells were determined *in vivo*. H1650 xenograft tumor volumes in mice treated with gefitinib, vandetanib, or vehicle ( $n = 8$ ) are shown in Fig. 1B. Vandetanib significantly suppressed tumor growth compared with gefitinib after day 15 ( $P < 0.01$ ) ( $P = 0.005$  at day 22). Protein expression profiles in H1650 cells after treatment with gefitinib or vandetanib are shown in Fig. 1C. Gefitinib more potently reduced pEGFR levels than did vandetanib. This was expected based on the kinase selectivity of vandetanib [25]. Levels of pMAPK, pAKT and pSTAT3 were similar in cells treated with either drug. pVEGFR2, pHER3 and pHER2 were not detected in H1650 cells, but were detected in PC-9 cells.

The *PTEN* gene cloned from PC-9 cells was successfully transfected into H1650 cells (Fig. 2A). The dose-response curves in parental H1650 cells and transfected H1650 cells (H1650/PTEN) treated with gefitinib for 72 h are shown in Fig. 2B. The  $IC_{50}$  value in H1650/PTEN cells ( $31.4 \pm 4.9 \mu\text{M}$ ) was similar to that in the parental cells ( $34.3 \pm 3.3 \mu\text{M}$ ). H1650 xenograft tumor volumes in mice receiving the indicated drug (15 mg/kg of gefitinib or 15 mg/kg of vandetanib;  $n = 6$ ) are shown in Fig. 2C. Gefitinib inhibited the growth of

H1650/PTEN xenograft tumors, but not H1650 parental xenograft tumors ( $P < 0.01$  at day 22). In contrast, vandetanib had similar effects on the growth of both types of xenograft tumors ( $n = 6$ ) (Fig. 2D).

Intrinsic PTEN expression in PC-9 cells was partially ablated by an shRNA-expression vector (Fig. 3A). The third clone from the left lane, which exhibited the lowest PTEN expression, was selected for further experiments. The drug sensitivities of PC-9 parental cells and PTEN-knockdown PC-9 cells are shown in Figs. 3B and 3C. Knockdown of PTEN led to about five times more resistance to gefitinib in PC-9 cells in terms of  $IC_{50}$  values, which for gefitinib were  $0.012 \pm 0.003 \mu\text{M}$  in PC-9 parental cells and  $0.063 \pm 0.03 \mu\text{M}$  in PTEN-knockdown PC-9 cells ( $P = 0.045$ ). Meanwhile, the  $IC_{50}$  value of vandetanib was  $0.086 \pm 0.01 \mu\text{M}$  in PC-9 parental cells and  $0.17 \pm 0.03 \mu\text{M}$  in PTEN-knockdown PC-9 cells ( $P < 0.01$ ). Contrary to our expectations, PTEN deficiency led to resistance *in vitro* not only to gefitinib but also to vandetanib.

## Discussion

We demonstrated that vandetanib exhibited better efficacy than gefitinib *in vitro*, at clinically achievable concentrations, against H1650 cells harboring both EGFR mutations and PTEN loss (Fig. 1A); the plasma concentration of vandetanib can be  $>2 \mu\text{M}$  [33]. Vandetanib also had a superior anti-tumor effect than gefitinib in the H1650 xenograft model.

Both gefitinib and vandetanib suppressed activation of EGFR and MAPK at concentrations of  $\leq 1 \mu\text{M}$  *in vitro* (Fig. 1C). In contrast, AKT phosphorylation was preserved at the same concentration. These results suggest that H1650 cells are not dependent on the EGFR-MAPK axis for survival. Ablation of PTEN in PC-9 cells carrying an *EGFR* mutation induced resistance to gefitinib (Fig. 3B). PTEN loss was previously reported to be associated with sensitivity to gefitinib in NSCLCs with EGFR mutations [21]. The reason that vandetanib was effective in PTEN-deficient and EGFR-mutant cells remains unclear. We examined the status of both VEGFR and RET, which are inhibited by vandetanib. VEGFR1 mRNA levels were higher in PC-9 cells than in H1650 cells (data not shown). pVEGFR2 (Fig. 1C) and VEGFR2 mRNA (data not shown) were not detected in H1650 cells. VEGFR3 and RET mRNAs were not detected in either cell line (data not shown). Thus, we could not explain why vandetanib was more effective than gefitinib based on the major targets of vandetanib (EGFR, VEGFR2, and RET). Therefore, vandetanib, but not gefitinib, might inhibit unknown targets *in vitro*.

Although PTEN loss did not affect gefitinib sensitivity (Fig. 2B), gefitinib was effective in the xenografts (Fig. 2C). The same experiments were repeated *in vivo* using H1650 and H1650/PTEN cells ( $n = 8$ ) (Supplementary Fig. 1). It was confirmed that gefitinib suppressed H1650/PTEN xenograft tumors. Thus, the efficacy of gefitinib might be affected by the deactivation of signaling molecules that act downstream of EGFR, supporting data

published previously [19].

A possible explanation is that the major effect of vandetanib in this model (Figs. 1B, 2D) was not inhibition of EGFR. The different effects of vandetanib and gefitinib might be due to differences in their ability to suppress VEGF/VEGFR signaling *in vivo*. We hypothesized that VEGF-A levels differ between H1650 and H1650/PTEN cells. Supernatants were collected after culture for 6 days. VEGF-A levels in supernatants were determined in triplicate by enzyme-linked immunosorbent assay (Human VEGF Quantikine; R&D Systems, Minneapolis, USA). VEGF-A secretion (mean  $\pm$  SD:  $1397 \pm 593$  pg/mL) by H1650/PTEN cells tended to be lower than that ( $1967 \pm 539$  pg/mL) by H1650 cells ( $P = 0.14$ ). VEGF-A production by tumor cells results in VEGFR2 activation on the neovasculature around tumors. Vandetanib may block this signal in our xenograft model by inhibiting VEGF/VEGFR in stromal cells. Actually, a low baseline plasma VEGF concentration had a significantly superior progression-free survival when treated with vandetanib monotherapy compared with gefitinib monotherapy [34].

Sos *et al.* reported that H1650 cells are erlotinib-resistant and retained high levels of pAKT despite inhibition of EGFR [19]. They silenced *PTEN* in PC-9 cells using lentiviral short hairpin RNAs and this led to resistance to erlotinib. We confirmed this using gefitinib instead of erlotinib. Erlotinib-mediated inhibition of EGFR can be rescued by activation of the PI3K/AKT/mTOR pathway in cells lacking PTEN expression. The mTOR pathway,

which is located downstream of EGFR, plays an important role in cell proliferation and maintenance of the malignant phenotype, especially in PTEN-deficient tumors. The effectiveness of compounds targeting mTOR, such as rapamycin, CCI-779 and everolimus, in NSCLC has been explored [35, 36]. The combination of PI3K/AKT/mTOR pathway inhibitors with an EGFR-TKI may be beneficial for the resistant tumor although our data suggest the potency of vandetanib.

The major point of the study is that PTEN-status alone does not explain the activity of vandetanib in EGFR-mutant lung cancers cells when employed a xenograft model using PTEN-transfected cells, as shown in Fig. 2D. The existence of mutated EGFR in lung cancer cells might be necessary, but not sufficient, to explain the overall *in vivo* efficacy of vandetanib, as shown in Fig. 2D vs. Fig. 3C. Thus, the dependency on the EGFR status should be further pursued. Bivona *et al.* knocked down the major NF- $\kappa$ B subunit RELA and found that RELA knockdown also induced erlotinib sensitivity in H1650 cells [37]. Its erlotinib-sensitizing effect was specific to mutant EGFR because no potentiating effect was seen in wild-type EGFR cells. Meanwhile, Kim *et al.* reported that expression of RELA of NF- $\kappa$ B decreased PTEN expression and resulted in increased AKT activation *in vitro* [38]. The interaction between PTEN/PI3K/AKT and NF- $\kappa$ B in EGFR-TKI resistance should be further investigated.

Although vandetanib was shown not to be useful for non-selected NSCLC in

all-comers trials [28-31], it may have effects on some NSCLCs that exhibit specific molecular targets, such as mutated EGFR, VEGFR2 [32] and KIF5B-RET fusions [39] in tumor cells. Vandetanib might be useful for NSCLC patients with EGFR mutations and PTEN loss. Clinical studies of such NSCLC cases selected by biomarkers are warranted.

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**Figure legends****Fig. 1** Effects of gefitinib and vandetanib on H1650 cells.

(A) Dose-response curve for H1650 cells. The IC<sub>50</sub> values of gefitinib and vandetanib were 34.3 ± 3.3 and 3.5 ± 1.2 μM, respectively ( $P < 0.01$ ). (B) H1650 xenograft tumor volumes in mice treated with gefitinib or vandetanib (15 mg/kg p.o. daily) or vehicle alone (n = 8, each). Vandetanib was more effective than gefitinib ( $P = 0.005$  at day 22). Differences in tumor volume were compared using Student's *t*-test. (C) Protein levels in H1650 cells after treatment with gefitinib or vandetanib. Gefitinib was a more potent suppressor of pEGFR than was vandetanib. Levels of pMAPK, pAKT, and pSTAT3 were similar in cells treated with either drug.

**Fig. 2** Effects of gefitinib and vandetanib on *PTEN*-transfected H1650 cells.

(A) The *PTEN* gene cloned from PC-9 cells was transfected into H1650 cells. PTEN protein expression was determined by Western blotting. (B) Dose-response curves for H1650 cells and H1650 cells transfected with PTEN (H1650/PTEN cells). (C) Xenograft tumor volumes in mice treated with gefitinib (n = 6, each). Gefitinib was more effective in H1650/PTEN cells than in H1650 parent cells. (D) Xenograft tumor volumes in mice treated with vandetanib (n = 6, each). Vandetanib had similar effects on both types of xenograft tumors.

**Fig. 3** Effect of PTEN knockdown in PC-9 cells.

(A) PTEN was partially ablated using an shRNA expression vector. The third clone from the left lane was selected for subsequent experiments. (B) Dose-response curves for parental PC-9 cells and PTEN-knockdown PC-9 cells. The  $IC_{50}$  values of gefitinib were  $0.012 \pm 0.003$   $\mu$ M in parental PC-9 cells and  $0.063 \pm 0.03$   $\mu$ M in PTEN-knockdown PC-9 cells ( $P = 0.045$ ). (C) Dose-response curves for parental PC-9 cells and PTEN-knockdown PC-9 cells. The  $IC_{50}$  values of vandetanib were  $0.086 \pm 0.01$   $\mu$ M in parental PC-9 cells and  $0.17 \pm 0.03$   $\mu$ M in PTEN-knockdown PC-9 cells ( $P < 0.01$ ).

### Supplementary Fig. 1

The experiment shown in Fig. 2C was repeated ( $n = 8$ , each). The results confirmed that gefitinib suppressed H1650/PTEN xenograft tumors.

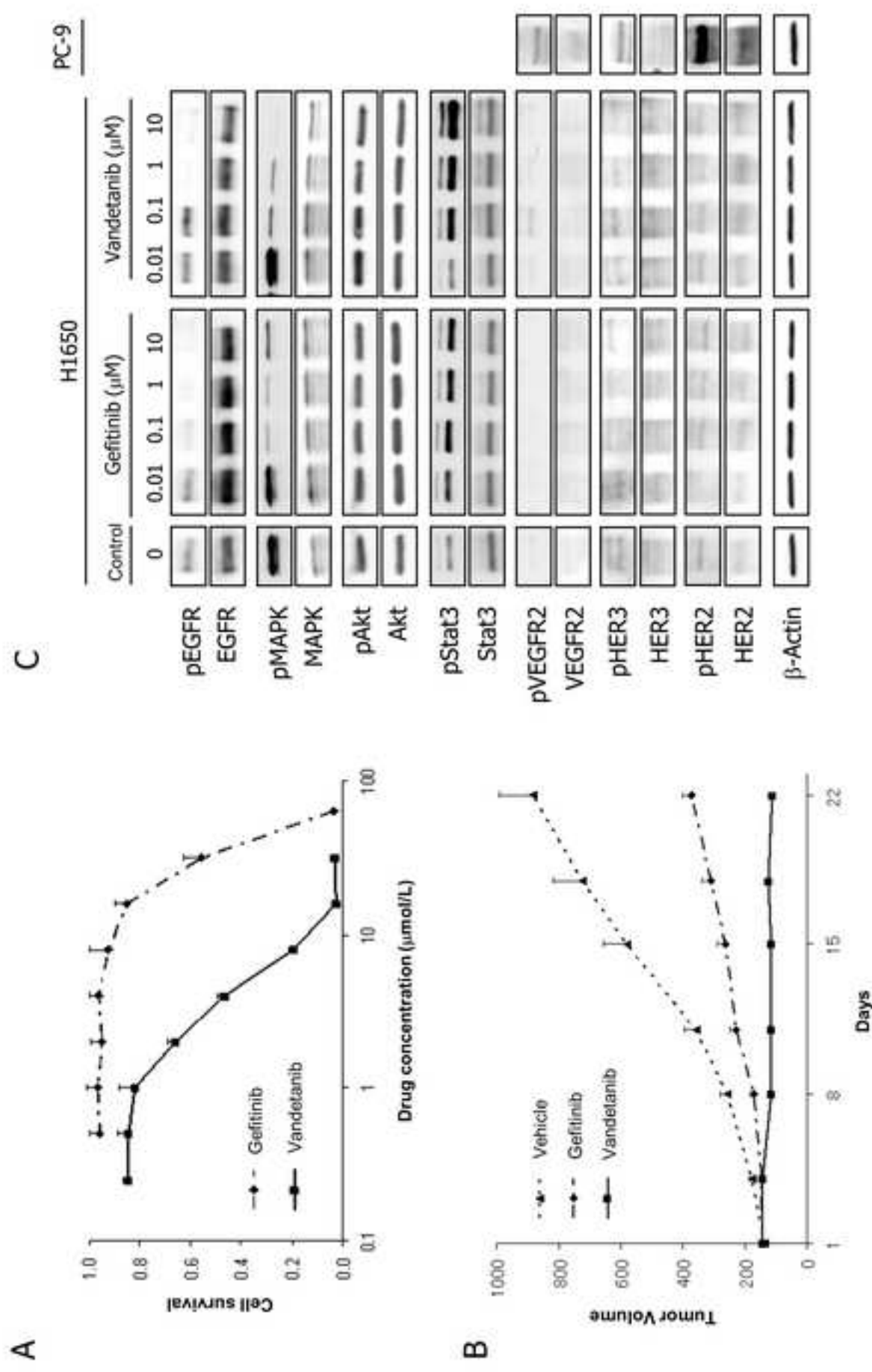


Figure 1

