ond phase 2 study in Japanese patients with NSCLC, second-line erlotinib treatment resulted in median OS of 13.5 months (410 days).[3]

We acknowledge that there are several limitations of this study, including the fact that this was a single-arm observational study with no control group, and the lack of a strict observation period, unlike a clinical trial. The lack of information on *EGFR* mutation status is also considered a limitation as this is known to strongly affect the efficacy of erlotinib. The lack of patient selection criteria may also be seen as a limitation; however, this may mean that our study population was more representative of the actual Japanese population than would be the case in a clinical trial, especially because of the large patient population in this study. The information on EGFR TKI-associated ILD in this study is thought to be decisive; it provides valuable information for treatment considerations and monitoring in Japanese patients with *EGFR* mutant or wild-type lung cancer.

Healthcare providers should carefully observe patients during treatment with erlotinib to ascertain whether the patient has any of the risk factors detailed in this analysis. After suspicion of the onset of ILD and diagnosis by CT, it is important to follow the patient's status continuously and carefully monitor their risk level. The final safety and efficacy data from the large-scale POLARSTAR surveillance study confirm that erlotinib has a well-characterized safety profile with proven efficacy in Japanese patients; however, the risk of ILD should still be monitored.

## Acknowledgments

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## Disclosure Statement

KN, SK, YO, TJ, MA, NY, and MF have all participated as independent advisory board members for erlotinib, reimbursed by Chugai Pharmaceutical Co. Ltd. YO also has an immediate family member who is an employee of Chugai Pharmaceutical Co. Ltd. HA, YI, ME, TJ, MK, KK, FS, HT, AG, and YF have all participated as independent ILD Review Committee members for erlotinib, reimbursed by Chugai Pharmaceutical Co. Ltd. AS and TI are full-time employees of Chugai Pharmaceutical Co. Ltd. This trial was designed, funded, and monitored by Chugai Pharmaceutical Co. Ltd. Data were gathered, analyzed, and interpreted by Chugai with input from all authors. The corresponding author had full access to the relevant data and took full responsibility for the final decision to submit the report for publication. Although technically classed as a clinical trial, the POLARSTAR study was a non-interventional surveillance study analyzing all NSCLC patients receiving erlotinib in Japan, therefore it was not registered as a phase II/III clinical trial would be.

## Abbreviations

| | |
|---|---|
| ADR | adverse drug reaction |
| AE | adverse event |
| CI | confidence interval |
| COPD | chronic obstructive pulmonary disease |
| CT | computed tomography |
| DAD | diffuse alveolar damage |
| ECOG | PS Eastern Cooperative Oncology Group performance status |
| EGFR | epidermal growth factor receptor |
| HR | hazard ratio |
| ILD | interstitial lung disease |
| NSCLC | non-small-cell lung cancer |
| OR | odds ratio |
| OS | overall survival |
| PFS | progression-free survival |
| POLARSTAR | Post-Launch All-patient-Registration Surveillance in Tarceva®-treated NSCLC patients |
| TKI | tyrosine-kinase inhibitor |

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# The anti-HER3 antibody patritumab abrogates cetuximab resistance mediated by heregulin in colorectal cancer cells

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## ABSTRACT

**We previously showed that tumor-derived heregulin, a ligand for HER3, is associated with both de novo and acquired resistance to cetuximab. We have now examined whether patritumab, a novel neutralizing monoclonal antibody to HER3, is able to overcome such resistance. Human colorectal cancer (DiFi) cells that are highly sensitive to cetuximab were engineered to stably express heregulin by retroviral infection, and the effects of cetuximab and patritumab on the resulting DiFi-HRG cells were examined. DiFi-HRG cells released substantial amounts of heregulin and showed resistance to cetuximab. Cetuximab alone inhibited EGFR and ERK phosphorylation in DiFi-HRG cells, but it had no effect on the phosphorylation of HER2, HER3, or AKT, suggesting that sustained AKT activation by HER2 and HER3 underlies cetuximab resistance in these cells. In contrast, patritumab in combination with cetuximab markedly inhibited the phosphorylation of EGFR, HER2, HER3, ERK, and AKT. The combination therapy also inhibited the growth of DiFi-HRG tumor xenografts in nude mice to a greater extent than did treatment with either drug alone. Activation of HER2-HER3 signaling associated with the operation of a heregulin autocrine loop confers resistance to cetuximab, and patritumab is able to restore cetuximab sensitivity through inhibition of heregulin-induced HER3 activation.**

## INTRODUCTION

Cetuximab, a chimeric human-mouse monoclonal antibody to the epidermal growth factor receptor (EGFR), has shown clinical efficacy in individuals with metastatic colorectal cancer (mCRC). However, a subset of mCRC patients fails to show an initial response (de novo resistance) to this agent, whereas others develop resistance after an initial response (acquired resistance). Well-established causes of de novo resistance to cetuximab include activating mutations in codon 12 or 13 of *KRAS* and in *BRAF* [1–4]. Various mechanisms responsible for acquired resistance to cetuximab in colorectal cancer have also been identified [5–7]. We previously established cetuximab-resistant cancer cells by exposing parental cells to increasing concentrations of cetuximab [8]. Analysis of these cells revealed that cell-derived heregulin confers cetuximab resistance through bypass signaling via HER2 (also known as ERBB2) and HER3 (also known as ERBB3). Heregulin is a ligand for HER3 and stabilizes the HER2-HER3 heterodimer [9]. We also found that high initial levels of serum heregulin protein and tumor heregulin mRNA were significantly associated with a poor clinical outcome in mCRC patients treated with cetuximab [8]. Furthermore, in patients who initially

achieved a partial response to cetuximab-based therapy, the serum concentration of heregulin after the development of clinical cetuximab resistance was significantly higher than that before treatment [8]. These preclinical and clinical data indicate that increased levels of heregulin are associated with both de novo and acquired resistance to cetuximab.

Patritumab (U3-1287) is a first-in-class, fully human monoclonal antibody directed to the extracellular domain (ECD) of HER3 that is currently in clinical development, as are other HER3-targeted antibodies such as MM-121 and LJM716 (MM-121 prevents ligand binding, whereas LJM716 specifically binds to an epitope formed by ECD domains II and IV in the closed conformation of HER3 [10]). Patritumab has been shown both to inhibit ligand-induced HER3 phosphorylation and to suppress the growth of pancreatic, non–small cell lung cancer, and colorectal cancer xenograft tumors [11, 12]. To identify strategies or agents capable of overcoming resistance to cetuximab induced by heregulin, we have now established sublines of the cetuximab-sensitive human colorectal cancer cell line DiFi that stably express heregulin derived from transfected cDNA. With the use of these cells, we investigated the effects of patritumab on cetuximab resistance mediated by cell-derived heregulin both *in vitro* and *in vivo*.

## RESULTS

### DiFi cells stably overexpressing heregulin show resistance to cetuximab

The human colorectal cancer cell line DiFi, which harbors wild-type alleles of *KRAS, BRAF*, and *PI3K*, is highly sensitive to cetuximab [13]. To investigate whether cell-derived heregulin might induce cetuximab resistance in DiFi cells, we established DiFi sublines that stably overexpress this protein (DiFi-HRG4, DiFi-HRG5, and DiFi-HRG6) or that stably harbor the corresponding empty vector (DiFi- Mock1) as a result of retroviral infection. Heregulin is a soluble growth factor that is synthesized as a transmembrane precursor molecule of 105 kDa. Cell surface proteases catalyze cleavage of the extracellular domain of this precursor, which is then released and functions as a ligand for HER3. Immunoblot analysis revealed the presence of the transmembrane form of heregulin in DiFi-HRG cells (with its abundance being greatest in DiFi-HRG4 cells), whereas no such band was detected in DiFi-Mock1 cells or the parental DiFi cells (Fig. 1A). Analysis of conditioned medium from these cell lines with an enzyme-linked immunosorbent assay (ELISA) also revealed the presence of substantial amounts of heregulin in the medium from all DiFi-HRG cell lines but not in that from DiFi-Mock1 or the parental cells (Fig. 1B). To assess the effect of cetuximab on cell growth, we exposed DiFi-HRG and DiFi-Mock1 cells to various concentrations of the drug for 5 days and then measured cell viability. All DiFi-HRG cell lines showed a reduced sensitivity to cetuximab compared with DiFi-Mock1 cells, with median inhibitory concentration ($IC_{50}$) values of > 100 μg/mL for the former cell lines and ~0.1 μg/mL for the latter (Fig. 1C). The DiFi-HRG cell lines also showed resistance to panitumumab, another antibody to EGFR (data not shown). These data thus suggested that DiFi-HRG cells are resistant to EGFR-targeted antibodies.

### Heregulin maintains HER3 and AKT phosphorylation and survivin expression in the presence of cetuximab in DiFi-HRG cell lines

To investigate possible differences in signal transduction among the DiFi isogenic lines, we examined the effects of cetuximab (10 μg/mL) on EGFR, HER2, HER3, AKT, and extracellular signal–regulated kinase (ERK) phosphorylation (Fig. 2A). Immunoblot analysis revealed that cetuximab markedly inhibited the phosphorylation of all of these proteins in DiFi-Mock1 cells. In contrast, whereas cetuximab substantially reduced the level of EGFR and ERK phosphorylation in DiFi-HRG cells, it had little effect on the phosphorylation of HER2, HER3, or AKT. We next examined the effects of cetuximab on expression of the apoptosis-related proteins BIM (a proapoptotic BH3-only protein) and survivin (a member of the inhibitor of apoptosis, or IAP, family). We previously showed that inhibition of the MEK-ERK signaling pathway induces BIM expression, and that inhibition of the PI3K-AKT pathway suppresses survivin expression, with both of these effects being independently required for tyrosine kinase inhibitor (TKI)–induced apoptosis in lung cancer cells positive for *EGFR* mutation [14], breast cancer cells positive for *HER2* amplification [15], and gastric cancer cells positive for *MET* amplification [16]. Consistent with these observations, we found that cetuximab induced both up-regulation of BIM and down-regulation of survivin in DiFi-Mock1 cells, resulting in generation of the cleaved form of poly(ADP-ribose) polymerase (PARP), a characteristic of apoptosis (Fig. 2B). In contrast, in DiFi-HRG cell lines, whereas cetuximab induced BIM expression, it had little effect on the abundance of survivin or PARP cleavage (Fig. 2B), suggesting that sustained AKT signaling and survivin expression confer resistance to cetuximab in these cell lines.

### The HER3 neutralizing antibody patritumab abrogates cetuximab resistance induced by heregulin

To investigate further the role of HER3 and heregulin in the resistance of DiFi-HRG cell lines to cetuximab, we exposed DiFi-HRG4 cells to cetuximab, the fully human HER3-targeted monoclonal antibody patritumab, or the combination of both agents. We found that neither antibody alone substantially affected cell proliferation, whereas the combination of both agents

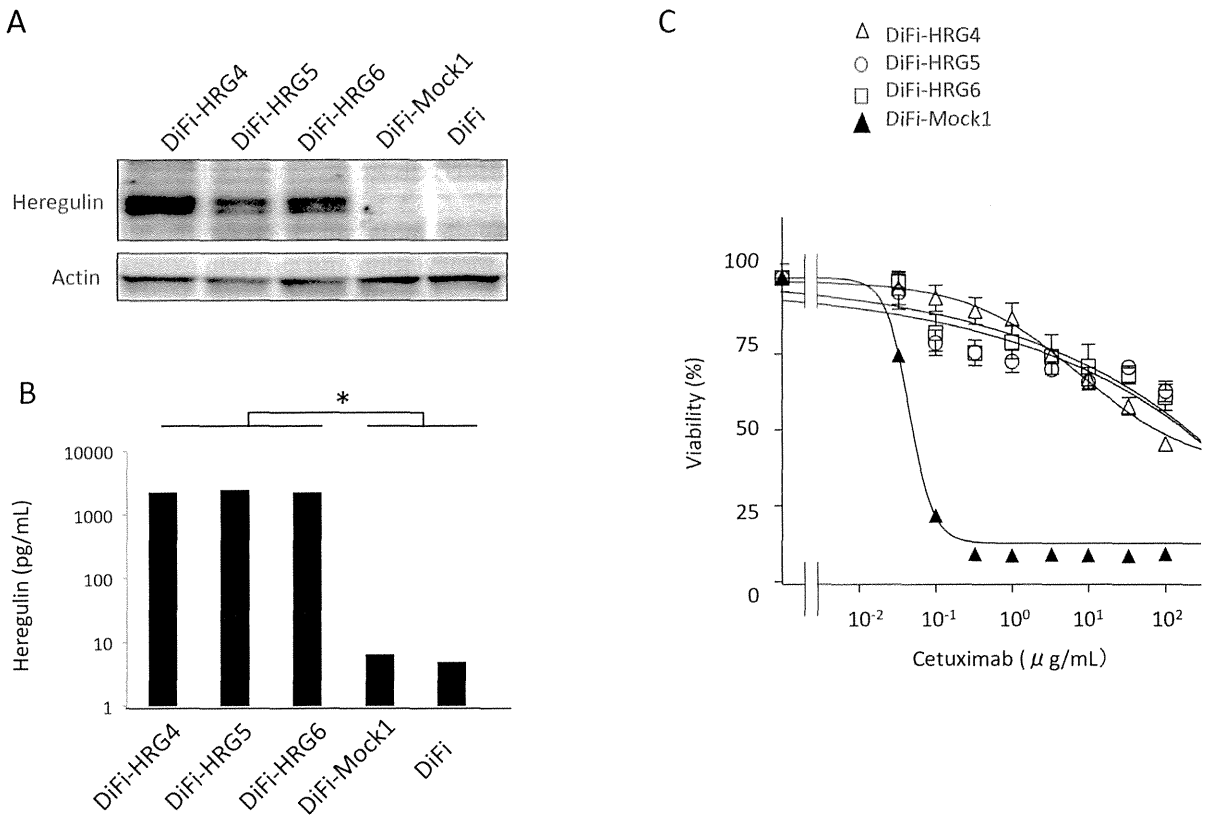


**Figure 1: Characterization of DiFi isogenic cell lines.** **(A)** DiFi isogenic cell lines (DiFi, DiFi-Mock1, DiFi-HRG4, DiFi-HRG5, and DiFi-HRG6) were cultured overnight in medium containing 10% serum and then incubated for 24 h in serum-free medium, after which the cells were lysed and subjected to immunoblot analysis with antibodies to heregulin and to β-actin (loading control). **(B)** Culture supernatants from cells cultured as described in Materials and Methods were assayed for heregulin with an ELISA. Data are means ± SE from three independent experiments. $^*P < 0.05$ (Student's *t* test) for comparison of each DiFi-HRG line with DiFi-Mock1 or DiFi cells. **(C)** Cells were treated with cetuximab at the indicated concentrations for 5 days, after which cell viability was assessed. Data are means ± SE from three independent experiments.

induced marked inhibition of cell growth (Fig. 3A). We next examined the effects of these antibodies on apoptosis in DiFi-Mock1 and DiFi-HRG4 cells. An annexin V binding assay revealed that cetuximab alone induced a substantial level of apoptosis in DiFi-Mock1 cells but not in DiFi-HRG4 cells (Fig. 3B, C), suggesting that the operation of a heregulin autocrine loop in these latter cells inhibits cetuximab-induced apoptosis. However, exposure of DiFi-HRG4 cells to the combination of patritumab (10 μg/mL) and cetuximab (10 μg/mL) resulted in a marked increase in the proportion of apoptotic cells (Fig. 3B, C), suggesting that patritumab sensitizes DiFi-HRG cells to cetuximab such that the extent of apoptosis induced by both antibodies in these cells is similar to that induced by cetuximab alone in DiFi-Mock1 cells.

We also examined the effects of patritumab alone or in combination with cetuximab on intracellular signaling. Immunoblot analysis showed that patritumab alone had little effect on such signaling in DiFi-Mock1 cells. In contrast, patritumab alone markedly inhibited the phosphorylation of HER3 and AKT, without affecting that of ERK, in DiFi-HRG4 cells (Fig. 3D). The combination of patritumab and cetuximab markedly attenuated the phosphorylation of EGFR, HER2, HER3, AKT, and ERK in DiFi-HRG4 cells (Fig. 3D). It also induced the cleavage of PARP in these cells to an extent similar to that observed in DiFi-Mock1 cells treated with cetuximab alone, and this effect was accompanied by both up-regulation of BIM and down-regulation of survivin expression (Fig. 3E). These results thus indicated that cetuximab resistance induced by heregulin is abrogated by patritumab through attenuation of AKT-suvivin signaling in DiFi-HRG4 cells.

## Cell-derived heregulin induces cetuximab resistance and patritumab restores cetuximab sensitivity in tumor xenografts *in vivo*

To examine whether cell-derived heregulin induces cetuximab resistance as well as the efficacy of combined

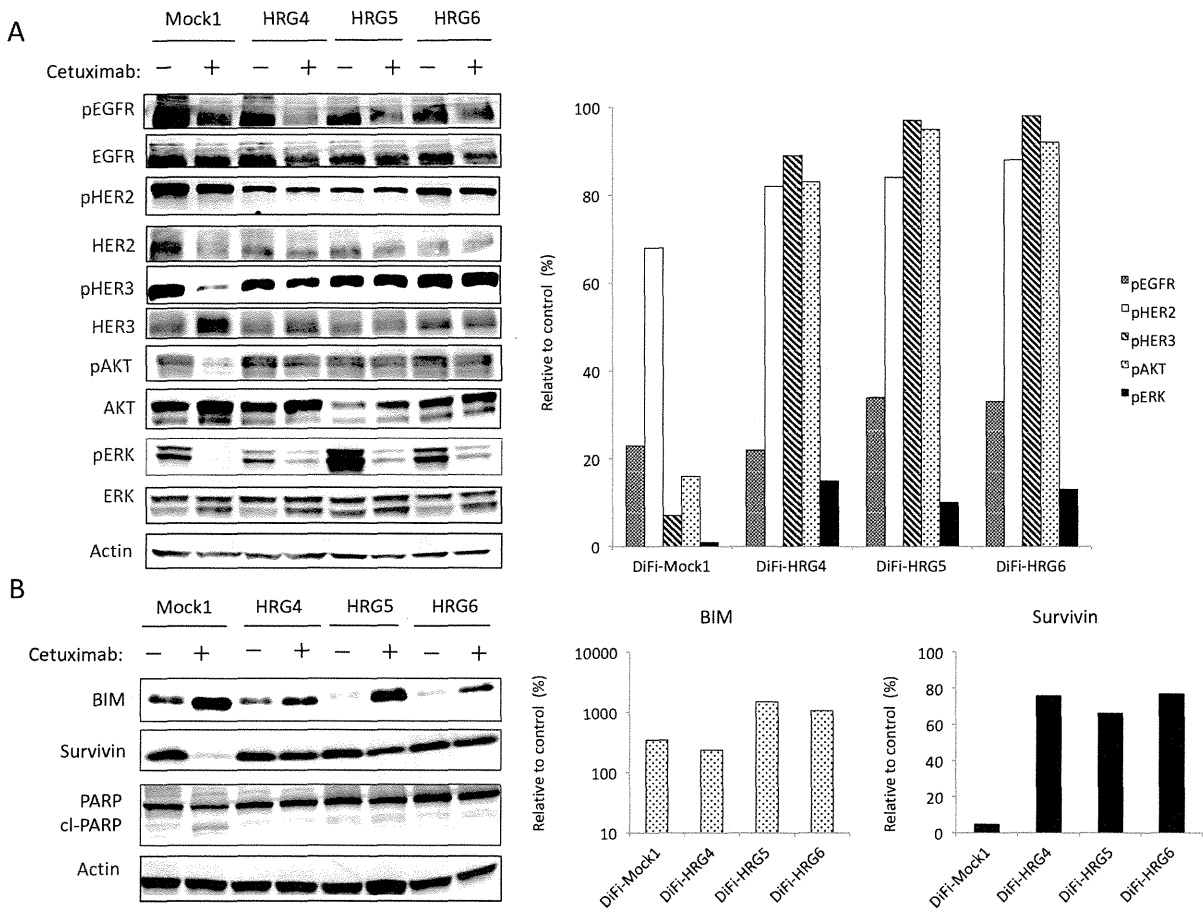


**Figure 2: Effects of cetuximab on intracellular signaling and the expression of apoptosis-related proteins in DiFi isogenic cell lines.** DiFi-Mock1, DiFi-HRG4, DiFi-HRG5, or DiFi-HRG6 cells were cultured overnight in medium containing 10% serum and then incubated for 6 h **(A)** or 24 h **(B)** in serum-free medium with or without cetuximab (10 μg/mL), after which cell lysates were prepared and subjected to immunoblot analysis with antibodies to phosphorylated (p) or total forms of the indicated proteins (left panels). A band corresponding to the cleaved (cl) form of PARP is indicated. The intensity of the bands corresponding to phosphorylated forms of EGFR, HER2, HER3, AKT, and ERK (A) or to BIM and survivin (B) was normalized by that of the corresponding total proteins or β-actin, respectively, and then expressed relative to the corresponding value for control cells not exposed to cetuximab (right panels).

treatment with patritumab and cetuximab *in vivo*, we injected nude mice with DiFi-Mock1 or DiFi-HRG4 cells to allow the formation of tumor xenografts. Whereas cetuximab alone markedly inhibited the growth of DiFi-Mock1 xenografts (Fig. 4A), DiFi-HRG4 xenografts were resistant to this drug (Fig. 4B). Patritumab alone had little effect on the growth of tumors formed by either cell line. However, the combination of cetuximab and patritumab induced substantial regression of DiFi-HRG4 xenografts (Fig. 4B). These results thus suggested that heregulin produced by colorectal cancer tumors harboring wild-type *KRAS* induces cetuximab resistance, and that combination therapy with cetuximab and patritumab overcomes such resistance *in vivo*.

## DISCUSSION

Resistance to cetuximab is a major problem in the treatment of colorectal cancer. Although various mechanisms of cetuximab resistance have been identified [1–7, 17–20], the optimal treatment strategies for mCRC patients who show resistance to this drug remain unclear. We previously showed that tumor-derived heregulin mediates cetuximab resistance in preclinical models [8]. High levels of heregulin were also associated with a poor clinical outcome in mCRC patients treated with cetuximab-based regimens [8]. Moreover, increased heregulin levels were observed in such patients after the development of clinical resistance to cetuximab-based

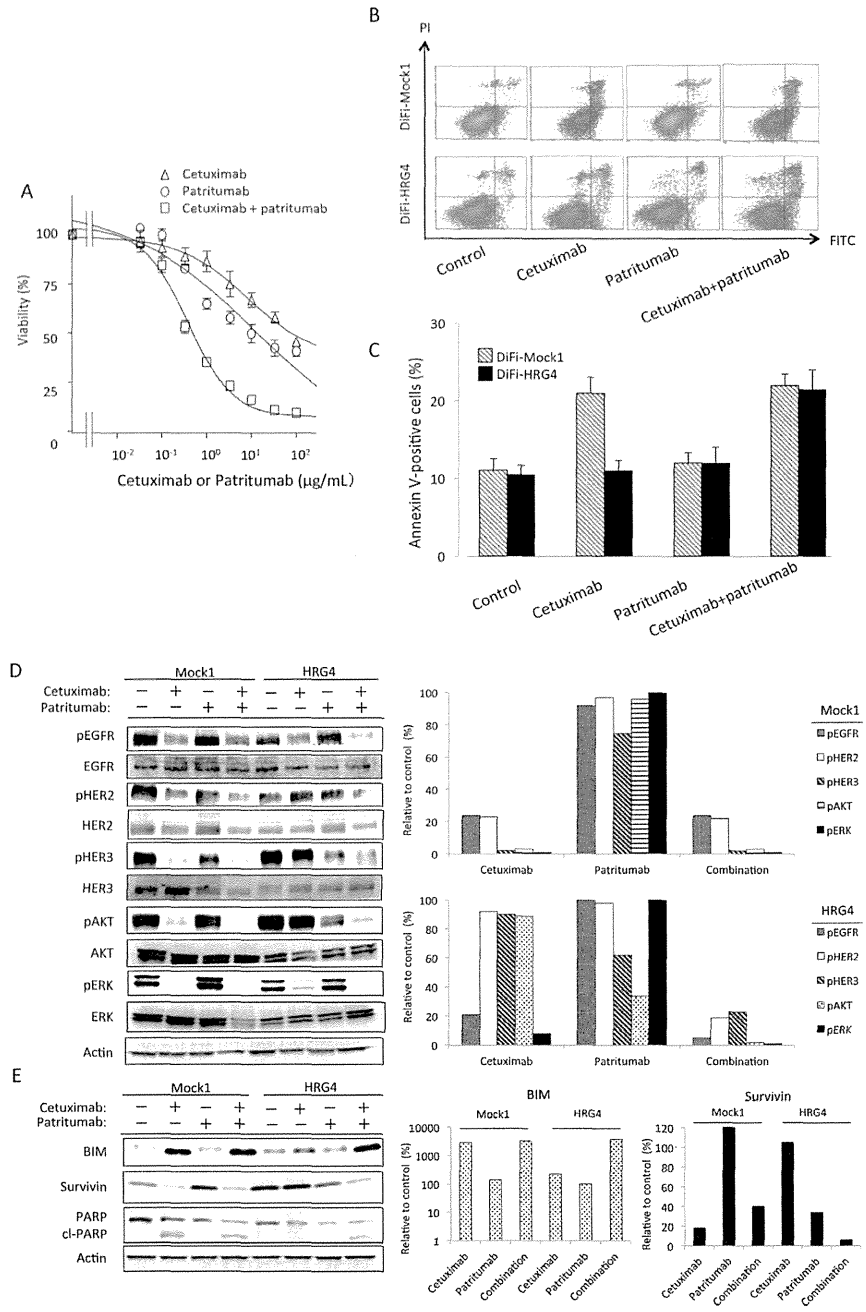


**Figure 3: Effect of patritumab on heregulin-mediated cetuximab resistance in DiFi-HRG cells *in vitro*.** **(A)** DiFi-HRG4 cells were incubated for 5 days with cetuximab alone, patritumab alone, or the combination of both drugs at the indicated concentrations, after which cell viability was assessed. Data are means ± SE from three independent experiments. **(B, C)** DiFi-Mock1 or DiFi-HRG4 cells were cultured overnight in medium containing 10% serum and then incubated for 48 h in the absence or presence of cetuximab alone (10 μg/mL), patritumab alone (10 μg/mL), or the combination of both drugs in serum-free medium, after which the number of apoptotic cells was determined by staining with propidium iodide (PI) and fluorescein isothiocyanate (FITC)–labeled annexin V followed by flow cytometry. Representative flow cytometric profiles are shown in (B), and quantitative data (means ± SE of three independent experiments) are shown in (C). **(D, E)** DiFi-Mock1 or DiFi-HRG4 cells were cultured overnight in medium containing 10% serum and then incubated for 6 h (D) or 48 h (E) in the absence or presence of cetuximab alone (10 μg/mL), patritumab alone (10 μg/mL), or the combination of both drugs in serum-free medium, after which cell lysates were prepared and subjected to immunoblot analysis with antibodies to the indicated proteins (left panels). The intensity of the bands corresponding to phosphorylated forms of EGFR, HER2, HER3, AKT, and ERK (D) or to BIM and survivin (E) was normalized by that of the corresponding total proteins or β-actin, respectively, and then expressed relative to the corresponding value for control cells not exposed to drug (right panels).

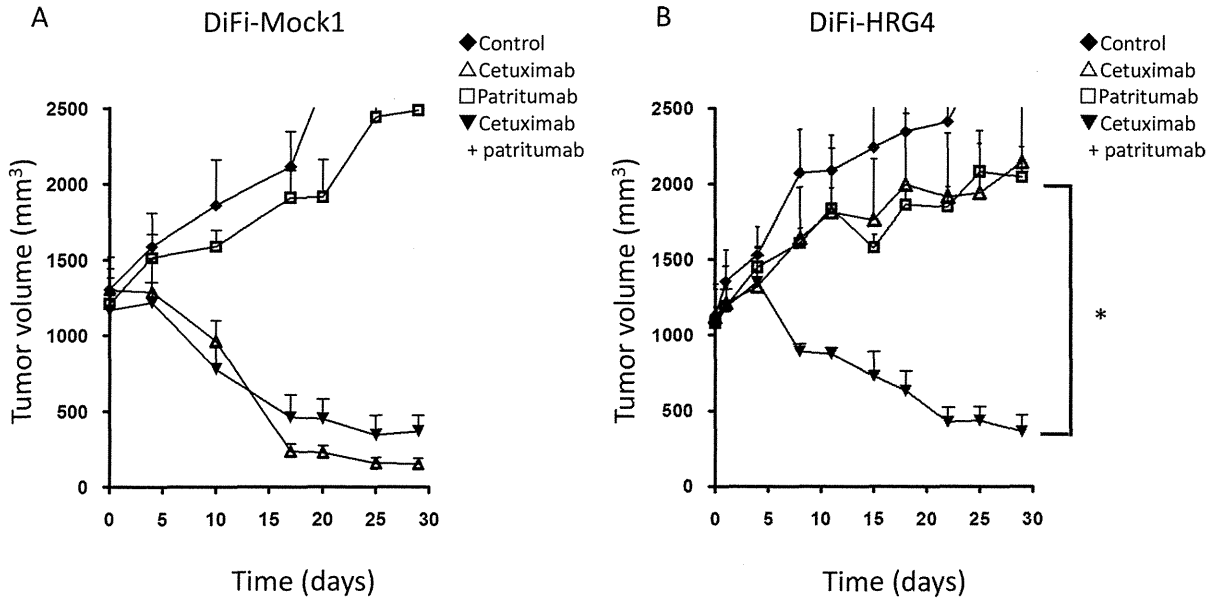


**Figure 4: Effects of cetuximab, patritumab, and the combination of both drugs on the growth of DiFi-HRG tumor xenografts *in vivo*.** Nude mice with tumor xenografts established by subcutaneous injection of DiFi-Mock1 **(A)** or DiFi-HRG4 **(B)** cells were treated for 4 weeks with vehicle (control), cetuximab (1.0 mg/body), patritumab (1.0 mg/body), or both drugs, as described in Materials and Methods. Tumor volume was determined at the indicated times after the onset of treatment. Data are means ± SE from six mice per group. $*P < 0.05$ for comparison of the combination of both drugs with cetuximab alone or patritumab alone (Student's *t* test).

therapy [8]. Effective treatment options to overcome cetuximab resistance mediated by heregulin are thus urgently required.

We have here established heregulin-overexpressing sublines of DiFi cells (DiFi-HRG cells) and shown that these cells are resistant to cetuximab both *in vitro* and *in vivo*. Whereas the amount of the transmembrane form of heregulin was substantially higher in DiFi-HRG4 cells than in DiFi-HRG5 or DiFi-HRG6 cells (Fig. 1A), these three cell lines appeared to release similar amounts of the soluble form of heregulin into the culture medium (Fig. 1B). This difference in the relative abundance of the transmembrane and soluble forms of heregulin among DiFi-HRG cell lines might reflect a difference in the activity of cell surface proteases among the cell lines. Alternatively, the production of the transmembrane form of heregulin in DiFi-HRG4 cells might exceed the capacity of such proteases. To investigate the mechanism responsible for cetuximab resistance in DiFi-HRG cells, we examined differences in signal transduction between these cells and DiFi-Mock1 cells. In DiFi-Mock1 cells, cetuximab inhibited the phosphorylation of EGFR, HER2, HER3, AKT, and ERK as well as up-regulated BIM expression and down-regulated survivin expression, resulting in the induction of apoptosis. By contrast, in DiFi-HRG cell lines, whereas cetuximab inhibited EGFR and ERK phosphorylation, leading to BIM induction, it did not affect HER2, HER3, or AKT phosphorylation or survivin expression. Given that down-regulation both of AKT signaling and of the expression of its downstream target survivin is required for apoptosis induced by inhibition of receptor tyrosine kinases [14–16], our data suggest that sustained AKT-survivin signaling in the presence of cetuximab is responsible for the resistance of DiFi-HRG cell lines to this drug. To investigate further the relation between AKT signaling and the operation of a heregulin autocrine loop, we examined the effects of patritumab, a neutralizing monoclonal antibody to HER3, in DiFi-HRG4 cells. We found that exposure of these cells to patritumab in combination with cetuximab resulted in inhibition of EGFR, HER2, HER3, AKT, and ERK phosphorylation as well as in both up-regulation of BIM expression and down-regulation of survivin expression, leading to the induction of apoptosis. These results indicate that AKT signaling is triggered by heregulin binding to HER3.

Given that HER3 (a kinase-dead receptor) manifests impaired kinase activity [21], it requires dimerization with other HER family members to activate signaling after ligand binding [22, 23]. HER2 has been implicated as a dimerization partner of HER3, and heregulin stabilizes the HER2-HER3 heterodimer [9]. In this context, we examined the effects of lapatinib, a TKI for EGFR and HER2, in DiFi-HRG4 cells. Inhibition of EGFR and HER2 by lapatinib resulted in down-regulation of HER3, AKT, and ERK phosphorylation as well as in the induction of BIM and suppression of survivin expression in these cells, thereby triggering apoptosis (Supplementary Fig. S1A, S1B). These

findings suggest that, in DiFi-HRG cells, HER3 is trans-phosphorylated by HER2 as a result of heregulin-induced HER2-HER3 heterodimerization, which in turn leads to the activation of AKT signaling [24] (Fig. 5A, 5B, 5C). The heterodimerization partner of HER3 is thus likely switched from EGFR to HER2 as a result of the overexpression of heregulin in DiFi-HRG cells. We also found that patritumab alone inhibited the phosphorylation of HER3 and AKT as well as down-regulated survivin expression in DiFi-HRG4 cells but not in DiFi-Mock1 cells (Fig. 3D, 3E). These results suggest that patritumab may prevent ligand-dependent HER3 phosphorylation by blocking HER2-HER3 heterodimerization, whereas it has little effect on ligand-independent HER3 phosphorylation.

In our DiFi-HRG xenograft model, we showed that combination therapy with patritumab and cetuximab inhibited tumor growth to the same extent as did cetuximab alone in the DiFi-Mock1 xenograft model. Antagonism of the heregulin-HER3 interaction by patritumab thus represents an effective strategy to abrogate cetuximab resistance induced by heregulin derived from tumor cells. Given that elevated circulating levels of heregulin are associated with both de novo and acquired cetuximab resistance in mCRC patients [8], our model systems based on stable overexpression of heregulin are clinically relevant and should prove useful for establishing strategies to overcome cetuximab resistance mediated by the heregulin autocrine loop. Indeed, a recent phase I/II study with refractory colorectal cancer patients revealed potential antitumor activity of the combination of cetuximab and pertuzumab, a HER2-targeted antibody that blocks ligand-dependent HER2-HER3 heterodimerization. However, this drug combination was not tolerable as a result of overlapping toxicities [25]. Given that the toxicity profile of patritumab differs from that of pertuzumab [26], the combination of cetuximab and patritumab warrants evaluation in the clinical setting. We also found that the $IC_{50}$ value for the antiproliferative effect of the combination of cetuximab and patritumab in DiFi-HRG4 cells was ~10 times as high as that for cetuximab alone in DiFi-Mock1 cells (Fig. 1C, Fig. 3A). The discrepancy between these *in vitro* data and our *in vivo* findings may suggest that antibody-dependent cellular cytotoxicity [27] involving NK cell activation plays a role in tumor growth inhibition by the combination of both agents. Moreover, combination therapy with these two IgG1 antibodies may result in an enhanced antitumor activity mediated by cytotoxic T lymphocytes [28] in the clinical setting. Given the recent evidence implicating the importance of interactions between therapeutic antibodies and the immune system in the efficacy of antibody treatment [20], further investigation of such mechanisms is warranted.

In conclusion, we have shown that consecutive activation of HER2-HER3 and AKT by heregulin in an autocrine-dependent manner confers resistance to cetuximab, and that patritumab restores cetuximab sensitivity in tumors with heregulin-induced cetuximab resistance. Further studies of combination therapy with patritumab and cetuximab are thus warranted in mCRC patients with heregulin-induced resistance to EGFR-targeted antibodies.

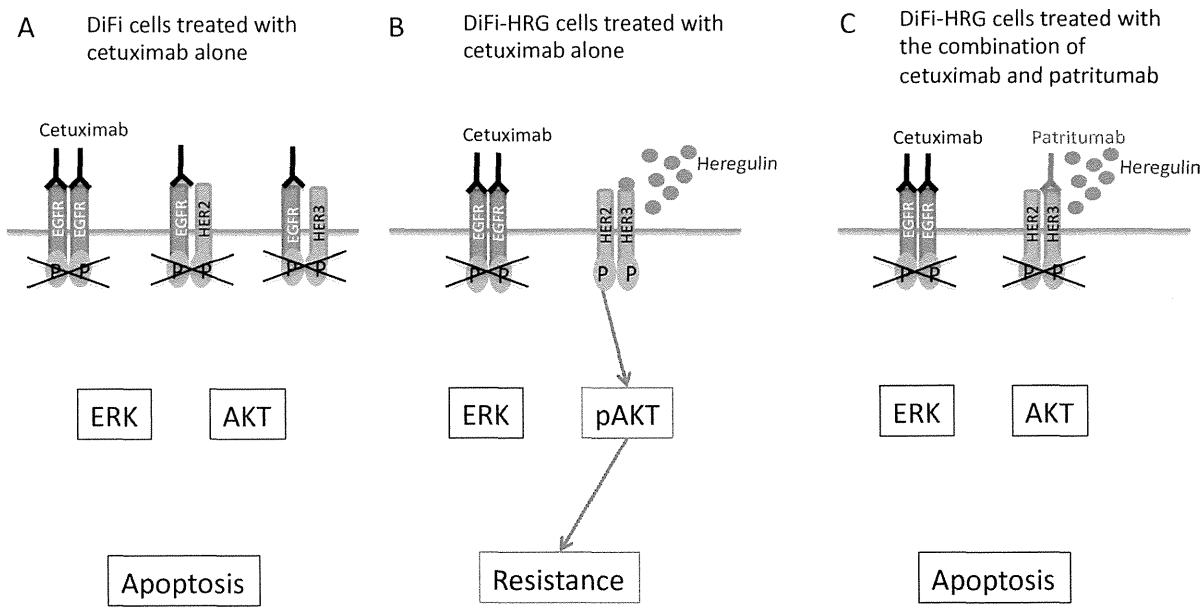


**Figure 5: Model for intracellular signaling underlying the induction of apoptosis by cetuximab and patritumab in colorectal cancer cells.** **(A)** DiFi colorectal cancer cells treated with cetuximab alone. **(B)** DiFi cells that stably overexpress heregulin (DiFi-HRG cells) are resistant to cetuximab as a result of HER2-HER3 heterodimerization and AKT activation induced by heregulin. **(C)** Patritumab abrogates cetuximab resistance mediated by the heregulin autocrine loop in DiFi-HRG cells.

## MATERIALS AND METHODS

### Cells and reagents

The DiFi human colorectal cancer cell line was kindly provided by P. A. Janne (Dana Farber Cancer Institute). DiFi cells were maintained under a humidified atmosphere of 5% $CO_2$ in air at 37°C in Dulbecco's modified Eagle's medium (DMEM) containing high glucose and supplemented with Ham's F-12 and 10% fetal bovine serum (FBS). Cetuximab was obtained from Merck Serono, and patritumab was kindly provided by Daiichi-Sankyo (Tokyo, Japan). Recombinant human heregulin (NRG1-β1/HRG1-β1 extracellular domain) was obtained from R&D Systems.

### Establishment of cells stably overexpressing heregulin

A full-length cDNA encoding human heregulin (NRG1, GenBank accession no. NM_013956) was obtained from Origene (Rockville, MD). The amplification product was verified by sequencing after its cloning into the pCR-Blunt II-TOPO vector (Invitrogen). The heregulin cDNA was then excised from pCR-Blunt II-TOPO and transferred to the pQCXIH retroviral vector (Clontech), and retroviruses encoding heregulin were produced and used to infect DiFi cells as described [29]. Cells stably expressing heregulin were then isolated by selection with hygromycin (Invivogen) at 500 μg/mL.

### Cell growth inhibition assay

Cells were transferred to 96-well flat-bottomed plates and cultured for 24 h before exposure to various concentrations of cetuximab or patritumab in medium containing 1% FBS for 120 h. Cell Counting Kit-8 solution (Dojindo, Kumamoto, Japan) was then added to each well, and the cells were incubated for 3 h at 37°C before measurement of absorbance at 490 nm with a Multiskan Spectrum instrument (Thermo Labsystems). Absorbance values were expressed as a percentage of that for nontreated cells, and the $IC_{50}$ of cetuximab for inhibition of cell growth was determined.

### Heregulin ELISA

The concentration of heregulin in cell culture supernatants was measured with the use of a sandwich ELISA (NRG1-β1 DuoSet, R&D Systems) as previously described [30]. Cells were seeded in six-well plates at a density of $0.5 \times 10^6$ cells per well in DMEM supplemented with 10% FBS. After the cells had achieved confluence, the medium was replaced with 5 ml of DMEM supplemented with 0.1% FBS, the cells were incubated for 48 h, and the culture supernatants were collected for assay of heregulin.

### Immunoblot analysis

Cells were washed twice with ice-cold phosphate-buffered saline (PBS) and then lysed with 1 × Cell Lysis Buffer (Cell Signaling Technology) consisting of 20 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 1 mmol/L EDTA (disodium salt), 1 mmol/L EGTA, 1% Triton X-100, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L β-glycerophosphate, 1 mmol/L $Na_3VO_4$, leupeptin (1 μg/mL), and 1 mmol/L phenylmethylsulfonyl fluoride. The protein concentration of the lysates was determined with a bicinchoninic acid assay kit (Thermo Fisher Scientific), and equal amounts of protein were subjected to SDS-polyacrylamide gel electrophoresis on a 7.5% gel (Bio-Rad). The separated proteins were transferred to a nitrocellulose membrane, which was then incubated with Blocking One solution (Nacalai Tesque) for 20 min at room temperature before incubation overnight at 4°C with primary antibodies. Antibodies to heregulin (NRG1-β1), to phosphorylated EGFR (phospho-Tyr$^{1068}$), to phosphorylated HER2 (phospho-Tyr$^{1248}$), to phosphorylated HER3 (phospho-Tyr$^{1289}$), to phosphorylated or total forms of AKT, to phosphorylated ERK, to PARP, and to BIM were obtained from Cell Signaling Technology; those to total HER3, to total ERK, and to survivin were from Santa Cruz Biotechnology; those to total EGFR were from Zymed/Invitrogen; those to total HER2 were from Millipore; and those to β-actin were from Sigma. The membrane was then washed with PBS containing 0.05% Tween 20 before incubation for 1 h at room temperature with horseradish peroxidase–conjugated secondary antibodies (GE Healthcare). Immune complexes were finally detected with ECL detection reagents (GE Healthcare).

### Annexin V binding assay

The binding of annexin V to cells was measured with the use of an Annexin-V-FLUOS Staining Kit (Roche). Cells were harvested by exposure to trypsin-EDTA, washed with PBS, and centrifuged at 200 × *g* for 5 min. The cell pellets were resuspended in 100 μL of Annexin-V-FLUOS labeling solution, incubated for 10 to 15 min at 15° to 25°C, and then analyzed for fluorescence with a flow cytometer (FACSCalibur) and Cell Quest software (Becton Dickinson).

### Tumor growth inhibition assay *in vivo*

All animal experiments were performed in accordance with the Recommendations for Handling of Laboratory Animals for Biomedical Research compiled by the Committee on Safety and Ethical Handling Regulations for Laboratory Animal Experiments, Kinki University. The ethical procedures followed conformed to the UKCCCR guidelines for the welfare and use of

animals in cancer research [31]. The study was also reviewed and approved by the Animal Ethics Committee of Kinki University (approval no. KAME-22-018). Cells were injected subcutaneously into the axilla of 5- to 6-week-old female athymic nude mice (BALB/c nu/nu; CLEA Japan), and treatment was initiated when tumors in each group of six mice achieved an average volume of 1000 to 1200 $mm^3$. Treatment groups consisted of vehicle control, cetuximab (1.0 mg/body), patritumab (1.0 mg/body), and the combination of both agents. Cetuximab and patritumab were administered by intraperitoneal injection twice a week for 4 weeks, with control animals receiving a 0.5% (w/v) aqueous solution of hydroxyl propylmethyl cellulose as vehicle. Tumor volume was determined from caliper measurements of tumor length ($L$) and width ($W$) according to the formula $LW^2/2$. Both tumor size and body weight were measured twice weekly. For ethical reasons, animals were removed from the study if the tumor volume exceeded 2500 $mm^3$.

### Statistical analysis

Quantitative data are presented as means ± SE unless indicated otherwise. The significance of differences was evaluated with the unpaired two-tailed Student's $t$ test. A $P$ value of $< 0.05$ was considered statistically significant.

### Conflict of interest statement

The authors declare no conflict of interest.

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# Tolerability of Nintedanib (BIBF 1120) in Combination with Docetaxel: A Phase 1 Study in Japanese Patients with Previously Treated Non–Small-Cell Lung Cancer

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**Background:** This phase I, open-label study evaluated the safety/tolerability and maximum tolerated dose of second-line nintedanib combined with docetaxel in Japanese patients with advanced non–small-cell lung cancer.

**Methods:** Eligible patients received docetaxel 60 or 75 mg/m$^2$ (day 1) plus nintedanib 100, 150, or 200 mg twice daily (bid; days 2–21) in 21-day cycles. Standard 3 + 3 dose escalations were performed separately in patient cohorts with a body surface area (BSA) of less than 1.5 m$^2$ (BSA <1.5) and BSA greater than or equal to 1.5, respectively.

**Results:** Forty-two patients (17 BSA <1.5, 25 BSA ≥1.5) were treated. The maximum tolerated dose of nintedanib was 150 and 200 mg bid in patients with BSA less than 1.5 and BSA greater than or equal to 1.5 (BSA ≥1.5), respectively, in combination with 75 mg/m$^2$ of docetaxel. Dose-limiting toxicities (all grade 3 hepatic enzyme elevations) occurred in 12 patients (six per cohort). Drug-related adverse events included neutropenia (95%), leukopenia (83%), fatigue (76%), alopecia (71%), decreased appetite (67%), and elevations in alanine aminotransferase (64%) and aspartate aminotransferase (64%). All hepatic enzyme elevations were reversible and manageable with dose reduction or discontinuation. Among 38 evaluable patients, 10 (26%) had a partial response and 18 (47%) had stable disease.

**Conclusion:** Continuous treatment with second-line nintedanib combined with docetaxel was manageable and showed promising signs of efficacy in Japanese patients with advanced non–small-cell lung cancer.

**Key Words:** Clinical trials, Phase I, Docetaxel, Japanese, Nintedanib, Non–small-cell lung cancer, Pharmacokinetics.

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Few treatment options are available for patients with advanced non–small-cell lung cancer (NSCLC) who fail first-line chemotherapy. Currently, the only licensed second-line therapies for individuals with NSCLC, who do not harbor identifiable driver oncogenes, such as sensitizing epidermal growth factor receptor (*EGFR*) gene mutations or anaplastic lymphoma kinase (*ALK*) gene translocations, are docetaxel, gemcitabine, pemetrexed (for nonsquamous NSCLC), and erlotinib.[1] Although these treatments are efficacious, survival benefits are modest. Hence, there is an urgent need for effective and well-tolerated second-line options.

Angiogenesis plays an important role in the development and differentiation of NSCLC.[2] Targeting vascular endothelial growth factor (VEGF) signaling appears to be particularly important in advanced NSCLC, given the proven efficacy of the VEGF-targeted monoclonal antibody bevacizumab as first-line therapy in large-scale trials.[3,4] However, to date no oral tyrosine kinase inhibitors of VEGF receptors have been approved for the treatment of advanced NSCLC. Mechanisms that support solid tumor angiogenesis include VEGF, fibroblast growth factor, and platelet-derived growth factor signaling pathways.[5–8] Nintedanib (BIBF 1120) is a potent, oral, small-molecule triple angiokinase inhibitor that targets VEGF receptors 1 to 3, platelet-derived growth factor

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receptors alpha and beta, and fibroblast growth factor receptors 1 to 3, besides RET and Flt3.[9] Preclinical experiments have shown that nintedanib can delay tumor growth and inhibit angiogenesis in various xenograft models of human cancer, including NSCLC.[9] More recently, the global LUME-Lung 1 phase III trial (Study 1199.13; NCT00805194) for previously treated advanced NSCLC demonstrated that treatment with a combination of nintedanib and docetaxel produced a significant and clinically meaningful improvement in overall survival compared with docetaxel and placebo in predefined patients with adenocarcinoma tumor histology.[10]

In a recent Japanese phase I study, the maximum tolerated dose (MTD) of nintedanib monotherapy was 200 mg bid, which is lower than the MTD of 250 mg bid for Caucasian patients.[11,12] Although the reason for this difference remains unclear, analogous differences in the tolerability of chemotherapy for advanced NSCLC between Japanese and US patients have been reported previously, and have been related to differences in genotypic variants between the two populations.[13] In addition, the standard dose of docetaxel 60 mg/m$^2$ commonly employed for Japanese patients with advanced NSCLC[14] is lower than the 75 mg/m$^2$ dose used for Western populations.[10,15] This phase I dose-escalation study (Study 1199.29; NCT00876460) was conducted to define the MTD of nintedanib combined with docetaxel, and to confirm the safety/tolerability profile of the combination in Japanese patients with advanced NSCLC following failure of first-line platinum-based chemotherapy.

## PATIENTS AND METHODS

### Study Population

Patients aged 20 to 74 years with histologically or cytologically confirmed, advanced stages IIIB to IV or recurrent NSCLC (any histology) who had received one platinum-based chemotherapy regimen (not containing docetaxel) were enrolled. Patients had an Eastern Cooperative Oncology Group performance status of 0 to 1, a life expectancy exceeding 3 months, and adequate organ function. Exclusion criteria included: active brain metastases; gastrointestinal disorders that could interfere with the absorption of the study drug; history of major thrombotic or clinically relevant major bleeding event in the past 6 months; clinically significant hemoptysis in the past 3 months; active multiple primary neoplasms; or significant cardiovascular disease.

### Study Design

This open-label trial utilized a standard 3 + 3 dose-escalation design. Eligible patients received intravenous docetaxel at a dose of 60 mg/m$^2$ or 75 mg/m$^2$ on day 1, followed by continuous, oral nintedanib bid on days 2 to 21 in 21-day cycles. Nintedanib was started at a dose of 100 mg bid and escalated up to 200 mg bid in 50 mg bid intervals. Continuous nintedanib monotherapy was permitted in cases where docetaxel had to be permanently discontinued for reasons other than progression, and the patient had already received at least four treatment cycles of combination therapy.

Dose-limiting toxicity (DLT) was defined as nonhematologic toxicity greater than Common Terminology Criteria for Adverse Events (CTCAE) grade 3, excluding electrolyte abnormalities or isolated elevations of γ-glutamyl transpeptidase (γ-GT); grade 3 or higher gastrointestinal toxicity or hypertension despite optimal supportive care/intervention; grade 4 neutropenia for more than 7 days despite optimal supportive care; grade 4 febrile neutropenia of any duration; grade 2 or higher alanine aminotransferase (ALT) and/or aspartate aminotransferase (AST) elevations combined with grade 2 or higher bilirubin elevations; inability to resume nintedanib dosing within 14 days of stopping treatment due to treatment-related toxicity. DLTs observed in the first 21 days of treatment were used to determine MTD, defined as the highest dose at which incidence of DLTs in cycle 1 was less than or equal to 33.3%.

After testing nintedanib 100 mg bid plus docetaxel 60 mg/m$^2$ (N100/D60), nintedanib 150 mg bid plus docetaxel 60 mg/m$^2$ (N150/D60), and nintedanib 200 mg bid plus docetaxel 60 mg/m$^2$ (N200/D60) without considering body surface area (BSA), dose escalations were performed separately in two patient cohorts with a BSA of less than 1.5 m$^2$ (BSA <1.5) and greater than or equal to 1.5 m$^2$ (BSA ≥1.5), respectively. This protocol amendment was recommended by the external Efficacy and Safety Review Committee following early observation of a high incidence of DLTs in patients with a BSA of less than 1.5 m$^2$.

The institutional review board reviewed and approved the protocol and its amendments. The trial was conducted in compliance with the study protocol, the Declaration of Helsinki, and Good Clinical Practice guidelines. All patients provided written informed consent.

### Assessments

Adverse events (AEs) were assessed according to CTCAE version 3.0 throughout the trial and for 28 days after treatment cessation. All safety analyses were undertaken in patients who had received 1 dose or more of nintedanib. Objective tumor response was evaluated according to the Response Evaluation Criteria in Solid Tumors (RECIST 1.0). Tumor assessment was performed at screening and every 6 weeks on day 1 (within 7 days) of each odd-numbered treatment cycle (cycles 3, 5, etc.). Hematology and biochemistry assessments were undertaken at screening and at predefined intervals during the trial.

To investigate the possible effect of nintedanib on the pharmacokinetics (PK) of docetaxel, blood samples were taken predose and 1, 1.5, 2, 3, 4, 7, 24, and 48 hours postdose on days 1 to 3 of cycles 1 and 2. Sampling for PK characterization of nintedanib was carried out on days 2 to 3 of cycle 1, with samples taken predose, 1, 2, 3, 4, 6, 7, 10, and 24 hours after the morning dose. Samples for evaluation of trough concentrations of nintedanib were taken on days 8 and 15 of the first two cycles, and on days 1 to 3 during cycle 2, before the morning dose. All PK analyses were carried out using WinNonlin software, applying a noncompartmental approach.

### Statistical Analysis

The primary end points were the determination of the MTD of nintedanib in combination with docetaxel at doses

of 60 or 75 mg/m$^2$, and the assessment of the frequency and severity of AEs. Secondary end points included PKs of nintedanib and docetaxel, best tumor response and progression-free survival (PFS). Descriptive statistics are presented.

## RESULTS

### Patients

A total of 43 patients with advanced NSCLC were enrolled into this study from March 2009 to August 2012. One patient discontinued due to a non-DLT adverse event before the first dose of nintedanib was administered and was excluded from the study. Baseline characteristics, except for gender and clinical stage, were similar between the two BSA cohorts (Table 1).

At the time of the database lock (June 11, 2013), all 42 patients had discontinued combination treatment. Reasons for discontinuation included progressive disease ($n$ = 22), AEs ($n$ = 14), and withdrawal of consent ($n$ = 3). Three patients continued to be treated with nintedanib monotherapy after discontinuation of docetaxel due to drug-related AEs (grade 1 and 2 peripheral neuropathy in two patients, and grade 2 pleural effusion in one patient). Median (range) number of days of treatment administered was 126.5 (7–1339).

### Maximum Tolerated Dose and Dose-Limiting Toxicities

The allocation of patients to treatment during the study is summarized in Figure 1. Of the 42 patients who received nintedanib treatment, three patients were excluded from the DLT assessment due to low compliance with study treatment: one excluded patient had a non-DLT adverse event,

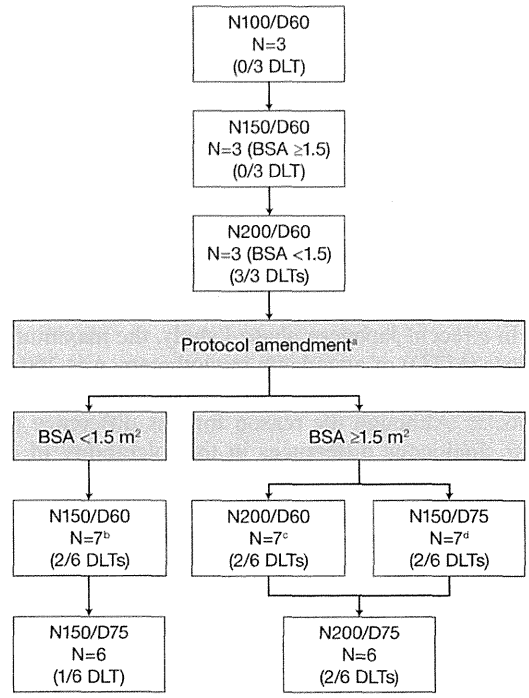


**FIGURE 1.** Patient flow. N100/D60, nintedanib 100 mg bid plus docetaxel 60 mg/m$^2$; N150/D60, nintedanib 150 mg bid plus docetaxel 60 mg/m$^2$; N150/D75, nintedanib 150 mg bid plus docetaxel 75 mg/m$^2$; N200/D60, nintedanib 200 mg bid plus docetaxel 60 mg/m$^2$; N200/D75, nintedanib 200 mg bid plus docetaxel 75 mg/m$^2$. [a]Protocol amendment by the Efficacy and Safety Review Committee, which recommended separate assessments of dose levels for patients with a body surface area (BSA) <1.5 m$^2$ and ≥1.5 m$^2$. [b]One patient was replaced due to low compliance with study drugs administration with a non-dose-limiting toxicity adverse event (pneumonia). [c]One patient was replaced due to insufficient data to evaluate the duration of grade 4 neutropenia as a dose-limiting toxicity. [d]One patient was replaced due to early withdrawal of consent.

**TABLE 1.** Patient Characteristics at Baseline and Treatment Allocation

| | Patients with BSA <1.5 m$^2$ ($n$ = 17) | Patients with BSA ≥1.5 m$^2$ ($n$ = 25) | All Patients ($n$ = 42) |
|---|---|---|---|
| Age, years | | | |
| Median (range) | 65 (45–72) | 62 (47–73) | 64 (45–73) |
| Gender, $n$ (%) | | | |
| Male | 6 (35) | 23 (92) | 29 (69) |
| Female | 11 (65) | 2 (8) | 13 (31) |
| ECOG performance score, $n$ (%) | | | |
| 0 | 6 (35) | 8 (32) | 14 (33) |
| 1 | 11 (65) | 17 (68) | 28 (67) |
| Clinical stage, $n$ (%) | | | |
| IIIB | 1 (6) | 6 (24) | 7 (17) |
| IV | 16 (94) | 19 (76) | 35 (83) |
| Histology, $n$ (%) | | | |
| Adenocarcinoma | 14 (82) | 19 (76) | 33 (79) |
| Squamous cell carcinoma | 3 (18) | 5 (20) | 8 (19) |
| Large-cell carcinoma | 0 | 1 (4) | 1 (2) |

bid, twice daily; BSA, body surface area; D, docetaxel; DLT, dose-limiting toxicity; ECOG, Eastern Cooperative Oncology Group; N, nintedanib.

the second patient withdrew consent before the completion of cycle 1, and there were insufficient data to confirm a DLT occurrence in the third patient. Three patients were enrolled in the N100/D60 cohort, three patients in the N150/D60 cohort, and three patients in the N200/D60 cohort, without consideration of their BSA. No DLT was observed for the first and second cohorts (N100/D60 and N150/D60). At 200 mg bid (N200/D60), all three patients experienced DLTs (ALT, AST, and γ-glutamytranferase increases in two patients, and ALT and AST increase in one patient) that were fully reversible (Table 2). All three patients who experienced DLTs at N200/D60 had BSA less than 1.5, whereas the three patients treated with N150/D60 who did not experience DLTs had BSA greater than or equal to 1.5. In a previous investigation of nintedanib monotherapy in Japanese patients,[11] all DLTs at 200 mg bid were observed in patients whose BSAs were smaller than those of patients without observed DLTs. The external Efficacy and Safety Review Committee recommended the protocol amendments for reassessment of

**TABLE 2.** Observed Dose-Limiting Toxicities in Treatment Cycle 1 at Each Nintedanib Dose Level Among Evaluable Patients with BSA <1.5 $m^2$ or BSA ≥1.5 $m^2$

| Cohort | Nintedanib Dose (mg bid) | Docetaxel Dose ($mg/m^2$) | No. of DLTs/Patients[a] | Nature of DLT |
|---|---|---|---|---|
| — | 100 | 60 | 0/3[b] | — |
| BSA <1.5$m^2$ | 150 | 60 | 2/6 | (1) ALT and AST elevation; (2) ALT elevation |
| | | 75 | 1/6 | (1) ALT and AST elevation |
| | 200 | 60 | 3/3 | (1) ALT, AST, and γ-GT elevation; (2) ALT, AST, and γ-GT elevation; (3) ALT and AST elevation |
| BSA ≥1.5$m^2$ | 150 | 60 | 0/3 | — |
| | | 75 | 2/6 | (1) ALT and γ-GT elevation; (2) ALT elevation |
| | 200 | 60 | 2/6 | (1) ALT, AST and γ-GT elevation; (2) ALT and γ-GT elevation |
| | | 75 | 2/6 | (1) ALT, AST, and γ-GT elevation; (2) ALT elevation |

[a]Patients eligible for evaluation of dose-limiting toxicity.
[b]BSA in 100 mg bid group: <1.5 $m^2$, $n = 1$; ≥1.5 $m^2$, $n = 2$.
ALT, alanine aminotransferase; AST, aspartate aminotransferase; bid, twice daily; BSA, body surface area; DLT, dose-limiting toxicity; γ-GT, gamma glutamyltransferase.

the N150/D60 dose in patients with BSA less than 1.5, and for subsequent dose escalations to be performed separately for cohorts with BSA less than 1.5 and BSA greater than or equal to 1.5, respectively.

As shown in Table 2, of 12 patients with BSA less than 1.5 treated in the N150/D60 or the nintedanib 150 mg bid plus docetaxel 75 $mg/m^2$ (N150/D75) cohorts, three patients experienced DLTs (liver enzyme elevations that were reversible with dose reduction or discontinuation); two in the N150/D60 cohort and one in the N150/D75 cohort. Of 12 patients with BSA greater than or equal to 1.5 treated in the N200/D60 and the nintedanib 200 mg bid plus docetaxel 75 $mg/m^2$ (N200/D75) cohorts, respectively, two of six patients in each cohort experienced DLTs (reversible liver enzyme elevations). In eight of 12 patients who developed DLTs, nintedanib was reintroduced with dose reduction following rapid recovery of liver enzyme levels; one patient required a second dose reduction (Fig. 2). The MTD of nintedanib was thus 150 and 200 mg bid combined with 75 $mg/m^2$ of docetaxel in the BSA less than 1.5 and BSA greater than or equal to 1.5 cohorts, respectively.

## Safety Profile of Nintedanib

Of the 42 patients who received combination treatment, the most frequent drug-related AEs (all CTCAE grades) were neutropenia, leukopenia, fatigue, alopecia, decreased appetite, ALT/AST elevations, diarrhea, and γ-GT elevations (Table 3). The only grade 4 AEs were neutropenia ($n = 37$) and leukopenia ($n = 9$). Liver enzyme elevations were asymptomatic, and manageable with dose reduction or discontinuation. Among drug-related AEs commonly observed with other VEGF-targeted tyrosine kinase inhibitors, grade 1 or 2 rash was observed in 17 patients, grade 2 proteinuria in one patient, and grade 1 bleeding in seven patients; hypertension, perforation, and thromboembolism were not observed in this study.

Two patients died during the study period. One of these deaths occurred in a male patient (53 years of age; BSA = 1.92 $m^2$), who was previously treated concurrently with radiation to the mediastinum and systemic chemotherapy (vinorelbine plus cisplatin) until 19 months before beginning the present study treatment (N200/D60) for metastatic disease in mediastinal lymph nodes and an abdominal para-aortic lymph node. He responded to the study treatment

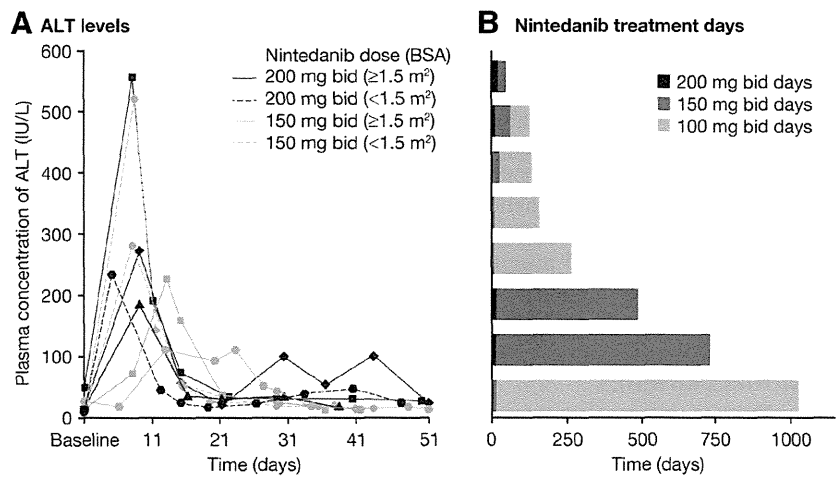


**FIGURE 2.** Change in alanine aminotransferase (ALT) values in all eight patients with dose reduction on nintedanib by dose-limiting toxicity (DLT) during first treatment course and nintedanib treatment days. BSA, body surface area.

ALT, alanine aminotransferase; bid, twice daily; DLT, dose-limiting toxicity

**TABLE 3.** Frequency of Patients with Drug-Related AEs (≥20% Incidence) Across all Dose Groups in all Treatment Courses by Body Surface Area

| | Patients with BSA <1.5 m² (*n* = 17) | | Patients with BSA ≥1.5 m² (*n* = 25) | | All patients (*n* = 42) | |
|---|---|---|---|---|---|---|
| *n* (%) | CTCAE grade 3–4 | All CTCAE grades | CTCAE grade 3–4 | All CTCAE grades | CTCAE grade 3–4 | All CTCAE grades |
| Hematologic | | | | | | |
| Neutropenia | 17 (100) | 17 (100) | 23 (92) | 23 (92) | 40 (95) | 40 (95) |
| Leukopenia | 10 (59) | 14 (82) | 17 (68) | 21 (84) | 27 (64) | 35 (83) |
| Anemia | 0 | 4 (24) | 0 | 6 (24) | 0 | 10 (24) |
| Nonhematologic | | | | | | |
| Fatigue | 0 | 15 (88) | 0 | 17 (68) | 0 | 32 (76) |
| Alopecia | 0 | 12 (71) | 0 | 18 (72) | 0 | 30 (71) |
| Decreased appetite | 1 (6) | 13 (76) | 0 | 15 (60) | 1 (2) | 28 (67) |
| Diarrhea | 0 | 6 (35) | 1 (4) | 16 (64) | 1 (2) | 22 (52) |
| Dysgeusia | 0 | 6 (35) | 0 | 11 (44) | 0 | 17 (40) |
| Rash | 0 | 8 (47) | 0 | 9 (36) | 0 | 17 (40) |
| Nausea | 0 | 7 (41) | 0 | 8 (32) | 0 | 15 (36) |
| Vomiting | 0 | 9 (53) | 0 | 5 (20) | 0 | 14 (33) |
| Stomatitis | 0 | 4 (23) | 0 | 8 (32) | 0 | 12 (29) |
| Peripheral sensory neuropathy | 1 (6) | 3 (18) | 0 | 7 (28) | 1 (2) | 10 (24) |
| Edema | 0 | 5 (29) | 0 | 4 (16) | 0 | 9 (21) |
| Laboratory abnormalities | | | | | | |
| ALT increased | 6 (35) | 13 (76) | 6 (24) | 14 (56) | 12 (29) | 27 (64) |
| AST increased | 5 (29) | 13 (76) | 2 (8) | 14 (56) | 7 (17) | 27 (64) |
| γ-GT increased | 3 (18) | 10 (59) | 4 (16) | 12 (48) | 7 (17) | 22 (52) |
| ALP increased | 1 (6) | 9 (53) | 0 | 9 (36) | 1 (2) | 18 (43) |

ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BSA, body surface area; CTCAE, Common Terminology Criteria for Adverse Events; γ-GT, gamma glutamyltransferase.

(partial response), and the combination treatment was continued until cycle 27. Notable on-treatment AEs were grade 3 to 4 neutropenia and grade 1 fatigue, with no AEs of bleeding observed before the fatal event. On day 12 of cycle 27, the patient died with bleeding suggestive of hemoptysis. The second death occurred in a woman (69 years; BSA = 1.29 m²) who had progressed after first-line platinum-based chemotherapy, and received a total of three cycles of N150/D75 in the present study. On the planned day 1 of cycle 4, a grade 1 AST elevation was observed, docetaxel administration was postponed, and nintedanib treatment was interrupted. Eight days after nintedanib interruption, the study treatment was postponed again because of a grade 1 AST elevation despite no abnormalities in any other vital signs. Fourteen days after nintedanib interruption, the patient died. Based on the details available, the most probable reason for death for both patients was underlying advanced progressive lung cancer. However, the information was not sufficient to clarify the reasons for their events.

## Pharmacokinetics

Despite interpatient variability, nintedanib AUC and $C_{max}$ increased in an almost dose-proportional manner following single-dose administration (Supplemental Table S1, SDC 1, http://links.lww.com/JTO/A737). Plasma concentrations of nintedanib reached maximum levels 2 to 3 hours postadministration and then declined, with a half-life of 8 to 9 hours.

PK analysis revealed no apparent interactions between nintedanib and docetaxel. The AUC and $C_{max}$ for nintedanib (non-dose-normalized) in this study were similar to those observed in a previous Japanese phase I study of single-agent nintedanib.[11] Similarly, coadministration of nintedanib did not affect docetaxel PKs (Supplemental Table S2, SDC 1, http://links.lww.com/JTO/A737; Supplemental Figure S1, SDC 2, http://links.lww.com/JTO/A738).

Dose-normalized PK parameters ($C_{max,norm}$, $AUC_{0\ 12,norm}$, and $AUC_{0\ \infty,norm}$) were compared among patients with BSA less than 1.5 and BSA greater than or equal to 1.5 patients. Although geometric mean values of nintedanib $C_{max,norm}$, $AUC_{0\ 12,norm}$, and $AUC_{0\ \infty,norm}$ were slightly higher in patients with BSA less than 1.5 than in patients with BSA greater than or equal to 1.5, the wide overlap of individual patient values indicated no significant differences in nintedanib exposure between the two patient cohorts (Figure 3).

## Efficacy

Four of 42 patients were excluded from the efficacy evaluation for objective response according to RECIST because they had no post-treated tumor measurement due to treatment discontinuation during cycle 1; discontinuation

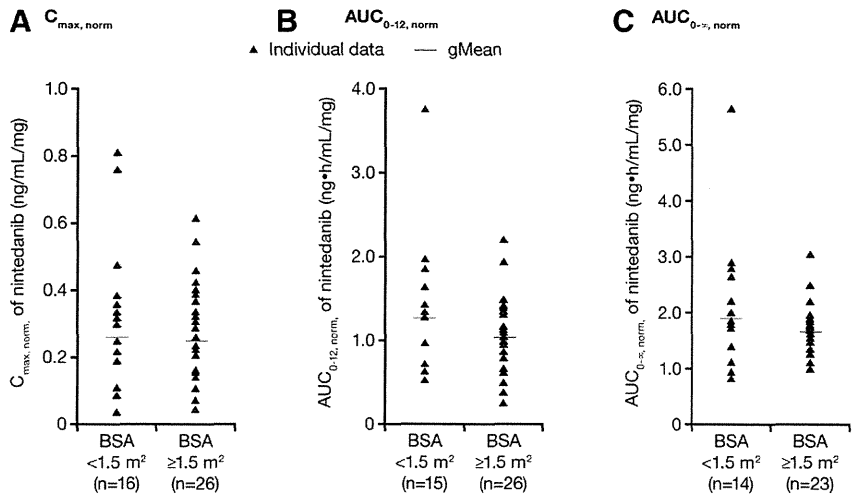


**FIGURE 3.** $C_{max, norm}$ (*A*), $AUC_{0-12, norm}$ (*B*), and $AUC_{0-\infty, norm}$ (*C*) of nintedanib following single oral administration of nintedanib 100, 150, or 200 mg in patients with a body surface area <1.5 m² or ≥1.5 m². $AUC_{0-\infty, norm}$, dose-normalized area under the concentration–time curve (0–∞ hours); $AUC_{0-12, norm}$, dose-normalized area under the concentration–time curve (0–12 hours); $C_{max, norm}$, dose-normalized peak concentration; gMean, geometric mean; BSA, body surface area.

was related to DLTs in three patients and early withdrawal of consent in one patient. Among 38 assessable patients, 10 had a partial response (two patients in the N150/D60 cohort, five in the N150/D75 cohort, two in the N200/D60 cohort, and one in the N200/D75 cohort), yielding an overall response rate of 26.3% (95% confidence interval [CI]: 13.4–43.1%) (Supplemental Table S3, SDC 1, http://links.lww.com/JTO/A737). All 10 responders had nonsquamous histology: nine with adenocarcinoma and one with large-cell carcinoma. A further 18 patients (47.4%) had stable disease, yielding a disease control rate of 73.7%. Median PFS was 5.7 months [95% CI: 4.3–8.3 months].

## DISCUSSION

This phase I trial was conducted to determine the MTD of nintedanib in combination with docetaxel in Japanese patients with advanced NSCLC who had previously received platinum-based chemotherapy. The MTD of nintedanib was 150 and 200 mg bid in patients with BSA less than 1.5 and BSA greater than or equal to 1.5 in combination with 75 mg/m² of docetaxel, respectively. The protocol was amended so that patients were divided according to BSA (<1.5 m² and ≥1.5 m²) due to the occurrence of an unexpectedly high number of DLTs in patients with a lower BSA (i.e., <1.5 m²). All DLTs were grade 3 liver enzyme elevations (ALT, AST, or γ-GT), and were completely reversible with dose reduction or discontinuation. A reduced dose of nintedanib was successfully reintroduced following rapid recovery of enzyme levels for eight of 12 patients who had developed liver enzyme level-related DLTs.

All three patients with BSA less than 1.5 treated with nintedanib 200 mg experienced DLTs, whereas only four of 12 patients with BSA greater than or equal to 1.5 treated at the same dose developed DLTs. This is consistent with our previous phase I study of nintedanib monotherapy, in which three of four patients with BSA less than 1.5 in the 200 mg bid cohort developed DLTs (grade 3 hepatic enzyme elevations), whereas DLTs were not reported in eight patients with BSA greater than or equal to 1.5 treated at the same dose.[11]

Studies with other small-molecule tyrosine kinase inhibitors also suggest that dosing according to BSA might be meaningful. For example, a low BSA has been associated with a high incidence of severe toxicities and DLTs in patients treated with sunitinib.[16,17] Furthermore, a reduced dose of 300 mg/day imatinib in low-BSA patients with chronic myeloid leukemia showed equivalent efficacy to the standard dose.[18,19] A large-scale PK analysis of imatinib identified a weak inverse correlation between trough concentration of imatinib and BSA.[20] Based on these observations, larger-scale investigations are warranted to identify optimal initial dosing of nintedanib, especially in low-BSA patients.

In addition to liver enzyme elevations, common drug-related AEs included hematologic toxicities, alopecia, and gastrointestinal AEs. Many of these toxicities are commonly observed during docetaxel administration.[15] These AEs were reversible and could usually be managed effectively with supportive therapies (except for alopecia). The mild-to-moderate gastrointestinal AEs and asymptomatic, reversible liver enzyme increases are consistent with the established safety/tolerability profile of nintedanib in NSCLC and other tumor types.[10,11,21–25] AEs associated with many other VEGF-targeted tyrosine kinase inhibitors, such as grade 3–4 skin toxicities, hypertension, bleeding, perforation, thromboembolism, and proteinuria,[26] were either absent or infrequent in this study.

The PK profile of nintedanib following docetaxel administration was very similar to that seen in our phase I nintedanib monotherapy study.[11] This suggests that docetaxel has no clinically relevant effect on the PK of nintedanib. Analyses of blood samples taken on day 1 of cycle 1 with docetaxel alone, and day 1 of cycle 2 of docetaxel/nintedanib showed that coadministration of nintedanib did not affect the PK of docetaxel. This is consistent with findings from a phase I study of nintedanib/docetaxel in patients with prostate cancer.[25] In the present study, we found no clear differences in PK data from patients with BSA less than 1.5 and BSA greater than or equal to 1.5. This could be due to the small sample size, so population-based PK analyses of nintedanib are needed.

Our study showed that 26% of patients achieved an objective response to nintedanib/docetaxel, with a median PFS of 5.7 months. This high level of antitumor activity is

consistent with data from the global LUME-Lung 1 trial of nintedanib/docetaxel in NSCLC, where a statistically significant improvement in PFS was observed in all patients, and a significant extension in overall survival was seen in patients with adenocarcinoma.[10]

In conclusion, the MTD for continuous daily treatment with nintedanib plus docetaxel (75 mg/m$^2$) was 150 and 200 mg bid in patients with BSA less than 1.5 and BSA greater than or equal to 1.5, respectively. There were no clinically relevant PK interactions between nintedanib and docetaxel. DLTs were observed in one-third of enrolled patients, and there were two fatal events including hemoptysis; therefore, careful observation of patients receiving nintedanib in combination with docetaxel is required in future investigations.

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ORIGINAL REPORT

# Randomized Phase III Trial Comparing Weekly Docetaxel Plus Cisplatin Versus Docetaxel Monotherapy Every 3 Weeks in Elderly Patients With Advanced Non–Small-Cell Lung Cancer: The Intergroup Trial JCOG0803/WJOG4307L

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See accompanying editorial on page 534 and article on page 567

## ABSTRACT

### Purpose
This phase III trial aimed to confirm the superiority of weekly docetaxel and cisplatin over docetaxel monotherapy in elderly patients with advanced non–small-cell lung cancer (NSCLC).

### Patients and Methods
Chemotherapy-naïve patients with stage III, stage IV, or recurrent NSCLC age ≥ 70 years with a performance status of 0 or 1 who were considered unsuitable for bolus cisplatin administration were randomly assigned to receive docetaxel 60 mg/m$^2$ on day 1, every 3 weeks, or docetaxel 20 mg/m$^2$ plus cisplatin 25 mg/m$^2$ on days 1, 8, and 15, every 4 weeks. The primary end point was overall survival (OS).

### Results
In the first interim analysis, OS of the doublet arm was inferior to that of the monotherapy arm (hazard ratio [HR], 1.56; 95% CI, 0.98 to 2.49), and the predictive probability that the doublet arm would be statistically superior to the monotherapy arm on final analysis was 0.996%, which led to early study termination. In total, 276 patients with a median age of 76 years (range, 70 to 87 years) were enrolled. At the updated analysis, the median survival time was 14.8 months for the monotherapy arm and 13.3 months for the doublet arm (HR, 1.18; 95% CI, 0.83 to 1.69). The rates of grade ≥ 3 neutropenia and febrile neutropenia were higher in the monotherapy arm, and those of anorexia and hyponatremia were higher in the doublet arm.

### Conclusion
This study failed to demonstrate any survival advantage of weekly docetaxel plus cisplatin over docetaxel monotherapy as first-line chemotherapy for advanced NSCLC in elderly patients.

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## INTRODUCTION

Lung cancer is the leading cause of cancer-related death in most developed countries. Non–small-cell lung cancer (NSCLC) accounts for 85% of all lung cancers, and more than 50% of patients with NSCLC already have advanced disease at diagnosis.[1] The number of elderly patients with lung cancer has also increased, and the median age at diagnosis is 70 years.[2]

The Elderly Lung Cancer Vinorelbine Italian Study, in which single-agent vinorelbine was compared with the best supportive care, first demonstrated the benefits of chemotherapy in elderly patients with advanced NSCLC.[3] In the Multicenter Italian Lung Cancer in the Elderly Study, a combination of vinorelbine plus gemcitabine did not improve survival over vinorelbine or gemcitabine alone and only increased the toxicity frequency.[4] Therefore, single-agent vinorelbine or gemcitabine was established as the standard treatment for elderly patients with NSCLC. We compared docetaxel (every 3 weeks) with vinorelbine in the West Japan Thoracic Oncology Group (the former name of the West Japan Oncology Group [WJOG]) 9904 study, which revealed significantly superior responses and better survival in the docetaxel arm.[5]

However, platinum-doublet chemotherapy has been recommended for patients with NSCLC with a performance status (PS) of 0 or 1,[6-8] and several retrospective subgroup analyses of large phase III trials have shown that the efficacy of platinum-doublet chemotherapy is similar in selected elderly patients and younger patients.[9,10] However, drug excretion or metabolic abilities generally decline because of age-related insufficiencies, especially in renal function. Therefore, modifications of anticancer drug dosages or schedules are recommended in chemotherapy for elderly patients with cancer.[11] In Japan, phase I[12] and II trials of weekly docetaxel plus cisplatin (DP) were conducted in elderly patients with NSCLC. The phase II study revealed a response rate (RR) of 52% (95% CI, 31% to 67%), a median survival time of 15.8 months, and no grade 4 toxicity.[13] On the basis of these promising results, we conducted a randomized phase III trial, the Japan Clinical Oncology Group (JCOG) 0207 trial, to compare DP with single-agent docetaxel. For the control arm, we chose weekly split docetaxel to investigate the effects of added cisplatin. In the second interim analysis, the overall survival (OS) seemed to be more favorable in the DP arm; however, an unexpected large difference was observed in the subgroup of patients age less than 75 years.[14] Therefore, considering the potential disadvantage of single-agent docetaxel therapy in this subgroup, we terminated the study and designed a new phase III trial in which the control arm received bolus infusions of docetaxel every 3 weeks, based on the West Japan Thoracic Oncology Group 9904 study.[5]

## PATIENTS AND METHODS

### Patients

Patients eligible for this study included chemotherapy-naïve patients with histologically or cytologically confirmed stage III (no indication for definitive radiotherapy), stage IV, or recurrent NSCLC who were age ≥ 70 years, with an Eastern Cooperative Oncology Group PS of 0 or 1 and adequate organ functioning, but who were unsuitable for bolus cisplatin administration. Considering that the age group of 70 to 74 years included those who were suitable and unsuitable for bolus cisplatin administration, we classified the reasons for administration unsuitability in this age group into six categories and examined patients for these conditions before enrollment. The pre-enrollment evaluation is described in the Appendix and Appendix Table A1 (online only). Prior radiotherapy, except for the primary lesion, was permitted if it had been completed at least 2 weeks before enrollment onto the study. Patients with symptomatic brain metastasis, active malignancy within the previous 5 years, superior vena cava syndrome, massive pleural effusion or ascites, critical vertebral metastasis, uncontrolled hypertension or diabetes, severe heart disease, active infection, hepatitis virus B surface antigen seropositivity, pulmonary fibrosis, polysorbate 80 hypersensitivity, or steroid dependence were excluded.

The study protocol was reviewed and approved by the JCOG Protocol Review Committee, WJOG executive board, and institutional review boards of each participating institution before study initiation. All patients provided written informed consent before enrollment.

### Study Design and Treatment Plan

Eligible patients were randomly assigned to either the docetaxel arm (docetaxel 60 mg/m$^2$ infused over 60 minutes on day 1 every 3 weeks) or the DP arm (docetaxel 20 mg/m$^2$ infused over 60 minutes plus cisplatin 25 mg/m$^2$ infused over 15 to 20 minutes on days 1, 8, and 15 every 4 weeks). Patients were randomly assigned via the minimization method to balance the arms with the institution, disease stage (III *v* IV or recurrence), and age (≥ *v* < 75 years). In the DP arm, treatment was skipped under the following conditions: total leukocyte count less than 2,000/$\mu$L, platelet count less than 50,000/$\mu$L, creatinine level ≥ 1.5 mg/dL, and presence of fever or grade ≥ 3 nonhematologic toxicity (except constipation, weight loss, cough, hoarseness, and hyponatremia) on day 8 or 15. In both arms, subsequent cycle treatment was administered when the patients met the following conditions: total leukocyte count ≥ 3,000/$\mu$L, absolute neutrophil count ≥ 1,500/$\mu$L, platelet count ≥ 100,000/$\mu$L, serum creatinine level less than 1.5 mg/dL, total bilirubin level less than 2.0 mg/dL, ALT/AST ≤ 100 IU/L, and PS 0 to 2. Administration procedures, dose reduction criteria, and methods are detailed in the Appendix. Both treatments were repeated until the detection of disease progression or appearance of unacceptable toxicity. Radiographic tumor evaluations were performed and assessed, according to RECIST (version 1.0),[15] by each investigator at least every two cycles. Laboratory examinations were performed at least once a week in both arms, and toxicity was assessed before every cycle and classified in accordance with the National Cancer Institute Common Terminology Criteria for Adverse Events (version 3.0). Second-line treatment was administered at the investigator's discretion; however, cross-over to the other treatment arm was not permitted.

### Quality-of-Life Assessment

Quality of life (QOL) was assessed by symptom scores, using the seven items of the Lung Cancer subscale of the Functional Assessment of Cancer Therapy–Lung.[16] The patients scored themselves immediately after providing informed consent and after completing the second and third treatment cycles. The proportions of patients with improved scores between the baseline and the end of the third cycle in each arm were compared. Missing data after treatment initiation were considered as indicating no improvement. In addition, we compared least squared means of the total scores from repeated measures analysis of variance with treatment arm, time, and their interaction and the 95% CI at each time point.

### Supplementary Ad Hoc Analysis

Additional data collection and ad hoc analysis were performed. Data on the active epidermal growth factor receptor (*EGFR*) mutation status (exon 19 deletion or L858R point mutation) and poststudy treatments were collected because these were considered factors that could potentially affect survival.

### Statistical Analysis

OS was the primary trial end point. The secondary end points included RRs, progression-free survival (PFS), symptom scores, and toxicities. The study was designed to provide results with a statistical power of 80%, using a one-sided $\alpha = .05$ to detect a 33% increase in median survival from 10 to 13.3 months. A total of 364 patients was required, accrued over a 4-year period with a 1-year follow-up period. Assuming a 5% rate of ineligible patients and patients lost to follow-up, the study sample size was set at 380 patients. OS, PFS, and responses were assessed in all eligible patients on an intent-to-treat basis. OS and PFS, which are defined in the Appendix, were estimated using the Kaplan-Meier method and were compared using the stratified log-rank test, according to age. Hazard ratios (HRs) of the treatment effects were estimated using the Cox proportional hazards model. RRs were compared using Fisher's exact test.

Two interim analyses were planned, the first after 50% of the patients were enrolled and the second after enrollment was completed. In these interim analyses, the primary end point, OS, was evaluated after adjustment for multiple comparisons, according to the Lan and DeMets method.[17] The O'Brien-Fleming–type $\alpha$ spending function was used. *P* values presented for the primary analysis were one-sided, in accordance with the trial design, whereas the other analysis values were two-sided. All analyses were performed using SAS software, release 9.1 (SAS Institute, Cary, NC). This study is registered with University Hospital Medical Information Network Clinical Trials Registry (www.umin.ac.jp/ctr/; identification No.: UMIN000001424).

## RESULTS

The first interim analysis was performed in September 2010 and included data from 221 patients. Information time, defined as the

  