The Fibroblast Growth Factor Receptor Genetic Status as a Potential Predictor of the Sensitivity to CH5183284/Debio 1347, a Novel Selective FGFR Inhibitor

Yoshito Nakanishi¹, Nukinori Akiyama¹, Toshiyuki Tsukaguchi¹, Toshihiko Fujii¹, Kiyosaki Sakata¹, Hitoshi Sase¹, Takehito Isebe², Kenji Morikami², Hidetoshi Shindoh¹, Toshiyuki Mio, Hirosato Ebiike¹, Naoki Taka², Yuko Aoki¹, and Nobuya Ishii¹

Abstract

The FGF receptors (FGFR) are tyrosine kinases that are constitutively activated in a subset of tumors by genetic alterations such as gene amplifications, point mutations, or chromosomal translocations/rearrangements. Recently, small-molecule inhibitors that can inhibit the FGFR family as well as the VEGF receptor (VEGFR) or platelet-derived growth factor receptor (PDGFR) family displayed clinical benefits in cohorts of patients with FGFR genetic alterations. However, to achieve more potent and prolonged activity in such populations, a selective FGFR inhibitor is still needed. Here, we report the identification of CH5183284/Debio 1347, a selective and orally available FGFR1, FGFR2, and FGFR3 inhibitor that has a unique chemical scaffold. By interacting with unique residues in the ATP-binding site of FGFR1, FGFR2, or FGFR3, CH5183284/Debio 1347 selectively inhibits FGFR1, FGFR2, and FGFR3 but does not inhibit kinase insert domain receptor (KDR) or other kinases. Consistent with its high selectivity for FGFR enzymes, CH5183284/Debio 1347 displayed preferential antitumor activity against cancer cells with various FGFR genetic alterations in a panel of 327 cancer cell lines and in xenograft models. Because of its unique binding mode, CH5183284/Debio 1347 can inhibit FGFR2 harboring one type of the gatekeeper mutation that causes resistance to other FGFR inhibitors and block FGFR2 V564F–driven tumor growth. CH5183284/Debio 1347 is under clinical investigation for the treatment of patients harboring FGFR genetic alterations. Mol Cancer Ther; 13(11); 2547–58. ©2014 AACR.

Introduction

In recent years, the use of molecular-targeted agents is increasingly adopted in basic and clinical cancer research with some of these drugs, improving patient survival times. In this context, a number of agents that target tyrosine kinases have been launched, such as the EGFR inhibitor erlotinib, the anti-HER2 antibody trastuzumab, the BCR-ABL inhibitor imatinib, the B-RAF inhibitor vemurafenib, and the ALK inhibitor crizotinib. Each of these agents has demonstrable efficacy when used in patient cohorts that are stratified on the basis of the genetic status of their respective molecular targets. However, such agents cannot cover all tumors, thus a medical need remains for novel agents against tumors harboring novel genetic alterations.

Many tumors have genetic alterations in members of the receptor tyrosine kinase (RTK) family, which includes FGFR receptors (FGFR). The FGFR family consists of FGFR1, FGFR2, FGFR3, and FGFR4, and each member is bound by a subset of 22 FGF ligands. The MAPK and PI3K/AKT pathways are critical downstream mediators of FGFR signaling. In cancer, constitutive FGFR signaling is activated by gene amplification, point mutations, or chromosomal translocations/rearrangements in several tumor types and is known to be involved in cell growth, angiogenesis, cell migration, invasion, and metastasis (1). Amplification of FGFR1 is one of the key genetic alterations in squamous cell lung carcinoma and hormone receptor–positive breast cancer (2–4). In addition, FGFR2 is also amplified in gastric and breast cancers (5, 6). Point mutations of FGFR2 and FGFR3 are mainly observed in endometrial cancer and bladder cancer, respectively (7–9). Furthermore, the explosion in next-generation sequencing technology has revealed several chromosomal translocations/rearrangements of FGFR1, FGFR2, and FGFR3 in glioblastoma, bladder cancer, and breast cancer as well as in other tumor types (10–12). Thus, there is a clear clinical need for FGFR-selective inhibitors.
Several multitypered kinase inhibitors that inhibit FGFRs as well as VEGF receptors (VEGFR) and platelet-derived growth factor receptors (PDGFR), such as cediranib (13), dovitinib (14), and E-3810 (15), are currently under clinical trials and show some clinical benefits in FGFR genetic alteration–positive patients. For instance, dovitinib showed three unconfirmed partial responses (PR) in hormone receptor–positive breast cancer (16). However, the drugs are also potent inhibitors of kinase insert domain receptor (KDR), which likely underlies the grade 3/4 hypertension and dose-limiting toxicity observed during clinical trials of these inhibitors (17, 18). Because these inhibitors are unlikely to achieve the required exposure to sufficiently inhibit FGFR in tumors in a clinical setting, more selective FGFR inhibitors are required.

Currently, several FGFR-selective inhibitors, such as AZD4547 (19) and NVP-BGJ398 (20), are under clinical trials targeting patients who have FGFR genetic alterations. These inhibitors, as well as the FGFR-selective inhibitor PD173074, share a common 3,5-dimethoxy-phenyl moiety. Thus, it is possible that a common single mutation would confer resistance to these inhibitors. A potential strategy to mitigate this issue is the rational design of a novel inhibitor with a different chemical scaffold. Proof of principle is that resistance to the anaplastic lymphoma kinase (ALK) inhibitor crizotinib was overcome by the design of alectinib/CH5424802, a highly selective ALK inhibitor with a different chemical scaffold (21).

In this study, we generated a highly selective FGFR1, FGFR2, and FGFR3 inhibitor with a new chemical scaffold and investigated its activity in a cell growth inhibition assay against a large panel of tumors with FGFR genetic alterations and in several xenograft models. We also evaluated the potential of this novel agent to inhibit the activity of V564F, a gatekeeper mutant of FGFR2, which is resistant to previously developed FGFR inhibitors.

Materials and Methods

Reagents

CH5183284/Debio 1347 and AZD4547 were synthesized at Chugai Pharmaceutical Co. Ltd., according to the procedures described in patent publications WO2011016528 and WO2008075068. NVP-BGJ398, PD173074, cediranib, and dovitinib were purchased from Active Biochem and Sigma-Aldrich.

Protein kinase assay

Protein kinases (listed in Supplementary Materials and Methods) were purchased from Carna Biosciences or Millipore Corporation. The inhibitory activity against each kinase was evaluated as described previously (22).

Cell proliferation assay

Cell lines were obtained from the ATCC, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), Health Science Research Resources Bank (HSRRB), Asterand Inc., and Health Protection Agency Culture Collections (HPACC). All cell lines were authenticated by the cell banks with cytogenic analysis, DNA profiling, or growth properties and were propagated for less than 6 months after resuscitation. Also, all cell lines were cultured according to supplier instructions. The cell lines were added to the wells of 96-well plates containing 0.076 to 10,000 nmol/L CH5183284/Debio 1347 and incubated at 37°C. After 4 days of incubation, Cell Counting Kit-8 solution (Dojindo Laboratories) was added, and, after incubation for several more hours, absorbance at 450 nm was measured with the iMark Microplate-Reader (Bio-Rad Laboratories). The antiproliferative activity was calculated using the formula (1 – T/C) × 100 (%), where T and C represent absorbance at 450 nm of the cells treated with drugs (T) and that of untreated control cells (C). The IC50 values were calculated using Microsoft Excel 2007.

Western blot analysis

Cells were treated with 0.1% DMSO or CH5183284/Debio 1347 for 2 hours and were lysed with Cell Lysis Buffer (Cell Signaling Technology) containing protease and phosphatase inhibitors. The grafted tumors were homogenized using a BioMasher (K.K. Ashisuto) before lysis. The lysates were denatured with sample buffer solution with reducing reagent for SDS-PAGE (Life Technologies) and were then subjected to SDS-PAGE. After electrophoresis, Western blot analysis was performed as described previously (23). The antibodies used for this study are available in Supplementary Materials and Methods.

Telemetry study in rats

All in vivo studies were approved by the Chugai Institutional Animal Care and Use Committee. Male Wistar rats (340–390 g; Japan Slc Inc.) implanted with a telemetry transmitter were used for the assessment of effects on blood pressure (BP; ref. 24). Vehicle (0.5% carmellrose sodium, 0.5% polysorbate 20, and 0.9% benzyl alcohol in purified water) or CH5183284/Debio 1347 (10 and 30 mg/kg) were administered by oral gavage once a day for 4 consecutive days. Data for blood pressure were automatically analyzed and continuously recorded at 5-min intervals. Baseline blood pressure was determined by the 24-hour mean of blood pressure before administration, and change in blood pressure from the baseline value (ABP) is represented as mean ± SD. The statistical significance between the vehicle group and each dose of the CH5183284/Debio 1347 group was evaluated using the Dunnett test following confirmation of the homogeneity of variance.

Mouse xenograft study

All in vivo studies were approved by the Chugai Institutional Animal Care and Use Committee. Female BALB/c nu/nu mice (CAnN.Cg-Foxn1nu/CrlNuCrj) were obtained from Charles River Laboratories and kept under specified pathogen-free conditions. Cells (4 × 106 to...
1.1 × 10^3) were suspended in 100 to 200 μL serum-free culture medium and injected subcutaneously into the right flank of the mice. Tumor size was measured using a gauge twice per week, and tumor volume (TV) was calculated using the following formula: TV = \(\frac{a\times b^2}{2}\), where \(a\) is the length of the tumor and \(b\) is the width. Once the tumors reached a volume of approximately 200 to 300 mm³, animals were randomized into groups (n = 3, 4, or 5 in each group), and treatment was initiated. CH5183284/Debio 1347 or AZD4547 were orally administered once a day.

**Immunohistochemistry**

Xenograft tumors were extracted, fixed in formalin, and embedded in paraffin. Sections were stained with antibodies against phospho-FRS (ab78195, Abcam), phospho-FGFR (#3471), phospho-ERK (#4780), and phospho-S6 (#4858) purchased from Cell Signaling Technology. Immunohistochemistry was performed using the DISCOVERY XT automated staining platform (Ventana Medical Systems).

**Crystallization and structural determination of the FGFR1–CH5183284 complex**

Protein crystallography was performed by Roche. A crystal structure of the kinase domain of human FGFR1 (residues 462–763) in complex with CH5183284/Debio 1347 was determined at 2.2 Å resolution. Details are described in Supplementary Materials and Methods. For crystallographic data and refinement statistics, see Supplementary Table S1.

**Stable Ba/F3 transfectants**

The TEL-FGFR2 wild-type (WT), which is a fusion protein of the dimerization domain of the TEL transcription factor and cytosolic domain of FGFR2, and TEL-FGFR2 V564F were inserted into a pCXND3 vector (Kaketsuken). TEL-FGFR2 V564F was generated by PCR-based site-directed mutagenesis. Ba/F3-TEL-FGFR2 WT and the V564F cell lines were generated by transfecting Ba/F3 mutant using the NucleoFector device (Amaxa); stable transfectants with pCXND3 TEL-FGFR2 WT and the V564F mutant using the NucleoFector device (Amaxa); stable transfectants were then isolated from the cultured medium without IL3.

**Results**

**Selective inhibition of FGFR1, FGFR2, and FGFR3 kinase activity by CH5183284/Debio 1347**

To obtain an FGFR-selective inhibitor, we performed high-throughput screening against a chemical library of Chugai Pharmaceutical Co. Ltd. and identified a lead compound that inhibited a relatively broad range of kinases but had a different chemical scaffold from other known FGFR inhibitors. We then structurally modified and improved the pharmacokinetics profile and selectivity against FGFR1, FGFR2, and FGFR3. Finally, we generated a 1-(1H-benimidazol-5-yl)-5-aminopyrazole derivative, CH5183284/Debio 1347, as a potent, selective, and orally available FGFR1, FGFR2, and FGFR3 inhibitor (Fig. 1A). Our analysis of the FGFR inhibition kinetics of CH5183284/Debio 1347 in biochemical enzyme assays revealed an ATP-competitive inhibitory profile against FGFR1 (Supplementary Fig. S1). To investigate the selectivity of CH5183284/Debio 1347 against FGFR1, FGFR2, and FGFR3, we screened CH5183284/Debio 1347 for activity against the FGFR family members and its selectivity among 20 tyrosine kinases and 14 serine/threonine kinases in a cell-free system (Table 1). The IC₅₀ values of CH5183284/Debio 1347 on the enzyme activity of FGFR1, FGFR2, FGFR3, and FGFR4 were 9.3, 7.6, 22, and 290 nmol/L, respectively. Because the IC₅₀ for KDR kinase was 2,100 nmol/L, these data indicate that CH5183284/Debio 1347 could distinguish FGFR1, FGFR2, and FGFR3 from FGFR4 and KDR at the enzyme level. As for the other 33 kinases, CH5183284/Debio 1347 did not inhibit them at comparable degrees to the inhibition observed for FGFR1, FGFR2, or FGFR3. To evaluate kinase selectivity further, we used a KINOMEScan panel (DiscoveRx) consisting of 442 WT and mutant kinases. At 100 nmol/L, CH5183284/Debio 1347 only bound to five kinases in the panel, including FGFR1, FGFR2, and FGFR3 (more than 80% inhibition to an ATP analog binding; Supplementary Table S2). We also confirmed the selectivity for FGFR1, FGFR2, and FGFR3 with this ATP analog in a competitive binding assay. The binding affinity (Kᵦ) of CH5183284/Debio 1347 for FGFR1, FGFR2, FGFR3, FGFR4, and KDR was 30, 20, 25, 740, and 960 nmol/L, respectively (using the DiscoveRx panel).

To evaluate the FGFR1, FGFR2, and FGFR3 selectivity in cells, we tested the effects of the compound on autophosphorylation of several RTKs, including FGFRs. As shown in Fig. 1B, CH5183284/Debio 1347 prevented autophosphorylation of FGFR1, FGFR2, and FGFR3 at 100 to 300 nmol/L in the DMS114 (FGFR1 amplification), SNU-16 (FGFR2 amplification), and KM511 [It4;14 translocation and FGFR3 Y373C mutation] cell lines. In contrast, CH5183284/Debio 1347 could not suppress autophosphorylation of FGFR4, KDR, PDGFRα, or c-KIT at the same concentration in MDA-MB-453 (FGFR4 mutation), human umbilical vein endothelial cells (HUVEC), NCI-H1703 (PDGFRα amplification), and Kasumi-1 (c-KIT N822K mutation).

To further demonstrate the selectivity of CH5183284/Debio 1347 for FGFR versus KDR, we conducted FGF- or VEGF-dependent proliferation assay in HUVECs (25, 26). The IC₅₀ of CH5183284/Debio 1347 was 29 nmol/L for FGF-dependent proliferation and 780 nmol/L for VEGF-dependent proliferation (Fig. 1C). In addition, CH5183284/Debio 1347 did not inhibit VEGF-induced tube formation of HUVECs (Supplementary Fig. S2). Because inhibition of VEGF signaling leads to hypertension (17, 18), we also evaluated the effect of CH5183284/Debio 1347 on blood pressure in telemetry-instrumented rats. Four consecutive daily oral administrations of 10 or 30 mg/kg of CH5183284/Debio 1347 did not lead to significant changes in blood pressure compared with
vehicle-dosed animals (Fig. 1D). Of note, at a dose of 30 mg/kg, the CH5183284/Debio 1347 plasma exposure in rats (AUC, 102 μg/h/mL) was 4-fold higher than the exposure inducing a greater than 100% tumor growth inhibition in the SNU-16 mouse xenograft model (AUC, 26 μg/h/mL). In the same model, we have confirmed the elevation of blood pressure by cediranib treatment, multi-targeted KDR inhibitor (24). Therefore, CH5183284/Debio 1347 has no biologically significant effect on blood pressure at multiples of the efficacious dose. We also infer that Debio 1347 does not target KDR to a significant extent in vivo, as no increase in blood pressure (a feature of other FGFR inhibitors that also target KDR) was observed in our experiments. These results indicate that CH5183284/Debio 1347 selectively inhibits FGFR1, FGFR2, and FGFR3 kinase activity.

Unique interactions between the FGFR1 and the CH5183284/Debio 1347

To understand the mechanism of selectivity of CH5183284/Debio 1347 for FGFR1, FGFR2, and FGFR3 over other kinases, we solved the three-dimensional structure of a complex of CH5183284/Debio 1347 and the bacterially expressed protein kinase domain of human FGFR1 (residues 462–763). This revealed that CH5183284/Debio 1347 binds to the ATP-binding site of FGFR1 in the DFG-in mode through five hydrogen bonds. Two of these five hydrogen bonds occur between the benzimidazole moiety of CH5183284/Debio 1347 and a backbone nitrogen atom of Asp641 and a side chain oxygen atom of Glu531 of FGFR1. The remaining three hydrogen bonds were identified between the hinge binder of CH5183284/Debio 1347 and the hinge region of FGFR1 at Glu562, Tyr563, and Ala564 (Fig. 2A). Although AZD4547, NVP-BGJ398, and PD173074 have a common structure, 3,5-dimethoxy-phenyl moiety, CH5183284/Debio 1347 does not have it but a unique benzimidazole moiety to interact with the FGFR1 at the back pocket, so several unique interactions were suggested (Fig. 2B). One of the unique interactions is an interaction between Phe642 in FGFR1 and the methyl group of the benzimidazole moiety of CH5183284/Debio 1347. The second is an interaction between sulfur atom of Met535 in FGFR1 and the nitrogen atom of the benzimidazole moiety of CH5183284/Debio 1347. In the case of KDR, a leucine is used at the same position and a reasonable interaction is
and the aromatic ring because CH5183284/Debio 1347 has wider space between FGFR1s served among FGFRs and NVP-BGJ398 or PD173074, CH5183284/Debio 1347. Although this interaction is conserved among FGFRs and NVP-BGJ398 or PD173074, CH5183284/Debio 1347 (Fig. 2C). It would be one of the reasons why CH5183284/Debio 1347 has selectivity to KDR. The third unique residues, CH5183284/Debio 1347 obtains FGFR selective kinase inhibitory activity, and this interaction mode is different from other FGFR inhibitors.

FGFR4 has a cysteine residue in the middle of the hinge region (Cys552), whereas FGFR1, FGFR2, and FGFR3 have a tyrosine residue at the same position (Tyr563 in FGFR1, Tyr566 in FGFR2, and Tyr557 in FGFR3). This tyrosine residue in FGFR1 makes parallel-displaced π-π interactions with the indole moiety of CH5183284/Debio 1347. In contrast, CH5183284/Debio 1347 would not be able to make π-π interactions with FGFR4 at this position. This is consistent with the lower FGFR4 enzymatic inhibition by CH5183284/Debio 1347 as compared with the other FGFR family members.

The selective FGFR inhibition leads the selective antiproliferative activity against the cancer cells harboring the FGFR genetic alterations

Comparison of an encyclopedia of cell lines and their susceptibilities to drugs facilitates the identification of biomarkers, such as gene amplifications, point mutations, and chromosomal translocation/rearrangements, that can predict the efficacy of drugs (27, 28). Following this rationale, we predicted that a selectivity of CH5183284/Debio 1347 for FGFR1, FGFR2, and FGFR3 would manifest as a selective activity to cell lines with activating alterations in these FGFRs. To test this, the antiproliferative activity of CH5183284/Debio 1347 was assessed against a large panel of 327 human tumor cell lines that were genetically profiled (Fig. 3; Supplementary Table S3). CH5183284/Debio 1347–sensitive cancer cell lines harboring genetic alterations in FGFR accounted for 20 of 24 (83%) of the lines examined. Among these lines, four have copy number variations (CNV) of FGFR1 (>2.2-fold), two have a chromosomal translocation of FGFR1 (FGFR1OP-FGFR1), six have CNVs of FGFR2 (>2.2-fold), three have point mutations in FGFR2 (S252W, K310R, N549K), three have chromosomal translocation/rearrangements of FGFR3 (FGFR3-TACC3, FGFR3-BAIAP2L1), and two have point mutations in FGFR3 (S249C, Y373C). Together, these data indicate that the FGFR-selective inhibitor CH5183284/Debio 1347 has selective antiproliferative activity against cancer cell lines harboring genetic alterations in FGFR.

FGFR-selective antitumor activity of CH5183284/Debio 1347 in vivo

To confirm the selective antitumor activity of CH5183284/Debio 1347 against cancers harboring FGFR genetic alterations in vivo as well as in vitro, we evaluated its

Table 1. Kinase inhibitory activity of CH5183284/Debio 1347

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NOTE: Detailed description of in vitro kinase inhibitory assays in the presence of CH5183284/Debio 1347 is presented in Materials and Methods.
in vivo efficacy in xenograft mouse models. CH5183284/Debio 1347 showed significant antitumor activity against xenografts with FGFR genetic alterations such as KG1 [leukemia, FGFR1OP-FGFR1 fusion; maximum tumor growth inhibition (TGI), 134%], SNU-16 (gastric cancer, FGFR2 amplification; maximum TGI, 147%), NVP-BGJ398-hFGFR1 (PDB code: 3TT0), and PD173074-hFGFR1 (PDB code: 2FGI; ref. 48). The distance between the centroid of aromatic ring of Phe642 and the methyl group attached to benzimidazole moiety is 0.37 nm, between Cα atom of Val661 and X-position carbon atom of benzimidazole moiety is 0.37 nm, and between sulfur atom of Met535 and Y-position nitrogen atom of benzimidazole moiety is 0.38 nm. The ball-and-stick model of NVP-BGJ398 (cyan) and PD173074 (green) are overlaid on CH5183284/Debio 1347 (pink). C, superposition of CH5183284/Debio 1347-hFGFR1 (pink) and the binding model of CH5183284/Debio 1347 to KDR (green). Side chain of Phe564 residue is shown as a stick model, colored in cyan.

**Figure 2.** X-Ray structure of the FGFR1 and CH5183284/Debio 1347 complex. A, interaction of CH5183284/Debio 1347 with FGFR1 at the ATP-binding site (PDB ID 3WJ6). CH5183284/Debio 1347 is shown as a ball-and-stick model, colored by element type (C in purple, O in red, and N in blue). Hydrogen bonds are indicated by red dashes. B, superposition of CH5183284/Debio 1347-hFGFR1, NVP-BGJ398-hFGFR1 (PDB code: 3TT0), and PD173074-hFGFR1 (PDB code: 2FGI; ref. 48). The distance between the centroid of aromatic ring of Phe642 and the methyl group attached to benzimidazole moiety is 0.37 nm, between Cα atom of Val661 and X-position carbon atom of benzimidazole moiety is 0.37 nm, and between sulfur atom of Met535 and Y-position nitrogen atom of benzimidazole moiety is 0.38 nm. The ball-and-stick model of NVP-BGJ398 (cyan) and PD173074 (green) are overlaid on CH5183284/Debio 1347 (pink). C, superposition of CH5183284/Debio 1347-hFGFR1 (pink) and the binding model of CH5183284/Debio 1347 to KDR (green). Side chain of Phe564 residue is shown as a stick model, colored in cyan.

**Figure 3.** Selective antiproliferative activity of CH5183284/Debio 1347 against cancer cell lines harboring genetic alterations in FGFR. Cells were treated with various concentrations of CH5183284 for 4 days. The viable cells were counted with WST-8. IC50 values were arranged from lowest to highest and divided into tumor types. Red, genetic alterations in FGFR1; orange, genetic alterations in FGFR2 amplification; maximum TGI, 147%), MFE-280 (endometrial cancer, FGFR2 S252W mutation; maximum TGI, 100%), UM-UC-14 (bladder cancer, FGFR3 S249C mutation; maximum TGI, 116%), and RT112/84 (bladder cancer, FGFR3-TACC3 fusion; maximum TGI, 125%).
Debio 1347 was maintained (IC50 for FGFR2 V564F vs. tissues (Fig. 5C). In contrast, the drugs in the TEL-FGFR2 WT–driven Ba/F3 xenograft was also confirmed by Western blotting (Supplementary S5). Suppression of phosphorylation of TEL-FGFR2 and S5). Suppression of phosphorylation of TEL-FGFR2 and downstream was suppressed phospho-FGFR for at least 7 hours in SNU-16 tumors. These data are consistent with the in vitro observations. We then investigated the suppression of FGFR signaling in tumor tissues by conducting Western blotting and immunohistochemistry after single administration of the drug. CH5183284/Debio 1347 suppressed phospho-FGFR for at least 7 hours in SNU-16 xenograft tissue (Fig. 4B), as well as the downstream signaling, as indicated by a reduction in phospho-ERK, phospho-ERK, and phospho-S6 (Fig. 4C). These results suggest that CH5183284/Debio 1347 has selective antitumor activity against cancers harboring FGFR genetic alterations both in vitro and in vivo through suppression of the FGFR signaling pathway.

Effects of CH5183284/Debio 1347 on FGFR2 with gatekeeper mutations
Gatekeeper mutations in kinases, such as T790M in EGFR, T315I in ABL, and L196M in ALK, are responsible for acquired resistance to small-molecule inhibitors (29–31). We therefore investigated the inhibitory activity of CH5183284/Debio 1347 against several gatekeeper mutants of FGFR2. First, we compared the kinase activity of FGFR2 WT with three gatekeeper mutants of FGFR2 (V564F, V564I, and V564L), which could be generated with a single base substitution at the codon corresponding to the gatekeeper residue. Also, these residues are frequently used in other kinases. When these mutants were expressed in HEK293 cells, they exhibited higher FGFR2 kinase activities than FGFR2 WT (Supplementary Fig. S3). We then examined the capability of the compound to inhibit the three FGFR2 gatekeeper mutants and found that inhibition was sustained against the FGFR2 V564F mutant, but not against FGFR2 V564I and V564L. In contrast, AZD4547 was ineffective against all three gatekeeper mutants (Supplementary Fig. S4). To expand the investigation to other FGFR inhibitors, we established TEL-FGFR2 WT- and TEL-FGFR2 V564F–driven Ba/F3 cell lines. Although the efficacy of AZD4547, NVP-BGJ398, PD173074, or cediranib against the FGFR2 V564F mutant–harboring Ba/F3 cells was significantly diminished (IC50 for FGFR2 V564F vs. IC50 for FGFR2 WT: ~216- to 8,460-fold), the efficacy of CH5183284/Debio 1347 was maintained (IC50 for FGFR2 V564F vs. IC50 for FGFR2 WT: 7.3-fold; Fig. 5A; Supplementary Fig. S5). Suppression of phosphorylation of TEL-FGFR2 and TEL-FGFR2 V564F in the cells by CH5183284/Debio 1347 was also confirmed by Western blotting (Supplementary Fig. S5B). In addition, CH5183284/Debio 1347 was effective against the TEL-FGFR2 V564F–driven Ba/F3 in vivo; in contrast, AZD4547 failed to inhibit tumor growth (Fig. 5B). Consistent with the TGI profile, suppression of TEL-FGFR2 V564F phosphorylation by CH5183284/Debio 1347, but not by AZD4547, was observed in the xenograft tissues (Fig. 5C). In contrast, the in vivo efficacy of both drugs in the TEL-FGFR2 WT–driven Ba/F3 xenograft model and their ability to suppress FGFR2 activation in the tumor tissues were comparable (Supplementary Fig. S6).

To clarify the mechanism by which CH5183284/Debio 1347 was able to inhibit the FGFR2 V564F mutant, we generated an in silico model of the FGFR2-CH5183284/Debio 1347 complex. Models of CH5183284/Debio 1347, AZD4547, and NVP-BGJ398 binding to FGFR2 V564F were constructed from the crystal structural information of FGFR1 with CH5183284/Debio 1347 (PDB code 3WT6), FGFR1 with the AZD4547 analog (PDB code 4F65; ref. 32), and FGFR1 with NVP-BGJ398 (PDB code 3TT0; ref. 20), respectively. This modeling suggested that there is close van der Waals contact between Phe564 and the 7 position of the benzimidazole moiety of CH5183284/Debio 1347. In contrast, the 3,5-dimethoxy-phenyl moiety present in both AZD4547 and NVP-BGJ398 would result in steric clashes with Phe564. These differences may explain why CH5183284/Debio 1347, but not AZD4547 or NVP-BGJ398, can inhibit V564F (Fig. 5D).

We also examined the inhibitory activity in an enzymatic assay against FGFR2 N549H, which has been reported as a dovitinib-resistant mutation (33). All FGFR inhibitors that we tested were comparably less effective against FGFR2 N549H similar to dovitinib; however, these still kept some inhibitory activity (Supplementary Fig. S7).

Discussion
The FGFR family is frequently activated by diverse genetic alterations in cancer, and therefore, FGFR inhibitors are likely to be effective in patients stratified on the basis of the presence of FGFR genetic alterations. Several multitargeted kinase inhibitors that inhibit FGFRs, VEGFRs, and PDGFRs have shown some clinical benefits in patients with FGFR aberrations (16). However, dosage of these inhibitors is limited by adverse effects, rendering the “safe” dose range suboptimal for FGFR inhibition and suppression of tumor growth. This creates a need for FGFR-selective inhibitors that with the potential to inhibit FGFR more significantly in tumors.

To overcome this issue, several FGFR selective inhibitors have been developed. But these inhibitors share a common 3,5-dimethoxy-phenyl moiety. Thus, it is possible that a common single mutation would confer resistance to these inhibitors. For instance, erlotinib and gefitinib have the same moiety and it causes the resistance to EGFR T790M (34). A potential strategy to mitigate this issue is the rational design of a novel inhibitor with a different chemical scaffold. Then, we generated CH5183284/Debio 1347, an FGFR1-, FGFR2-, and FGFR3-selective inhibitor. The key residues in the CH5183284/Debio 1347 binding site are Val561 at the gatekeeper residue and Phe642 and Met535 in a back pocket. Only 15 of 490 human protein kinases use valine as a gatekeeper, which partly explains the relative selectivity of CH5183284/Debio 1347. Interestingly, although KDR uses valine as a gatekeeper, it is not inhibited by CH5183284/Debio 1347. We speculate that a methionine in the back pocket, such as Met535 in FGFR1,
also contributes to the selectivity to KDR. KDR has a leucine at the same position as Met535 of FGFR1. By interacting with Val561, Met535, and Phe642, which interacts with methyl group of benzimidazole moiety, CH5183284/Debio 1347 could achieve selectivity for binding to FGFR1, FGFR2, and FGFR3. This binding mode is different from other FGFR selective inhibitors, so CH5183284/Debio 1347 would overcome the resistance of other FGFR selective inhibitors. Furthermore, the selectivity among the FGFR family may be explained by the structure at the middle of the hinge region, likely because only FGFR4 contains a cysteine residue in this region. In fact, our in silico modeling suggests that CH5183284/Debio 1347 cannot participate in a productive interaction with this cysteine, leading to weaker kinase-inhibitory activity in this case.

For our investigation into genetic alterations that might prove useful as predictive biomarkers, we used the publicly available databases of genetic information (35), as well as our own encyclopedia of cell lines and a large antiproliferative assay system. This confirmed that FGFR genetic alterations act as predictive biomarkers of CH5183284/Debio 1347 efficacy. The mechanism underlying the sensitivity of 4 of 24 cell lines has not been clarified yet, but we consider that it might involve novel genetic alterations in FGFR, such as an intragenic duplication of the FGFR kinase domain (36) or constitutive FGFR activation via epigenetic mechanisms.

Figure 4. Selective antitumor activity of CH5183284/Debio 1347 in mouse models of cell lines harboring genetic alterations in FGFR. A, antitumor activity of CH5183284/Debio 1347. Mice bearing KG1, SNU-16, MFE280, UM-UC-14, RT112/84, or MKN-45 cells were treated with CH5183284/Debio 1347 orally once daily for 11 days at the indicated doses. Tumor volume and body weight change for each dose group were measured. Data are shown as mean ± SD (n = 4–5). B, time course of phospho-FGFR inhibition in xenograft tissue. Mice bearing SNU-16 cells were orally treated with a single dose of 30 mg/kg of CH5183284/Debio 1347, and xenograft tumors were extracted at 0, 2, 4, 7, and 24 hours after dosing and analyzed by Western blotting. C, vehicle or 100 mg/kg of CH5183284/Debio 1347 were administered, and SNU-16 xenograft tissues were resected 4 hours after administration and embedded in paraffin. Treated samples were immunostained.
In contrast, few CH5183284/Debio 1347–insensitive cancer cell lines have genetic alterations in FGFR (4.6%: 14 of 303), and six of 14 cell lines have point mutations, such as FGFR1 (S125L), FGFR2 (V12M, R612T, E636K), or FGFR3 (F384L, K650E). However, except for FGFR3 K650E, these mutations are not associated with activation of FGFRs; rather, the FGFR3 F384L mutant was demonstrated to be devoid of transforming activity in NIH-3T3 cells (37) and, although the J82 cell line has an FGFR3 K650E mutation, FGFR3 is not detectable at the mRNA and protein levels (38). In breast cancer, approximately 10% of patients have amplification of FGFR1 (3), and dovitinib showed some clinical benefits in these patients (16). We have tested six breast cancer cell lines with amplification of FGFR1, but these cell lines were insensitive to CH5183284/Debio 1347. One possible explanation for this resistance mechanism is a genetic alteration in another signaling pathway. In fact, the MDA-MB-134IV cell line has a K-RAS G12R mutation, JM1T-1 has HER2 amplification and a PIK3CA C420R mutation, and CAMA-1 and ZR-75-1 have PTEN D92H and L108R mutations, respectively. Because these genetic alterations could activate downstream pathways and bypass the FGFR signal blockade, these cell lines would exhibit resistance to an FGFR inhibitor (4, 39, 40). Recently, everolimus combined with an aromatase inhibitor improved progression-free survival in patients with hormone receptor–positive advanced breast cancer who...
relapsed from nonsteroidal aromatase inhibitors (41). FGFR1 amplification is enriched in this population, and synergism in the combination of an anti-estrogen antagonist with an FGFR inhibitor has been suggested (4). We therefore suggest that a clinical study of an FGFR inhibitor combined with an aromatase inhibitor in hormone receptor and FGFR1 gene amplification double-positive patients is warranted.

One of the most frequent causes of resistance to protein kinase inhibitors is a gatekeeper mutation (29–31). To overcome this, several small-molecule kinase inhibitors that can inhibit gatekeeper mutant kinases are now under clinical development, such as neratinib and afatinib for EGFR T790M and alectinib/CH5424802 for ALK L1196M (21, 42). Although a gatekeeper mutation of FGFR has not been identified in patients yet, the gatekeeper mutant V555M of FGFR3 or V564I of FGFR2 were identified as mutations resistant to AZD4547 or dovitinib in preclinical experiments (33, 43). Therefore, we evaluated the activity of CH5183284/Debio 1347 against the gatekeeper mutants of FGFR2. With a single base substitution at the codon corresponding to the gatekeeper residue, phenylalanine, isoleucine leucine, alanine, glycine, and aspartic acid could be generated. However, only 2 kinases use alanine or glycine, and no kinase uses aspartic acid as a gatekeeper residue in 490 kinases. In addition, it is known that the substitution of alanine or glycine for a gatekeeper residue results in diminished kinase activity and ATP affinity (44–46). Therefore, we only selected Phe, Ile, and Leu for our investigation. We demonstrated that gatekeeper mutants of FGFR2 have higher kinase activities than WT FGFR2 (Supplementary Fig. S2). Taken together, these data suggest that resistance conferred by a gatekeeper mutant of FGFR may appear in patients in the near future. Therefore, an inhibitor capable of blocking FGFRs with gatekeeper mutations will be required. Existing FGFR selective inhibitors, such as AZD4547, NVP-BGJ398, and PD173074, have the common 3,5-dimethoxy-phenyl moieties that would thus be able to productively interact with Phe564. Consistent with this, we found that CH5183284/Debio 1347 does not have the methoxy moieties and would thus be able to productively interact with Phe564. Consistent with this, we found that CH5183284/Debio 1347 has sustained inhibitory activity against the FGFR2 V564I gatekeeper mutant.

Because the binding site of FGFR inhibitors and the position of FGFR2 N549 are not close, we cannot explain with three-dimensional structure analysis why this mutation would affect the inhibitory activity in the enzymatic assay. Also, FGFR inhibitors, including CH5183284/Debio 1347, are effective on cancer cells harboring FGFR2 N549K mutation, such as AN3 CA or MFE-296 (Fig. 3; Supplementary Table S3). Therefore, it is still unclear whether this mutation would be a resistant mechanism, and this should be proved in the future clinical studies.

In summary, we suggest that CH5183284/Debio 1347 will provide therapeutic opportunities for patients who have FGFR genetic alterations and patients with acquired resistance to existing FGFR selective inhibitors that contain the common methoxy moieties. Recently, many kinds of FGFR genetic alterations have been identified using next-generation sequencing technologies (47). Thus, stratifying patients based on FGFR status using next-generation sequencing may improve the power of clinical trials, allowing a clinical response to be detected even in an early cohort. CH5183284/Debio 1347 is currently under phase I clinical investigation by Debiopharm International S.A. in selected patients harboring FGFR genetic alterations (NCT01948297).

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: Y. Nakanishi, N. Taka, Y. Aoki, N. Ishii
Development of methodology: Y. Nakanishi, N. Akiyama, T. Isobe, Y. Aoki
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Y. Nakanishi, N. Akiyama, T. Tsukaguchi, H. Sase, T. Isobe, H. Shindoh
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Y. Nakanishi, N. Akiyama, T. Fujii, K. Sakata, T. Isobe, K. Morikami
Writing, review, and/or revision of the manuscript: Y. Nakanishi, T. Isobe, K. Morikami, T. Mio, N. Taka, N. Ishii
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Y. Nakanishi, Y. Aoki
Study supervision: Y. Nakanishi, T. Mio, Y. Aoki, N. Ishii
Other (design and synthesis of the inhibitor): H. Ebiike

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References


The Fibroblast Growth Factor Receptor Genetic Status as a Potential Predictor of the Sensitivity to CH5183284/Debio 1347, a Novel Selective FGFR Inhibitor

Yoshito Nakanishi, Nukinori Akiyama, Toshiyuki Tsukaguchi, et al.


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