SUPPORTING INFORMATION

Design and synthesis of novel human epidermal growth factor receptor 2 (HER2)/epidermal growth factor receptor (EGFR) dual inhibitors bearing a pyrrolo[3,2-*d*]pyrimidine scaffold

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Molecular modeling studies

The amino acid sequences of human HER2 and EGFR were aligned by sequence homology using the ClustalW program. According to the alignment, a homology model of HER2 was constructed based on the EGFR crystal structure complexed with erlotinib (PDBcode:1M17) using the Homology module of Insight II (ver. 2000, Accelrys Inc., San Diego, California, USA). The pyrrolo[3,2-d]pyrimidine was docked into the HER2 model using Gold (ver. 2.0, Cambridge Crystallographic Data Centre, UK) with the standard default settings. The obtained structure was subjected to energy minimization for 1000 steps with the steepest descent minimizer, and subsequently 5000 steps with the conjugate gradient minimizer using the Discover-CVFF force field (ver. 980, Accelrys Inc., San Diego, California, USA).

HER2 and EGFR kinase assay

The cytoplasmic domain (amino acids 676-1255) of human HER2 and the cytoplasmic domain (amino acids 669-1210) of human EGFR were expressed as N-terminal peptide (DYKDDDD)-tagged protein using a baculovirus expression system. The expressed HER2 kinase and EGFR kinase were purified by anti-FLAG M2 affinity gel (Sigma-Aldrich, USA).

The HER2 and EGFR kinase assays were performed using radiolabeled [γ-^{32}P] ATP (GE Healthcare, USA) in 96-well plates. The kinase reactions were performed in 50 mmol/L Tris-HCl, pH7.5, 5 mmol/L MnCl2, 0.01% Tween-20 and 2 mmol/L DTT containing 0.9 μCi of [γ-^{32}P] ATP per reaction, 50 μmol/L ATP, 5 μg/mL poly-Glu-Tyr(4:1) and 0.25 μg/mL of purified Her2 or EGFR cytoplasmic domain in a total volume of 50 μL. To measure the IC_{50} value for enzyme inhibition, compounds were incubated with the enzyme for 5 minutes prior to the reaction at room temperature. Kinase reactions were initiated by adding ATP. After the kinase reaction incubated for 10 minutes at room temperature, reactions were terminated by the addition of 10% (final concentration) trichloroacetic acid. The [γ-^{32}P]-phosphorylated proteins were filtered in a harvest plate (Millipore, USA) with a cell harvester (PerkinElmer, USA) and washed free of [γ-^{32}P] ATP with 3% phosphoric acid. The plates were dried, followed by the addition of 25 μL of MicroScintO (PerkinElmer, USA). Radioactivity was counted by a Topcount scintillation counter (PerkinElmer, USA). IC_{50} values and 95% confidence intervals were calculated by nonlinear regression analysis.

Cell lines and culture

Cell lines BT-474 (HER2 over-expressing human breast cancer) were obtained from American Type Culture Collection. BT-474 cells were cultured in RPMI 1640, and medium was supplemented with 10% heat-inactivated fetal bovine serum. Human gastric cancer 4-1ST was obtained from Central Institutes for Experimental Animals (Kawasaki, Japan) and were maintained in vivo in mice.

Cell growth assay

BT-474 cells were seeded into 48-multiwell plates (3×10^4 cells/well) and allowed to attach overnight. The cells were treated continuously with compounds for 5 days after which live cell numbers were counted with a particle analyzer (CDA-500; Sysmex Corporation).

Animals

Female BALB/c nu/nu mice, or female nude rats (F344/N Jcl-rnu), each 5 weeks old, were obtained from Clea Japan, Inc. All animals were housed in rooms maintained at 24±1 °C with a 12-h light/12-h dark photo-cycle. Food (CE-2, Clea Japan, Inc.) and tetracycline water (5% w/v) was provided ad libitum during the experimental period.

Inoculation of BT-474 cells in nude mice

BT-474 xenografts were initiated by subcutaneous implantation of 1×10^7 cells, suspended in 100 μL of MATRIGEL solution (Becton Dickinson, USA), into the right flank of nude mice and allowed to develop to measurable size. On day 0 and day 7 after inoculation, each mouse received 0.25 mg estradiol dipropionate (ASKA Pharmaceutical Co., Ltd.) via intramuscular injection. The tumor-bearing animals were inspected for physical abnormalities and tumor growth 2 or 3 times per week, and animals bearing tumor volumes between 200 and 300 mm^3 were used for further study. At 21 days after the tumor cell inoculation, selected mice were separated into 3 groups and comparisons between the volumes of each group were carried out using the one-way analysis of ANOVA for values of $P<0.05$.

Inoculation of 4-1ST in nude mice or rats

Human gastric cancer, 4-1ST tumor tissues were excised from donor nude mice or rats bearing tumors and minced. Tumor fragments were implanted subcutaneously and allowed to develop to measurable size. Mice or rats bearing tumor volumes between 200 and 300 mm^3 were used for the further study. At 16 days after tumor inoculation, selected mice or rats were separated into 3 groups and comparisons between the volumes of each group were carried out using one-way ANOVA for values of $P<0.05$.

Antitumor activity in vivo

Selected compounds suspended in a 0.5% (w/v) methylcellulose solution were administered orally twice daily to mice or rats at the indicated dose (10 mL/kg) for 14 days. For the control group, 0.5% (w/v) methylcellulose solution was administered. Tumor volume was assessed in a blind manner and measured 2 or 3 times per week throughout the dosing period using digital calipers using the following formula:

Tumor volume (mm^3) = (a) × $(b)^2$ / 2

a = longer diameter, b = shorter diameter

Drug effects were expressed as ([(T/C (%)] = [volume of the treated tumor/average volume of control tumors] × 100). Anti-tumor activities of selected compounds were evaluated by comparison of the T/C (%) values at Day 31, the day following the last dosing day.

Kinase selectivity assay

The HER4 kinase assay was performed in the same method as the HER2 and EGFR kinase assays described above using 0.125 μg/mL of HER4 cytoplasmic domain purchased from Upstate (USA).

Assays for the other 17 tyrosine kinases were performed using the Alphascreen® system (Perkin Elmer, USA) in 384-well plates. The cytoplasmic domains of VEGFR2 were expressed as N-terminal FLAG-tagged proteins using a baculovirus expression system. The full-length proteins of FAK was expressed as N-terminal FLAG-tagged proteins using a baculovirus

expression system. FGFR3, VEGFR1(Flt1), PDGFRα, PDGFRβ, TIE2, c-Met, c-kit, Src, Lck, and IRK were purchased from Upstate (USA). FGFR1 was purchased from ProQinase (Germany). IGF-1R and CSK were purchased from BIOMOL (USA). Lyn A and Lyn B were purchased from Invitrogen (USA). The reaction conditions were optimized for each kinase: VEGFR2 (19 ng/mL of enzyme, 10 μmol/L ATP, 10 min reaction, PY-100 conjugated acceptor beads [PY-100]); VEGFR1 (20 ng/mL of enzyme, 0.5 μmol/L ATP, 5 min reaction, PT66 conjugated acceptor beads [PT-66[); FGFR1 (10 ng/mL of enzyme, 0.2 μmol/L ATP, 10 min reaction, PY-100); FGFR3 (20 ng/mL of enzyme, 20 μmol/L ATP, 10 min reaction, PY-100); PDGFRα (50 ng/mL of enzyme, 10 μmol/L ATP, 30 min reaction, PT66); PDGFRβ (50 ng/mL of enzyme, 20 μmol/L ATP, 60 min reaction, PT66); IRK (100 ng/mL of enzyme, 10 μmol/L ATP, 60 min reaction, PT66); TIE2 (20 ng/mL of enzyme, 2 μmol/L ATP, 10 min reaction, PT66); c-Met (1 ng/mL of enzyme, 2 μmol/L ATP, 10 min reaction, PT66); c-kit(10 ng/mL of enzyme, 20 μmol/L ATP, 20 min reaction, PT66); IGF-1R (10 ng/mL of enzyme, 10 μmol/L ATP, 20 min reaction, PT66); Src (0.33 ng/mL of enzyme, 2 μmol/L ATP, 10 min reaction, PY-100); Lck (100 ng/mL of enzyme, 2 μmol/L ATP, 30 min reaction, PY-100); CSK(3.2 ng/mL of enzyme, 2 μmol/L ATP, 10 min reaction, PY-100); FAK (62 ng/mL of enzyme, 2 μmol/L ATP, 60 min reaction, PT66); Lyn A (2 ng/mL of enzyme, 2 μmol/L ATP, 10 min reaction, PY-100); Lyn B (2.7 ng/mL of enzyme, 2 μmol/L ATP, 10 min reaction, PY-100). The tyrosine kinase reactions were performed in 50 mmol/L Tris-HCl, pH 7.5, 5 mmol/L MnCl2, 5 mmol/L MgCl2, 0.01% Tween-20 and 2 mmol/L DTT, 0.1 μg/mL biotinylated poly-Glu-Tyr (4:1) containing optimized concentration of enzyme, ATP as described above in a total volume of 25 μL. To determine IC_{50} values, the remaining kinase activities at 7 concentrations (0.01, 0.1, 1, 10, 100, 1000, and 10000 nmol/L) of compound were measured. Prior to the kinase reaction, test compound and enzyme were incubated for 5 minutes at room temperature. The reactions were initiated by adding ATP. After the reaction period as described above at room temperature, reactions were stopped by the addition of 25 μL of 100 mmol/L EDTA, 10 μg/mL Alphascreen streptavidine donor beads and 10 μg/mL acceptor beads described above in 62.5 mM HEPES, pH7.4, 250 mmol/L NaCl, and 0.1% BSA. The plates were incubated in the dark for more than 12 h and read by an EnVision 2102 Multilabel Reader (PerkinElmer, USA) or a Fusion α Plate Reader (Packard, USA). Wells containing the substrate and the enzyme without the compound were used as total reaction control. Wells containing the biotinylated poly-Glu-Tyr (4:1) and the enzyme without ATP were used as basal control.

Assays for 16 serine/threonine kinases were performed using radiolabeled [γ-33P] ATP (GE Healthcare, USA) in 96-well plates. p38α, ERK1, TAK1, ASK1, PKCθ, JNK1, MEK5, GSK3β, B-raf, PLK1, and TTK were expressed as N-terminal FLAG tagged protein using a baculovirus expression system. IKKβ and MEKK1 were expressed as C-terminal FLAG tagged protein using a baculovirus expression system. Aurora-B was expressed as N-terminal 6xHis tagged protein using a baculovirus expression system. MEK1 was expressed as N-terminal GST fusion protein using freestyle293 (Invitrogen, USA) expression. PKA was expressed using E.coli expression. The reaction conditions were optimized for each kinase: p38α (100 ng/well of enzyme, 1 μg/well of MBP (Wako, Japan), 0.1 μCi/well of [γ-33P] ATP, 60 min reaction at 30°C); ERK1 (100 ng/well of enzyme, 2 μg/well of MBP, 0.1 μCi/well of [γ-33P] ATP, 60 min reaction at 30°C); MEKK1 (25 ng/well of enzyme, 1 μg/well of MBP, 0.1 μCi/well of [γ-33P] ATP, 60 min reaction at 30°C); TAK1 (30 ng/well of enzyme, 1 μg/well of MBP, 0.1 μCi/well of [γ-33P] ATP, 60 min reaction at 30°C); ASK1 (30 ng/well of enzyme, 1 μg/well of MBP, 0.1 μCi/well of [γ-33P] ATP, 60 min reaction at 30°C); PKCθ (25 ng/well of enzyme, 2 μg/well of MBP, 0.1

μCi/well of [γ-33P] ATP, 60 min reaction at 30°C); JNK1 (10 ng/well of enzyme, 1 μg/well of c-Jun, 0.1 μCi/well of [γ-33P] ATP, 60 min reaction at 30°C); MEK5 (3 ng/well of enzyme, 1 μg/well of GST-ERK5(K83M), 0.3 μCi/well of [γ-33P] ATP, 30 min reaction at 30°C); GSK3β (100 ng/well of enzyme, 0.2 μg/well of pGS peptide, 0.1 μCi/well of [γ-33P] ATP, 30 min reaction at room temperature); IKKβ (20 ng/well of enzyme, 1 μg/well of IκBα, 0.1 μCi/well of [γ-33P] ATP, reaction at room temperature); B-raf (25 ng/well of enzyme, 1 μg/well of GST-MEK1(K96R), 0.1 μCi/well of [γ-33P] ATP, 20 min reaction at room temperature); MEK1 (100 ng/well of enzyme, 0.3 μg/well of GST-ERK1(K71A) 0.2 μCi/well of [γ-33P] ATP, 20 min reaction at room temperature); Aurora-B (50 ng/well of enzyme, 30 μmol/L of Aurora substrate peptide, 0.2 μCi/well of [γ-33P] ATP, 60 min reaction at room temperature); PLK1 (80 ng/well of enzyme, 3 μg/well of α-casein (usb, USA), 0.2 μCi/well of [γ-33P] ATP, 40 min reaction at room temperature); TTK (120 ng/well of enzyme, 0.3 μg/well of GST-MOBK1B, 0.2 μCi/well of [γ-33P] ATP, 10 min reaction at room temperature); PKA (3 nmol/L of enzyme, 1 μmol/L of PKA substrate peptide (Upstate, USA), 0.2 μCi/well of [γ-33P] ATP, 10 min reaction at room temperature). Except for the PKCθ reaction, the serine/threonine kinase reactions were performed in 25 mmol/L HEPES, pH 7.5, 10 mmol/L magnesium acetate, 1 mmol/L DTT and 0.5 μmol/L ATP containing optimized concentration of enzyme, substrate and radiolabeled ATP as described above in a total volume of 50 μL. For the PKCθ reaction, enzyme reactions were performed in 25 mmol/L HEPES, pH 7.5, 10 mmol/L magnesium acetate, 1 mmol/L DTT, lipid activator (Upstate, USA) and 0.5 μmol/L ATP containing optimized concentration of enzyme, substrate and radiolabeled ATP as described above in a total volume of 50 μL. To determine IC_{50} values, the remaining kinase activities at 5 concentrations (1, 10, 100, 1000, and 10000 nmol/L) of compound were measured. Prior to the kinase reaction, compound and enzyme were incubated for 5 minutes at reaction temperature. The kinase reactions were initiated by adding ATP. After the reaction period as described above, the reactions were terminated by the addition of 10% (final concentration) trichloroacetic acid. The [γ-33P]-phosphorylated proteins were filtrated in Harvest Plate (Millipore, USA) with a Cell Harvester (PerkinElmer, USA) and then free of [γ-33P] ATP was washed out with 3% phosphoric acid. The plates were dried, followed by the addition of 40 μL of MicroScintO (PerkinElmer, USA). Radioactivity was counted by a TopCount scintillation counter (PerkinElmer, USA). Wells containing the substrate and the enzyme without the compound were used as total reaction control. Wells containing the substrate and radiolabeled ATP without the enzyme were used as basal control. IC_{50} values were calculated by nonlinear regression analysis.

Pharmacokinetic study

Animals used in the studies summarized in Table 5 were 7-week old female Balb/c-A mice, or 8-week old male SD rats (CLEA Japan, Inc.). **34e** was suspended in 0.5 w/v% methylcellulose solution for oral administration at a dose of 50 mg/10 mL/kg. The concentration of **34e** in the plasma was determined by LC/MS/MS.

Tumor xenograft models

In the 4-1ST xenograft model studies presented in Figure 5, the tumor bearing animals were 9-week-old female F344/NJcl-rnu/rnu rats (CLEA Japan, Inc.). **34e** was suspended in 0.5 w/v% methylcellulose solution for oral administration twice daily for 14 days at doses of 6.25, 12.5 and 25 mg/10 mL/kg. Tumor samples were homogenized in a 4-fold volume of saline and the concentrations of **34e** in the plasma and tumor were determined by LC/MS/MS.

Mouse cassette dosing study

Animals used in the study (Tables 2-4) were female BALB/cAJcl mice (7-weeks old; CLEA Japan, Inc.). A mixture of 5 test compounds was suspended in 0.5 w/v% methylcellulose solution for oral administration at a dose of 10 mg each (10 mL/kg). Concentrations of compounds in the plasma were determined by LC/MS/MS.

Metabolic stability assay

Human hepatic microsomes were purchased from Xenotech, LLC (Lenexa, KS). An incubation mixture with a final volume of 0.1 mL consisted of microsomal protein in 50 mmol/L phosphate buffer (pH 7.4) and 1 μmol/L test compound. The concentration of hepatic microsomal protein was 0.2 mg/mL. An NADPH-generating system containing 50 mmol/L $MgCl_2$, 50 mmol/L glucose-6-phosphate, 5 mmol/L β-$NADP^+$ and 15 unit/mL glucose-6-phosphate dehydrogenase was prepared and added to the incubation mixture with a 10% volume of the reaction mixture. After the addition of the NADPH-generating system, the mixture was incubated at 37 °C for 0 and 20 min. The reaction was terminated by the addition of acetonitrile equivalent to the volume of the reaction mixture. All incubations were made in duplicate. The test compound in the reaction mixture was measured by HPLC. The Waters Alliance HPLC system was equipped with a separation module, a thermostatted column compartment, and a photodiode array detector (Waters, Milford, MA). The column was a Capcell Pak MG (75 x 4.6 mm I.D., Shiseido, Japan). The column temperature and the flow-rate were 40°C and 1.2 mL/min, respectively. The mobile phase A was 0.01 mol/L ammonium acetate and the mobile phase B was methanol. The time program for the gradient elution was as follows: the concentration of mobile phase B was linearly increased from 42% to 90% over a period of 8.5 minutes, 90% over a period of the next 4 minutes, after which the column was equilibrated to 42% for 5 minutes. For metabolic stability determinations, chromatograms were analyzed for parent compound disappearance from the reaction mixtures.

Structural analysis for compound 34e

The HER2 kinase domain was prepared as described previously.[1] The HER2/**34e** complex was prepared by co-concentration of 1 mM TAK-285 to a final protein concentration of 9.7 mg/mL. Crystals suitable for data collection were obtained by micro-seeding from a reservoir containing 15-20% PEG 3350, 150 mM sodium tartrate, 50mM di-ammonium tartrate, and either 100mM Bis-TRIS or PIPES (pH 6.5-7.0). Crystals were immersed in mother liquor solution containing 25% ethylene glycol and flash frozen with liquid nitrogen. Diffraction data were collected from cryocooled crystals at the Advanced Photon Source at Argone National Laboratory beamline ID23D. Data were reduced using the HKL2000 software package.[2] The HER2/TAK-285 structure was determined by molecular replacement with either MOLREP[3] of the CCP4 program suite utilizing in-house solved HER2 structure (PDB code: 3PP0) as a search model and refined with the program REFMAC.[4] Several cycles of model building with XtalView[5] and refinement were performed for improving the quality of the model. Data reduction and refinement statistics are summarized in Table 7. The coordinates and structure factors have been deposited in Protein Data Bank with accession code 3RCD.

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Tables

Table 7. Data reduction and refinement statistics for X-ray crystallography of the HER2/TAK-285 complex.

Data Collection	
X-ray source	APS-ID23D (GM/CA-CAT)
Wavelength (Å)	1.0
Space group	P1
Unit cell dimensions (Å)	a=50.5, b=64.9, c=92.4, α=90.4°, β=89.7°, γ=90.4°
Resolution (Å)	3.20
Unique reflections	19079
Redundancy	1.7
Completeness (%)	76.3 (42.6)
I/σ(I)	7.6 (2.6)
R_{sym} [a]	0.083 (0.211)
Refinement	
Reflections used	13792
RMS Bonds (Å)	0.008
RMS Angles (°)	1.240
Average B value ($Å^2$)	33.658
R-value[b]	0.224
R_{free} [b]	0.293

[a]Rsym = ΣhΣj |<I(h)> - I(h)j | / ΣhΣj <I(h)>, where <I(h)> is the mean intensity of symmetry-related reflections. [b]R-value = Σ | |Fobs| - |Fcalc| | / Σ |Fobs|. Rfree for 5% of reflections excluded from refinement. Values in parentheses are for the highest resolution shell.