Preclinical Activity of HER2-Selective Tyrosine Kinase Inhibitor Tucatinib as a Single Agent or in Combination with Trastuzumab or Docetaxel in Solid Tumor Models

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ABSTRACT

HER2 is a transmembrane tyrosine kinase receptor that mediates cell growth, differentiation, and survival. HER2 is overexpressed in approximately 20% of breast cancers and in subsets of gastric, colorectal, and esophageal cancers. Both antibody and small-molecule drugs that target HER2 and block its tyrosine kinase activity are effective in treating HER2-driven cancers. In this article, we describe the preclinical properties of tucatinib, an orally available, reversible HER2-targeted small-molecule tyrosine kinase inhibitor. In both biochemical and cell signaling experiments, tucatinib inhibits HER2 kinase activity with single-digit nanomolar potency and provides exceptional selectivity for HER2 compared with the related receptor tyrosine kinase EGFR, with a >1,000-fold enhancement in potency for HER2 in cell signaling assays. Tucatinib potently inhibits signal transduction downstream of HER2 and HER3 through the MAPK and PI3K/AKT pathways and is selectively cytotoxic in HER2-amplified breast cancer cell lines in vitro. In vivo, tucatinib is active in multiple HER2+ tumor models as a single agent and shows enhanced antitumor activity in combination with trastuzumab or docetaxel, resulting in improved rates of partial and complete tumor regression. These preclinical data, taken together with the phase-I tucatinib clinical trial results demonstrating preliminary safety and activity, establish the unique pharmacologic properties of tucatinib and underscore the rationale for investigating its utility in HER2+ cancers.

Graphical Abstract: http://mct.aacrjournals.org/content/molcanther/19/4/976/F1.large.jpg.

Introduction

HER2 gain-of-function mutations and gene amplification are observed in a subset of carcinomas, including breast, bladder, colorectal, non–small cell lung, esophageal, and gastric cancers (1–5). HER2 amplification, detected by IHC and FISH, has been observed in approximately 20% of invasive breast tumors (6–8), while HER2 overexpression has been observed in 6% to 37% of gastric cancers and 5% of colorectal cancers (9, 10). HER2 is a member of the human EGFR or HER receptor tyrosine kinase (RTK) subfamily, which includes 4 members: EGFR (HER1/erbB1), HER2 (neu/erbB2), HER3 (erbB3), and HER4 (erbB4; ref. 11). These proteins are structurally conserved with an extracellular ligand-binding domain and an intracellular kinase domain.

The activity of these receptors in tissues is regulated by ligand binding, which drives receptor dimerization (homodimers or heterodimers) and elicitation of downstream signaling via the activation of the C-terminal tyrosine kinase domain (2, 3). Although HER family proteins are structurally similar, HER2 lacks a ligand-binding site on its extracellular domain and has no known activating ligand, while HER3 displays extremely low or nonexistent protein kinase activity (11). As such, both HER2 and HER3 rely upon dimerization partners for active signaling, often forming heterodimers with each other (2, 3). Activation of the kinase activity of the HER RTKs results in the transphosphorylation of intracellular domains within homodimeric or heterodimeric complexes, leading to the scaffolding of adaptor protein networks and stimulation of downstream effectors, including activation of the MAPK and PI3K pathways (2, 3). HER2 is the preferred dimerization partner with other EGFR subfamily members, such as HER3, and subverts cellular signaling when overexpressed in tumor cells, resulting in constitutive activation of mitogenic and prosurvival signaling (2, 3, 12–14).

Significant progress has been made in treating HER2+ disease, particularly in breast cancer where multiple HER2-targeted drugs have been developed that have significantly prolonged the life of patients, particularly those with early-stage HER2+ breast cancer (3, 10, 15–18). However, up to a quarter of all patients treated with anti-HER2 therapy in the adjuvant setting relapse (15–18), underscoring the need for new drugs designed to treat HER2-driven malignancies in the later-stage setting. Currently, approved treatments for patients with HER2+ breast cancer include mAb therapies, such as trastuzumab and...
pertuzumab, the antibody–drug conjugate ado-trastuzumab emtansine, and the small-molecule tyrosine kinase inhibitors (TKI) lapatinib and neratinib (3, 19–21). Both lapatinib and neratinib demonstrate nearly equipotent inhibition of EGFR and HER2 (22, 23), which is thought to contribute to high frequencies of dermatologic and gastrointestinal adverse events (AE), necessitating dose interruptions and modifications (24, 25).

Considering the tolerability challenges imposed by the potent coinhibition of HER2 and EGFR by the approved HER2-targeted TKIs lapatinib and neratinib, there is an unmet need for novel TKIs that inhibit HER2 activity while sparing other EGFR subfamily proteins to facilitate dosing continuity, maintain efficacy, and increase tolerability. In this article, we provide a preclinical characterization of tucatinib, an orally bioavailable, small-molecule HER2-targeted TKI that has shown clinical activity in studies of HER2+ advanced cancers, including activity in brain metastases (19, 26–47).

**Materials and Methods**

**Biochemical assays**

HER2 biochemical assays were performed using HER2 aa 679–1255 produced as an N-terminal GST fusion protein (Prokinease #015). Assays contained 16.7 nmol/L HER2 enzyme and 0.2 μg/mL poly (Glu-Tyr) E4Y1 peptide substrate (Sigma). Assays were performed in 50 mmol/L HEPES, pH 7.5, 20 mmol/L MgCl₂, 0.5 mmol/L DTT, and 0.01% Tween using 22 μm/L ATP. Kinase activity was determined after 4-hour incubation at 25°C using ADP-Glo reagent (Promega). EGFR biochemical assays were performed using EGFR aa 668–755 produced as an N-terminal GST fusion protein (SignalChem #E10-112G). Assays contained 6 nmol/L enzyme and 0.2 μg/mL substrate peptide poly (Glu-Tyr), E4Y1. Assays were performed in 50 mmol/L HEPES, pH 7.5, 20 mmol/L MgCl₂, 5 mmol/L MnCl₂, 0.5 mmol/L DTT, and 0.01% Tween using 14.8 μm/L ATP. Kinase activity was determined after 2-hour incubation at 25°C using ADP-Glo reagent. 

IC₅₀ values for kinase activity inhibition were determined by fitting ADP-Glo assay data using a nonlinear 4-parameter variable slope formula (GraphPad Prism). Tucatinib potency was determined using data from a 12-point serial dilution (3-fold dilutions) of drug ranging from 0.00017 to 30 μm/L. Lapatinib potency was determined using data from a 12-point serial dilution (3-fold dilutions) of drug ranging from 0.0000393 to 6 μm/L. Lapatinib potency was determined using data from a 16-point serial dilution (2-fold dilutions) of drug ranging from 0.000137 to 4.5 μm/L.

The HER4 kinase assay was performed using N-terminal His6-tagged recombinant human ErbB4 (aa 706–991 expressed by baculovirus) and 7.5 μm/L ATP in assay buffer [50 mmol/L HEPES, pH 7.3, 5 mmol/L MgCl₂, 0.2 mmol/L MnCl₂, 0.1 mmol/L Na₂VO₄, final DMSO concentration 1% (v/v)]. Reagents were incubated on 0.25 mg/mL PGT-coated plates for 20 minutes at room temperature. The reaction mixture was removed by washing and the phosphorylated polymer substrate was detected with 0.2 μg/mL phosphotyrosine specific mAb (PY20) conjugated to horseradish peroxidase. After the addition of 1 mol/L phosphoric acid to stop the development, the chromogenic substrate color was quantified by spectrophotometry at 450 nm. IC₅₀ values were determined by fitting data using a nonlinear 4-parameter variable slope formula (GraphPad Prism). Tucatinib potency in the HER4 assay was determined using data from a 10-point serial dilution (3-fold dilutions) of drug ranging from 0.000508 to 10 μm/L.

**Kinase-inhibitory activity profiling screen**

Tucatinib-inhibitory activity against 223 kinases was analyzed using the KinaseProfiler service (Millipore). Compounds were run in duplicate at a concentration of ATP near the Kᵣ for each individual kinase according to the manufacturer’s specifications. Tucatinib activity across this panel of kinases is represented by a kinome tree illustration (Cell Signaling Technology), which was created using the KinMap web-based tool (www.kinhub.org/kinmap; ref. 29). Tucatinib activity was assessed at 1 and 10 μm/L.

**Phosphorylation analysis**

**HER2 and EGFR**

BT-474 and A431 cells (1.5 × 10⁵ cells in a 24-well) were seeded overnight and treated with increasing concentrations of tucatinib (0.1–10,000 nmol/L) per well for 2 hours. Samples were run with two replicates. Purified EGF (Cell Signaling Technology) was added to A431 cells for a final concentration of 10 ng/mL for 10 minutes prior to harvest. Lysates were prepared with buffer (supplied) containing protease and phosphatase inhibitors, and samples were processed using PathScan Sandwich ELISA kit (Cell Signaling Technology) according to manufacturer’s protocol. Assays were performed for total HER2 (Cell Signaling Technology, catalog no. 7310), phospho-HER2 (pan-Tyr, Cell Signaling Technology, catalog no. 7968), total EGFR (Cell Signaling Technology, catalog no. 7250), and phospho-EGFR (pan-Tyr, Cell Signaling Technology, catalog no. 7911). Phosphorylated protein levels were normalized to total protein levels. Analysis was performed within GraphPad Prism software, with IC₅₀ values generated from best-fit curves log(inhibitor) versus response—4-parameter variable slope formula. Error bars represent SD.

HER2, HER3, MEK, ERK, and AKT phosphorylation assays

BT-474 cells (30,000 per 96-well) were seeded overnight and treated with increasing concentrations of tucatinib for 2 hours at 37°C. Lysates were collected and phosphorylation of respective proteins was analyzed with phospho-ERK 1/2, phospho-MEK1, and phospho-AKT MapMate kits (Millipore) while phospho-HER2 and phospho-HER3 were detected by a custom phospho-RTK panel (Millipore) according to the manufacturer’s protocol. Samples were run on a Luminex LX200 instrument (R&D Systems) using xPONENT software.

**Cell lines**

BT-474 and A431 cells were obtained from ATCC in 2018 and 2013, respectively. Cell lines were demonstrated to be free of Mycoplasma by PCR evaluation and were authenticated by marker analysis prior to banking (completed by Idexx Bioanalytics). Cells were maintained in culture for a maximum of 7 weeks (7–15 passages).

**Quantitative flow cytometry analysis of cell surface receptor density**

HER2 and EGFR cell surface levels were determined using quantitative flow cytometry–based Qih kit (Agilent Dako, catalog no. K0078) executed according to manufacturer’s protocol. Cells were stained with anti-HER2 antibody (R&D Systems, catalog no. MAB1129) and anti-EGFR antibody (Abcam, catalog no. ab30) for Quantification.

**Cytotoxicity assays**

BT-474 (4,000 cells per 96-well) and A431 (5,000 cells per 96-well) cells were seeded overnight in duplicate and treated with increasing concentrations of tucatinib (0.15–10,000 nmol/L). Cell viability was measured at 96 hours using a CellTiter-Glo (CTG) Luminescent Cell
viability assay (Promega) following manufacturer’s protocol. Data were analyzed in GraphPad Prism, and EC50 values were generated from best-fit curves. Cytotoxicity against the panel of breast cancer cell lines were run in similar format and are described in greater detail in Supplementary Methods.

Caspase-3/7 apoptosis assays

BT-474 cells (50,000 per well) were seeded overnight and then treated with increasing concentrations of tucatinib (0.2–1,000 nmol/L) and trastuzumab (150 µg/mL; equivalent to 1 µmol/L) for 18 hours at 37°C. Caspase-Glo® 3/7 reagent and buffer (100 µL/well) was added and after 30-minute incubation, caspase-3/7 activity was quantified on a BMG PolarStar Luminator (BMG LABTECH).

Xenograft models

Cell line–derived (CDX) breast (BT-474) and gastric (NCI-N87) cancer cells were implanted subcutaneously into the flanks of female immunocompromised mice. Animals were treated with tucatinib (25, 50, or 100 mg/kg orally, every day), trastuzumab (20 mg/kg, intraperitoneally, every 3 days), doctaxel (10 mg/kg, intravenously, once weekly), or vehicle (30% Captisol, orally, everyday). Patient-derived (PDX) tumor fragments from solid tumors (breast, gastric, colorectal, and esophageal) were implanted subcutaneously into the flanks of immunocompromised mice. Mice were treated with tucatinib (50 mg/kg, orally, twice a day), trastuzumab (20 mg/kg, intraperitoneally, every 3 days or once weekly), tucatinib + trastuzumab, or vehicles (30% Captisol, orally, every day; PBS, intraperitoneally, twice a week). Animals were followed to the designated end of each experiment. Additional details are described in the Supplementary Methods. All in vivo studies adhered to Institutional Animal Care and Use Committee guidelines.

Statistical analysis

Data in xenograft studies are expressed as the mean ± SE. Statistical analyses were performed using data up to the last day that tumor volumes were measured for all groups. Statistical comparisons of tumor volumes in the efficacy studies were conducted using one-way ANOVA followed by Tukey multiple comparisons test to compare between all groups.

Tucatinib preparation

4-[(1,2,4]triazo]ol-1,5-][pyridin-7-yl]oxy)-3-methylphenyl)amine was prepared at Array Biopharma or Cascadian Therapeutics according to the following process description: step 1: 2-amino-4-hydroxy pyridine hydrochloride is reacted with 2 mol/L sodium hydroxide solution and water is added to yield 4-{[(1,2,4]triazo]-1,5-[pyridin-7-yl]-3-methylphenyl) amino)quinazolin-6-4,6-diamine (85%). Step 2: 1-(4-{[(1,2,4]triazo]-1,5-[pyridin-7-yl]-3-methylphenyl] amino)quinazolin-6-yl)-3-(1-hydroxy-2-methyl-2-propan-2-yl)thiourea was synthesized according to the processes described in the United States Patent Application Number US20170136022A1. Step 1: (E)-N'-(2-Cyano-4-[(1,2,4]triazo]-1,5-[pyridin-7-yl]-3-methylphenyl) hydrochloride in dimethylformamide at 83°C – 100°C was treated with increasing concentrations of tucatinib (0.2–1,000 nmol/L) and trastuzumab (100 µg/mL) for 18 hours at 37°C. Solvent swap to IPA to isolate (E)-N'-(2-Cyano-4-[(1,2,4]triazo]-1,5-[pyridin-7-yl]-3-methylphenyl) hydrochloride (95%). Step 2: (E)-N'(2-cyano-4-phenyl)-NN-dimethylformimidamide in THF is hydrogenated with 10% Pd/C at 35°C. Solvent swap to IPA to provide (E)-N'(2-cyano-4-phenyl)-NN-dimethylformimidamide (90%). Step 3: (E)-N'(4-amino-2-cyano-phenyl)-NN-dimethylformimidamide in THF is hydrogenated with 10% Pd/C at 35°C. Solvent swap to IPA to provide (E)-N'(4-amino-2-cyano-phenyl) hydrochloride (90%). Step 4: (Z)-N-(3-(1-hydroxy-2-methyl-2-propan-2-yl)thioureido)phenyl)-NN-dimethylformimidamide (80%).

Tucatinib is a potent and selective HER2 kinase inhibitor

Tucatinib was initially identified via a small-molecule discovery effort focused on HER2 and EGFR TKIs. On the basis of its chemical structure, which is conserved with other TKIs that contain a quinazoline core including lapatinib, erlotinib, and gefitinib (Fig. 1A), tucatinib is expected to bind to the ATP pocket of HER2 as a competitive, reversible inhibitor. However, unlike the related HER2-targeted TKI lapatinib (23), tucatinib demonstrates selectivity for HER2 compared with EGFR. In a kinase assay, the half maximal inhibitory constant (E)-N'(2-Cyano-4-[(1,2,4]triazo]-1,5-[pyridin-7-yl]-3-methylphenyl) hydrochloride (81%). Step 1: 2-amino-5-nitrobenzenediamine hemi-ethanolate (83%). Step 2: 1-(4-{[(1,2,4]triazo]-1,5-[pyridin-7-yl]-3-methylphenyl) amino)quinazolin-6-4,6-diamine (85%). Step 3: 1-(4-{[(1,2,4]triazo]-1,5-[pyridin-7-yl]-3-methylphenyl] amino)quinazolin-6-yl)-3-(1-hydroxy-2-methyl-2-propan-2-yl)thiourea was agitated in THF under basic conditions (2.5 N NaOH), followed by the addition of p-toluensulfon chloride. Water was charged to yield N4-{[(1,2,4]triazo]-1,5-[pyridin-7-yl]-3-methylphenyl]-N6-(4,4-dimethyl-4,5-dihydroxazol-2-yl)quinazoline-4, 6-diamine (92%). N4-{[(1,2,4]triazo]-1,5-[pyridin-7-yl]-3-methylphenyl}N6-(4,4-dimethyl-4,5-dihydroxazol-2-yl)quinazoline-4, 6-diamine was triturated in ethanol at greater than 65°C to provide N4-{[(1,2,4]triazo]-1,5-[pyridin-7-yl]-3-methylphenyl}-N6-(4,4-dimethyl-4,5-dihydroxazol-2-yl)quinazoline-4, 6-diamine hemi-ethanolate (83%).

Material used in tumor models was manufactured to GMP specifications. M(S(ESI): m/z 481 (M+H+); 1HNMR (DMSO-d6) δ (ppm) 1.31 (3H, 6H); 2.21 (2H, 3H), 4.08 (2H, 2H), 6.83 (d, 2.59 Hz, 1H), 7.00 (dd, 7.43, 2.59 Hz, 1H), 7.67 (d, 1H), 7.67 (d, br, 1H), 7.67 (d, br, 1H), 7.18 (d, 8.73 Hz, 1H), 7.87 (d, 8.73 Hz, 1H). 91.91 (s, 1H), 8.03 (s, br, 1H), 8.35 (s, br, 1H), 8.5 (s, 1H), 8.89 (d, 1H), 9.15 (s, 1H), 13CNMR (DMSO-d6) δ (ppm) 16.5 (CH3), 27.3 (2CH3), 56.6 (C), 78.3 (CH2), 98.2 (Ar-C), 108.0 (Ar-C), 116.4 (Ar-C), 121.9 (Ar-C), 122.1 (Ar-C), 125.6 (Ar-C), 128.7 (Ar-C), 130.4 (Ar-C), 131.1 (Ar-C), 130.4 (Ar-C), 138.1 (Ar-C), 146.1 (Ar-C), 147.9 (Ar-C), 151.9 (Ar-C), 152.8 (Ar-C), 155.2 (Ar-C), 156.3 (C), 157.6 (Ar-C), 160.4 (Ar-C). HPLC purity 99.67%.

Results

Tucatinib is a potent and selective HER2 kinase inhibitor

Tucatinib was initially identified via a small-molecule discovery effort focused on HER2 and EGFR TKIs. On the basis of its chemical structure, which is conserved with other TKIs that contain a quinazoline core including lapatinib, erlotinib, and gefitinib (Fig. 1A), tucatinib is expected to bind to the ATP pocket of HER2 as a competitive, reversible inhibitor. However, unlike the related HER2-targeted TKI lapatinib (23), tucatinib demonstrates selectivity for HER2 compared with EGFR. In a kinase assay, the half maximal inhibitory constant

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(IC$_{50}$) of tucatinib with HER2 was 6.9 nmol/L, 50-fold lower than that for EGFR, which showed an IC$_{50}$ of 449 nmol/L (Fig. 1B). Similarly, tucatinib demonstrated reduced potency in a HER4 biochemical assay relative to HER2, with an IC$_{50}$ of 310 nmol/L. Consistent with published data (22, 23), the IC$_{50}$ values for lapatinib and neratinib for HER2 and EGFR did not show HER2-selective kinase inhibition (Fig. 1B). A biochemical screen of 223 kinases confirmed tucatinib selectivity within the EGFR kinase family, with minimal inhibition of other kinases (Fig. 1C). Inhibition of non-EGFR kinases in the screen was limited with no kinase demonstrating more than 50% inhibition at a concentration of 1 μmol/L tucatinib and 7 kinases (KIT, EphA1, EphA2, Flt4, Lck, MERTK, PKD1) demonstrating more than 50% inhibition at a concentration of 10 μmol/L tucatinib.

**Tucatinib selectively inhibits HER2 signaling activity and cell proliferation in HER2-driven breast cancer cell lines**

To determine the potency of tucatinib in blocking HER2 phosphorylation in tumor cells, phosphorylation assays were performed in vitro using BT-474 (breast cancer) and A431 (skin cancer) cells. These cell lines were chosen for evaluating tucatinib’s cellular HER2 and EGFR activity based on the observation that BT-474 cells due to genomic amplification of the HER2 gene, resulting in high-level expression of HER2 protein on the cell surface (30), and that A431 cells overexpress EGFR due to genomic amplification of the EGFR gene (31). We confirmed these results by measuring the cell surface density of HER2 and EGFR using quantitative flow cytometry (Fig. 2A; Supplementary Table S1). In cell signaling assays, tucatinib potently inhibited phosphorylation of HER2 in BT-474 cells (IC$_{50}$ = 7 nmol/L)
but showed minimal inhibition of EGFR phosphorylation in A431 cells (IC_{50} > 10,000 nmol/L; Fig. 2B). Tucatinib treatment also strongly inhibited BT-474 cell proliferation (IC_{50} = 39 nmol/L) but had a much less potent effect on A431 cell proliferation, exhibiting an IC_{50} value nearly 500-fold higher (IC_{50} = 16,471 nmol/L; Fig. 2C). Similarly, tucatinib showed minimal activity in HER2-negative breast cancer cell lines (IC_{50} range, 4,938 to >25,000 nmol/L), whereas it demonstrated exponentially increasing potency in HER2 + breast cancer cell lines as HER2 surface receptor density increased (IC_{50} range, 23–431 nmol/L; Fig. 2D; Supplementary Table S1).

When compared directly with lapatinib or neratinib, only tucatinib demonstrated selectivity for HER2 compared with EGFR (Supplementary Fig. S1). In BT-474 cells, tucatinib inhibited HER2 phosphorylation with an IC_{50} of 7 nmol/L, which was similar to neratinib (IC_{50} = 2 nmol/L) and more potent than lapatinib (IC_{50} = 46 nmol/L; Supplementary Fig. S1). In contrast, tucatinib showed no appreciable inhibition of EGFR phosphorylation in A431 cells when dosed up to 1,000 nmol/L, whereas both neratinib (IC_{50} = 21 nmol/L) and lapatinib (IC_{50} = 36 nmol/L) potently inhibited EGFR phosphorylation (Supplementary Fig. S1). Similarly, in NCI-N87 HER2+ gastric

<table>
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<th>Cell line</th>
<th>HER2 Expression (Molecules)</th>
<th>EGFR Expression (Molecules)</th>
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<td>BT-474</td>
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<td>110</td>
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<td>A431</td>
<td>6,498</td>
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Figure 2.
Tucatinib selectively inhibits HER2-mediated signal transduction and is selectively cytotoxic in HER2-amplified cells. A, Surface expression of HER2 and EGFR receptors in BT-471 and A431 cell lines was assessed by quantitative flow cytometry. B, Inhibition of HER2 and EGFR by tucatinib in BT-474 and A431 tumor–derived cell lines. Duplicate samples were analyzed by ELISA recognizing a protein or phospho-specific protein (anti-HER2, anti-phospho-HER2, anti-EGFR, anti-phospho-EGFR). Results are expressed as total phospho-protein signal normalized to total protein ± SD. C, Cytotoxicity of tucatinib in HER2+ (BT-474) and EGFR-amplified (A431) cell lines. Cell viability (± SD) was measured from duplicate samples after 96 hours using the CellTiter Glo Luminescent Cell Viability Assay. D, Comparison of cellular IC_{50} and HER2 expression in breast cancer cell lines. HER2 expression levels were quantified in a panel of 22 breast cancer cell lines using quantitative flow cytometry. IC_{50} values were calculated for each cell line using a 10-point titration of tucatinib. Cell viability was measured after 96 hours using the CellTiter-Glo Luminescent Cell Viability Assay.
cancer cells, tucatinib potently inhibited HER2 phosphorylation (IC₅₀ = 4 nmol/L) but produced only a modest reduction in EGFR phosphorylation at 1,000 nmol/L. In contrast, lapatinib and neratinib both inhibited HER2 and EGFR phosphorylation in NCI-N87 cells with IC₅₀ values of 40 and 12 nmol/L, respectively.

**Tucatinib + trastuzumab combinations show enhanced inhibition of HER2 signaling activity**

HER2 signaling is mediated through activation of other EGFR subfamily members and several downstream effector pathways including MAPK/ERK and PI3K-AKT (12). Treatment of BT-474 cells with tucatinib inhibited phosphorylation of both HER2 and HER3 (Figs. 2B and 3A), a preferred dimerization partner of HER2 (2, 6). In addition, treatment of BT-474 cells with tucatinib inhibited phosphorylation of the downstream effector proteins MEK1 (S222), ERK1/2 (T185/Y187), and AKT (S473; Fig. 3A) with IC₅₀ values in the same range as HER2 inhibition. Trastuzumab binds to the extracellular domain of HER2, resulting in the inhibition of HER2/HER2 homodimerization, inhibition of downstream signaling through the PI3K–AKT pathway, and suppression of tumor cell proliferation (3, 32). On the basis of differing mechanisms of action by which each treatment inhibits HER2, we hypothesized that combination treatment of tucatinib + trastuzumab may lead to enhanced inhibition of HER2 downstream signaling activity. Treatment of BT-474 cells with tucatinib alone resulted in a dose-dependent reduction of AKT phosphorylation. Treatment of BT-474 cells with 150 µg/mL trastuzumab alone also reduced AKT phosphorylation, and when combined with increasing concentrations of tucatinib, resulted in further diminution of AKT phosphorylation (Fig. 3B). Consistent with increased inhibition of AKT phosphorylation, the combination of tucatinib + trastuzumab resulted in a 2-fold increase in caspase-3/7 activity (Fig. 3C). Overall, the above data show that tucatinib is a highly selective and potent HER2 inhibitor with strong antitumor cellular effects that are enhanced by combination treatment with trastuzumab.

**Tucatinib exhibits activity alone or in combination with trastuzumab or docetaxel in CDX xenograft models of HER2⁺ cancer**

We investigated the ability of tucatinib to prevent tumor cell growth in vivo in HER2⁺ breast and gastric cancer CDX xenograft models. Female immunocompromised mice implanted with BT-474 cells were treated with tucatinib and evaluated for response. Mice treated with tucatinib exhibited tumor growth delay at doses of 25 or 50 mg/kg tucatinib administered orally every day. This effect was similar to mice...
treated with trastuzumab monotherapy (Fig. 4A). In contrast, mean tumor volume (MTV) increased > 4-fold in mice treated with vehicle (Fig. 4A).

We then investigated the activity of tucatinib combined with docetaxel, a cytotoxic microtubule-targeted drug commonly used in patients with metastatic breast cancer (MBC; ref. 33). Using the BT-474 model, tumor-bearing mice treated with docetaxel exhibited tumor growth delay compared with the vehicle group. Mice treated with tucatinib monotherapy exhibited a similar reduction in tumor growth (Fig. 4B). When combined, tucatinib + docetaxel were significantly more active than either drug alone (P < 0.0001; Fig. 4B).

Given that tucatinib and trastuzumab were both individually effective at reducing tumor growth as single agents in the BT-474 breast cancer model (Fig. 4A), we next investigated whether tucatinib + trastuzumab would result in further tumor growth inhibition compared with monotherapy with either agent alone. In an independent experiment, tucatinib monotherapy again resulted in tumor growth inhibition that was similar to trastuzumab monotherapy (Fig. 4C). The combination of tucatinib + trastuzumab exhibited enhanced antitumor effects (Fig. 4C), resulting in 10 complete tumor regressions and 1 partial tumor regression in the group of 12 treated mice (Fig. 4C, Table 1). The treatment effect of the combination of tucatinib and trastuzumab was significant when compared with tucatinib alone (P < 0.0001) or trastuzumab alone (P < 0.0001). Overall, these data suggest that tucatinib is active against HER2+ breast tumors as both monotherapy and in combination with two standard-of-care therapies.

In addition to the BT-474 model, tucatinib was also evaluated in an NCI-N87 HER2+ gastric cancer cell line tumor model. In immunocompromised mice implanted with NCI-N87 cells, tumor growth was inhibited by tucatinib and included 4 partial tumor regressions (Fig. 4D). Mice treated with trastuzumab also exhibited a reduction in tumor growth, but no partial regressions (Fig. 4D). Mice treated with tucatinib + trastuzumab had an even greater antitumor response with higher rates of tumor regression noted (Fig. 4D; Table 1). These results were significant when compared with either tucatinib alone (P = 0.0015) or compared with trastuzumab alone (P < 0.0001). Collectively, these data suggest that tucatinib, alone and in combination with trastuzumab, effectively decreases in vivo tumor growth in the NCI-N87 gastric cancer model.

**Tucatinib exhibits in vivo activity alone or in combination with trastuzumab in HER2+ PDX models of breast, gastric, colorectal, and esophageal cancer**

To further investigate the preclinical efficacy of tucatinib, PDX models of HER2+ solid tumors derived from breast, gastric, colorectal, and esophageal cancers were tested. For each model, PDX tumor samples were chosen based on verified HER2 gene amplification (Supplementary Table S2), and tucatinib was evaluated alone or in combination with trastuzumab and compared with trastuzumab as a single agent.

Tucatinib as a single agent demonstrated activity in 3 breast (Fig. 5A), 3 gastric (Fig. 5B), 3 colorectal cancer PDX models (Fig. 5C), and 2 esophageal PDX models (Fig. 5D); %TGI values are...
Tucatinib Activity in Preclinical HER2^+ Solid Tumor Models

Table 1. In vivo efficacy of tucatinib as monotherapy or in combination with trastuzumab in various tumor models.

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Note: %TGI was calculated as [1 - (MTV in treatment group on day X - MTV in treatment group on day 0)/(MTV in vehicle group on day X - MTV in vehicle group on day 0)]. %TGI was typically calculated 0 to 2 days after the last day of tucatinib dosing, except for BT-474, CTG-0137, and CTG-0708 where %TGI was calculated on day 14, 15, and 24, respectively, due to rapid tumor growth in the vehicle group and animal sacrifices mandated by the protocol. Tumor regression criteria: PR, >50% reduction in tumor volume at least 2 consecutive measurements; CR, no detectable tumor for at least 2 consecutive measurements.

In the breast cancer PDX models, tucatinib + trastuzumab resulted in superior tumor growth inhibition (Fig. 5A) compared with trastuzumab alone (CTG-0708, P = 0.0431; CTG-0717, P = 0.0243) with partial responses noted in the CTG-0717 and CTG-0807 models (Table 1). Importantly, each of these breast PDX models are derived from patients that had shown clinical disease progression with trastuzumab combination regimens.

In the colorectal PDX models, tucatinib and trastuzumab treatment significantly reduced MTVs compared with vehicle (Table 1) and the combination of both drugs produced higher partial tumor regressions in 2 of 3 colorectal models (Table 1). In the CTG-0383 model, the combination of tucatinib with trastuzumab was significantly more active than trastuzumab alone (P = 0.0017). In the CTG-0309 model, the combination of tucatinib and trastuzumab was significantly more active than tucatinib alone (P = 0.0006) or trastuzumab alone (P < 0.0001).

In the esophageal PDX models, tucatinib + trastuzumab treatment significantly reduced MTVs compared with vehicle (Fig. 5D) and induced tumor regressions in CTG-0138 model (Table 1). In the CTG-0137 model, the combination of tucatinib and trastuzumab was significantly more active than tucatinib monotherapy (P = 0.0027) or trastuzumab monotherapy (P = 0.0117). In the CTG-0138 model, tucatinib monotherapy was more effective than trastuzumab monotherapy (P = 0.003) and the combination of tucatinib and trastuzumab was also significantly more active than trastuzumab monotherapy (P < 0.0001; Fig. 5D; Table 1). In total, these data, together with the results in breast and gastric CDX xenograft models, support the idea that tucatinib is active as single agent in diverse HER2^+ solid tumor models and that the combination of tucatinib with trastuzumab can enhance antitumor responses in many of these models.

Treatment of mice with tucatinib or the combination of tucatinib with trastuzumab was well tolerated with net positive weight gain over the course of the studies and similar to vehicle or trastuzumab single-agent treatment in many models (Supplementary Fig. S2).

Discussion

The advent of HER2-targeted therapies has resulted in a dramatic improvement in the survival of patients with HER2^+ breast cancer. In addition to breast cancer, other HER2^+ cancers, including gastric and colorectal, have been shown to respond to combination therapies that include HER2-targeted agents (5, 9, 10, 34). However, despite the effectiveness of these therapeutic strategies, there remains a need for new HER2-targeted therapies. The data presented here demonstrate that tucatinib is a novel, highly selective, and potent HER2-targeted TKI that exhibits activity in a variety of HER2^+ cancer models.

Biochemical assays demonstrate that tucatinib is a selective inhibitor of HER2 enzymatic activity, with reduced potency against EGFR.
This property distinguishes tucatinib from other HER2-targeted TKIs, including lapatinib and neratinib, which are potent inhibitors of both HER2 and EGFR (22, 23). In a broad kinome screen, it was shown that tucatinib potently targets only members of the EGFR RTK family when tested at drug concentrations that are 100- or 1,000-fold the biochemical IC₅₀ value for HER2 kinase inhibition. Similar selectivity for the EGFR kinase family was demonstrated with lapatinib, a structurally similar kinase inhibitor, but the HER2/EGFR inhibitor neratinib.

Figure 5.
Tucatinib monotherapy or in combination with trastuzumab significantly reduces tumor volume in HER2⁺ breast, gastric, colorectal, and esophageal PDX xenograft models. PDX xenograft models were developed from HER2⁺ breast (A), gastric (B), colorectal (C), and esophageal cancers. D, Tumor fragments implanted subcutaneously into the flank of female nude mice. Animals were treated with tucatinib (50 mg/kg, orally, twice a day), trastuzumab (20 mg/kg, intraperitoneally, every 3 days [gastric, colorectal, and esophageal] or once weekly [breast]), the combination of tucatinib + trastuzumab, or vehicle. Dosing continued for 28 days and tumor volume measurements were followed for up to 60 days after treatment initiation. Tumor volumes were measured at select timepoints throughout the experiment. Data are reported as MTV ± SEM.
The combination of tucatinib inhibited the phosphorylation of ERK1/2 at threonine 185 and 187, and MEK1 at serine 222. Of AKT phosphorylation at serine 473, and inhibition of phosphor-signaling, including a blockade of HER3 phosphorylation, inhibition of HER2 phosphorylation in the NCI-N87 line. These results are consistent with recently published data on lapatinib and neratinib. For example, lapatinib showed equivalent potency for inhibiting HER2 and EGFR in enzymatic and cell signaling assays and demonstrated similar potencies in blocking proliferation of either BT-474 or A431 cells in vitro (22). Likewise, neratinib also demonstrates similar inhibitory potencies in enzymatic assays comparing HER2 and EGFR, and in cell signaling assays for HER2 (BT-474 cells) and EGFR (A431 cells; ref. 23).

The enhancement in the relative potency for HER2 compared with EGFR in cell signaling assays (>1,000-fold) compared with enzymatic assays (~50-fold) may reflect differences in the structure of the ATP-binding pocket of ERK1/2 and MEK1 versus HER2, which may be a property of the covalent-binding mechanism of action (35).

The biological relevance of the HER2 selectivity of tucatinib was demonstrated when measured using tumor-derived cell lines containing HER2 or EGFR amplification. In HER2-amplified BT-474 cells, tucatinib blocked HER2 phosphorylation with an IC50 of 7 nmol/L, but at drug concentrations up to 10 μmol/L, there was no reduction in EGFR phosphorylation in EGFR-amplified A431 cells. This result was distinct from both lapatinib and neratinib, which potently inhibited both HER2 and EGFR phosphorylation in these two cell lines. Similar results were demonstrated using the NCI-N87 gastric cancer cell line, with tucatinib producing potent inhibition of HER2 phosphorylation with only a modest reduction in EGFR phosphorylation at 1 μmol/L drug. In contrast, lapatinib and neratinib potently inhibited both HER2 and EGFR phosphorylation in the NCI-N87 line. These results are consistent with published data on lapatinib and neratinib. For example, lapatinib showed equivalent potency for inhibiting HER2 and EGFR in enzymatic and cell signaling assays and demonstrated similar potencies in blocking proliferation of either BT-474 or A431 cells in vitro (22). Likewise, neratinib also demonstrates similar inhibitory potencies in enzymatic assays comparing HER2 and EGFR, and in cell signaling assays for HER2 (BT-474 cells) and EGFR (A431 cells; ref. 23).

In summary, we have demonstrated that tucatinib is a potent and highly selective HER2 kinase inhibitor with increased selectivity for HER2 compared with earlier-generation HER2-targeted TKIs such as lapatinib and neratinib. Pharmacologic data from both in vitro and in vivo studies demonstrate that tucatinib has antitumor activity, both as a single agent and in combination with either trastuzumab or lapatinib. These results are consistent with the findings from the phase-I clinical trial results with tucatinib as a single agent, where it was demonstrated that in patients with HER2+ solid tumors (n = 35), tucatinib produced an objective response rate (ORR) of 9% (19); in an expansion cohort of patients with HER2+ MBC (n = 22) treated with higher doses of tucatinib, the ORR was 14%, all of which were PRs (19).

The increased activity of dual targeting of HER2, either through combinations of two different HER2-targeted antibodies or by use of a HER2-targeted antibody and TKI, has been shown to improve outcomes in preclinical studies and in patients with HER2+ cancers (10, 38). In this study, the combinatorial activity of tucatinib + trastuzumab also produced improved antitumor activity in vivo in HER2+ CDX and PDX xenograft models of breast, gastric, colorectal, and esophageal cancers. Overall, there were substantially more partial and complete tumor regressions induced by tucatinib + trastuzumab combination therapy compared with either single agent alone. Across the CDX and PDX models, there were 25 complete tumor regressions; induced by the combination of tucatinib + trastuzumab, compared with 3 generated by tucatinib and 3 by trastuzumab alone. The overall regression rate for tucatinib in these studies was 26%, with a 15% regression rate for trastuzumab and a 66% regression rate for the combination of both agents.

These preclinical data support the contention that tucatinib is a potent HER2-selective TKI and that the co-expression of HER2 signal can drive robust antitumor activity across multiple models of HER2+ cancer. In particular, these results highlight the utility of combining tucatinib + trastuzumab, which may translate to produce enhanced clinical activity. These preclinical results and early clinical data support the hypothesis that selective inhibition of HER2 activity by tucatinib, in the absence of EGFR inhibition, can be an effective therapeutic approach in HER2+ cancers. This is an important point since currently available small-molecule TKI therapies for HER2+ breast cancer are equipotent HER2 and EGFR inhibitors, and are associated with skin and gastrointestinal toxicities that may reduce patient quality-of-life (24, 25, 39).

Although EGFR is expressed in HER2+ cancers (44), the role of EGFR expression in the outcome of metastatic patients treated with trastuzumab regimens is unclear (45). Similarly, the therapeutic benefit of inhibiting EGFR in HER2+ MBC has not been clearly defined. For example, in a phase I-II study of the combination of trastuzumab and gefitinib (an EGFR-targeted TKI) in HER2+ MBC, 250 or 500 mg/day doses of gefitinib were tested together with trastuzumab (46). All patients treated with the 500 mg/day dose of gefitinib developed grade 3/4 gastrointestinal toxicity and that dose combination was deemed beyond the MTD. At the tolerated dose of 250 mg/day, there was an ORR of 9% and a clinical benefit rate of 28%. An interim analysis of this trial indicated that the 250 mg gefitinib/trastuzumab combination did not merit further testing. These data also suggest that the potent inhibition of EGFR with dual EGFR/HER2 inhibitors could reduce drug tolerability and potentially restrict HER2 target saturation.

In summary, we have demonstrated that tucatinib is a potent and highly selective HER2 kinase inhibitor with increased selectivity for HER2 compared with earlier-generation HER2-targeted TKIs such as lapatinib and neratinib.
Disclosure of Potential Conflicts of Interest

A. Kulikian is a senior scientist and has ownership interest (including patents) in Seattle Genetics. P. Lee is a vice president, pharmacology/toxicology at Array BioPharma. J. Taylor is a staff scientist and has ownership interest (including patents) at Seattle Genetics. A. Forero-Torres is an executive medical director at Seattle Genetics. S. Peterson is a vice president, reports receiving a commercial research grant, and has ownership interest (including patents) in Seattle Genetics. No potential conflicts of interest were disclosed by the other authors.

References

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