**Supplementary Methods**

*Cell-Derived (CDX) and Patient-Derived Xenograft (PDX) Model Experiment Designs*

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
|  | **BT-474 (breast)** | **NCI-N87 (gastric)** | **Breast** | **Gastric** | **Colorectal** | **Esophageal** |
| CDX or PDX | CDX | CDX | PDX | PDX | PDX | PDX |
| Mouse model | Nu/Nu | Hsd:Athymic Nude-*Foxn1nu* | Hsd:Athymic Nude-*Foxn1nu* | NMRI-*Foxn1nu* | Nu/Nu | Nu/Nu |
| Cells/tumor fragments implanteda | 150 – 250 mm3 tumor fragments | 5 × 106 cells | ≈ 100 mm3 tumor fragments | ≈ 100 mm3 tumor fragments | ≈ 125 mm3 tumor fragments | 300 – 1000 mm3 tumor fragments |
| Implant location | Right flank | Right flank | Left flank | Right or left flank | Left flank | Left flank |
| Tucatinib dose and regimen | 25, 50, or 100 mg/kg PO, QD x21 | 50 mg/kg,  PO, BID x21 | 50 mg/kg,  PO, BID x28 | 50 mg/kg,  PO, BID x28 | 50 mg/kg,  PO, BID x28 | 50 mg/kg,  PO, BID x28 |
| Trastuzumab dose and regimen | 20 mg/kg,  IP, QW x3 | 20 mg/kg,  IP, BIW x3 | 20 mg/kg,  IP, QW x4 | 20 mg/kg,  IP, Q3D x 9 | 20 mg/kg,  IP, Q3D x9 | 20 mg/kg,  IP, Q3D x9 |
| Vehicle(s) dose and regimen | 30% Captisol, PO,  QD | 30% Captisol, PO, BID; PBS, IP, BIW | 30% Captisol, PO, BID | 30% Captisol, PO, BID; PBS, IP, Q3D | 30% Captisol, PO, BID; PBS, IP, Q3D | 30% Captisol, PO, BID; PBS, IP, Q3D |
| Duration of experiment, days | 23 | 27 | 28 | 42 | 43 – 60 | 27 – 44 |

BID, twice daily; BIW, twice weekly; IP, intraperitoneal; PBS, phosphate buffered saline; PO, oral; Q3D, every 3 days; QD, once daily; QW, every week.

a All cells/tumor fragments were implanted subcutaneously.

*Comparative Potency of Tucatinib, Neratinib, and Lapatinib*

BT-474 cells (25,000 per well), A431 cells (20,000 per well), or NCI-N87 cells (10,000 per well) were treated with increasing concentrations of tucatinib, neratinib, or lapatinib for 2 hours. Lysates were collected, and phosphorylation of HER2 and EGFR was quantified by a Luminex LX200 instrument (R&D Systems).

*Protein Kinase Enzymatic Assays*

HER2 enzymatic activity was determined using N-terminal GST-tagged HER2 amino acids 679-1255 (ProQinase #0108) with 22 µM ATP and 0.2 µg/µL poly (Glu-Tyr) E4Y1 in a buffer containing 50 mM HEPES pH 7.5, 3 mM MgCl2, 3 mM MnCl2, 0.5 mM DTT, and 0.01% Tween. Reactions were performed at 25oC for 2 hours, and kinase activity was determined using ADP-Glo reagent (Promega) and quantified using a LUMIstar Omega (BMG LABTECH) plate reader. Half maximal inhibitory concentration (IC50) values for lapatinib were determined based on an 18-point titration using 2-fold serial dilutions from 4.58 × 10-5 to 6µM. The IC50 for tucatinib was determined using a 12-point titration of drug with 3-fold serial dilutions starting from 1.7 × 10-4 to 30µM. EGFR enzymatic activity was determined using N-terminal GST-tagged EGFR amino acids 668-end (SignalChem #E110-112G) with 14.8 µM ATP and 0.2 µg/mL poly (Glu-Tyr) E4 Y1 in a buffer containing 50 mM HEPES pH 7.5, 20 mM MgCl2, 5 mM MnCl2, 0.5 mM DTT, and 0.01% Tween. Reactions were performed at 25oC for 2 hours, and kinase activity was determined using ADP-Glo reagent and quantified using a LUMIstar Omega plate reader. IC50 values for lapatinib or tucatinib were determined based on an 18-point titration using 2-fold serial dilutions from 4.12 × 10-5 to 5.4µM.

Enzyme kinetic analysis was performed by monitoring the production of phosphorylated product over time in an assay system containing 50 mM K+MOPS, pH 7.5, 0.005% Tween-20, 2 mM MnCl2, 100 µg/mL poly (Glu-Tyr) (phosphate acceptor), 2 µM [γ-33P]ATP (30 µCi/mL), 10 nM HER2, and various concentrations of tucatinib of up to 40 nM in a final volume of 50 µL. Assays were performed at 22°C in a microtiter plate and quenched by the addition of 50 µL of 25% trichloroacetic acid at specified times. Phosphorylated product was captured onto a glass fiber filter using a Tomtec Mach III cell harvester and, after adding scintillation cocktail, quantitated using a TopCount NXT microplate scintillation and luminescence counter (Hewlett Packard).

*HER2 Surface Receptor Density: Cell Lines and Culture Conditions*

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Cell Lines** | **Catalog Number** | **Acquisition & Authenticaton** | **Growth Media** | **Seeding Density (cells per well)** |
| AU-565 | ATCC #CRL-2351 | 2018 | cRPMI-1640 | 2000 |
| A431 | ATCC #CRL-1555 | 2013 | cDMEM | 5000 |
| BT-474 | ATCC #HTB-20 | 2018 | cRPMI-1640 | 4000 |
| BT-483 | ATCC #HTB-121 | 2017 | R20 + insulin | 2000 |
| CAMA-1 | ATCC #HTB-21 | 2017 | cEMEM | 2000 |
| DU-4475 | ATCC #HTB-123 | 2017 | cRPMI-1640 | 2000 |
| HCC-1419 | ATCC #CRL-2326 | 2018 | cRPMI-1640 | 2000 |
| HCC-1569 | ATCC #CRL-2330 | 2018 | cRPMI-1640 | 2000 |
| HCC-1954 | ATCC #CRL-2338 | 2018 | cRPMI-1640 | 2000 |
| HCC 202 | ATCC #CRL-2316 | 2018 | cRPMI-1640 | 5000 |
| HCC-2218 | ATCC #CRL-2343 | 2014 | cRPMI-1640 | 4000 |
| HS-578T | ATCC #HTB-126 | 2017 | cDMEM | 2000 |
| JIMT-1 | DSMZ #ACC-589 | 2018 | cDMEM | 500 |
| MCF7 (ATCC) | ATCC #HTB-22 | 2015 | cEMEM + insulin | 2200 |
| MCF7 (NCI) | NCI-PBCF-HTB22 | 2015 | cEMEM + insulin | 2200 |
| MDA-MB-231 | ATCC #HTB-26 | 2013 | cRPMI-1640 | 2200 |
| MDA-MB-436 | ATCC #HTB-130 | 2012 | cRPMI-1640 | 5000 |
| MDA-MB-468 | ATCC #HTB-132 | 2012 | cRPMI-1640 | 2200 |
| SK-BR-3 | ATCC #HTB-30 | 2013 | cRPMI-1640 | 2000 |
| T47D | ATCC #HTB-133 | 2013 | cRPMI-1640 | 2200 |
| UACC-812 | ATCC #CRL-1897 | 2018 | cRPMI-1640 | 2000 |
| UACC-893 | ATCC #CRL-1902 | 2018 | cRPMI-1640 | 10,000 |
| ZR-75-30 | ATCC #CRL-1504 | 2017 | cRPMI-1640 | 2000 |

Cell lines were obtained from ATCC, National Cancer Institute (NCI), or Leibniz Institute (DSMZ), as specified by catalog number. All 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 seven weeks (7-15 passages).

cRPMI-1640 media: RPMI-1640, 10% HiFBS, 1% sodium pyruvate, 1% L-GlutaMAX.

cEMEM media: EMEM, 10% HiFBS.

cDMEM media: DMEM, 10% HiFBS, 1% sodium pyruvate, 1% L-GlutaMAX.

cEMEM + insulin media: EMEM, 10% HiFBS, 0.01 mg/mL insulin.

R20 media: RPMI-1640, 20% HiFBS.

R20 + insulin media: RPMI-1640, 20% HiFBS, 0.01 mg/mL insulin.