shRNA Kinome Screen Identifies TBK1 as a Therapeutic Target for HER2+ Breast Cancer

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Abstract
HER2+ breast cancer is currently treated with chemotherapy plus anti-HER2 inhibitors. Many patients do not respond or relapse with aggressive metastatic disease. Therefore, there is an urgent need for new therapeutics that can target HER2+ breast cancer and potentiate the effect of anti-HER2 inhibitors, in particular those that can target tumor-initiating cells (TIC). Here, we show that MMTV-Her2/Neu mammary tumor cells cultured as nonadherent spheres or as adherent monolayer cells select for stabilizing mutations in p53 that "immortalize" the cultures and that, after serial passages, sphere conditions maintain TICs, whereas monolayer cells gradually lose these tumorigenic cells. Using tumorsphere formation as surrogate for TICs, we screened p53-mutant Her2/Neu+ tumorspheres versus monolayer cells with a lentivirus short hairpin RNA kinome library. We identified kinases such as the mitogen-activated protein kinase and the TGFβR protein family, previously implicated in HER2+ breast cancer, as well as autophagy factor ATG1/ULK1 and the noncanonical IκB kinase (IKK), TANK-binding kinase 1 (TBK1), which have not been previously linked to HER2+ breast cancer. Knockdown of TBK1 or pharmacologic inhibition of TBK1 and the related protein, IKKε, suppressed growth of both mouse and human HER2+ breast cancer cells. TBK1/IKKε inhibition promoted cellular senescence by suppressing p65–NF-κB and inducing p16Ink4a. In addition, TBK1/IKKε inhibition cooperated with lapatinib, a HER2/EGFR1–targeted drug, to accelerate apoptosis and kill HER2+ breast cancer cells both in culture and in xenografts. Our results suggest that patients with HER2+ breast cancer may benefit from anti-TBK1/IKKε plus anti-HER2 combination therapies and establish conditions that can be used to screen for additional TIC-specific inhibitors of HER2+ breast cancer. Cancer Res; 74(7); 2119–30. ©2014 AACR.

Introduction
HER2+ breast cancer is caused by overexpression/amplification of the HER2/ERBB2/NEU receptor tyrosine kinase and represent approximately 20% of breast tumors (1). About 72% of HER2+ breast cancers contain mutations or deletions in the tumor suppressor p53 (2). Patients with HER2+ breast cancer are treated with chemotherapy plus anti-HER2 inhibitors such as trastuzumab, a monoclonal antibody (mAb) directed against HER2 (3–6). Despite improvement in disease-free survival, adverse effects and emergence of drug-resistant metastases represent serious limitations. There is, therefore, an urgent need to identify novel drugs that can cooperate with anti-HER therapy to effectively kill HER2+ breast cancer.

Many cancer types exhibit hierarchical organization, whereby only a subset of tumor cells, termed tumor-initiating cells (TIC), sustains cancer growth (7, 8). These cells are functionally defined by their ability to induce secondary tumors following transplantation into recipient mice, and in certain cases by their ability to grow as spheres under nonadherent conditions (9, 10). In contrast, the tumor bulk comprises non-TICs, which descended from TICs but show reduced tumorigenic potential. TICs exhibit unique sensitivity to radiation and therapeutic drugs relative to non-TICs (11–13). Indeed, following conventional therapy, residual breast cancers are enriched for TICs (14). It was, therefore, suggested that therapeutic drugs should target TICs. However, there is also evidence that non-TICs can revert, albeit at low frequency, back to TICs (8) and, therefore, curative therapeutic regimens should target both compartments.

The identification of effective therapeutics is hindered by difficulty in obtaining primary tumor samples. One approach to circumvent this problem involves mouse models for specific...
subtypes. Bill Muller’s group has developed a mouse model for HER2+ breast cancer, MMTV-Her2/Neu (15), which has been successfully used to dissect genetic dependencies and pathways involved in metastatic dissemination (16, 17). This model was also used to identify TICs and tumorsphere-forming units (TFU; refs. 9, 18), and to generate a powerful prognostic signature for HER2+ breast cancer (19), demonstrating its utility in modeling the human disease. Here, we show that following several passages of Her2/Neu tumor cells as adherent or nonadherent cells, they become enriched for p53 mutations, and that tumorsphere but not monolayer cells maintain TICs. We then used these p53-mutant Her2+ tumor cells to screen for kinases that are essential for HER2+ breast cancer. The screen identified TANK-binding kinase 1 (TBK1) as essential. Genetic and pharmacologic inhibition of TBK1, and its close relative IKKe, either alone or together with the anti-EGFR/HER2 inhibitor, lapatinib, suppressed growth of human HER2+ breast cancer cell in vitro and in vivo. Our results identify TBK1/IKKe as novel therapeutic target for HER2+ breast cancer.

Materials and Methods

Animals, tumorspheres, and cell culture

MMTV-Her2/Neu mice on pure FvB background were maintained as per University Health Network (UHN) Animal Care Committee Guideline. Of note, 0.5- to 1-cm mammary tumors were minced and digested with 100 U/mL collagenase/hyaluronidase (STEMCELL Technologies) for 60 minutes at 37°C. Following negative selection with the EasySep Kit (STEMCELL Technologies), enriched Lin− mammary epithelial cells were plated onto ultra-low attachment 24-well plates (Corning, Costar) in Dulbecco’s Modified Eagle Medium (DMEM)/F-12 HAM medium containing basic fibroblast growth factor (bFGF), EGF, and B-27 supplement as described previously (9, 20) or onto regular plates with DMEM plus 10% FBS for monolayer cell cultures. HER2+ breast cancer cell lines—HCC1954 (American Type Culture Collection, ATCC; CRL-2338), SKBR3 (ATCC; HTB-30), and JIMT1 (DSMZ; # ACC-589)—were obtained from Dr. Tak W. Mak, UHN (Toronto, Ontario, Canada), and were not further authenticated. Upon receiving, cells were expanded, frozen, and fresh aliquots were used for experiments. HCC1954, SKBR3, and JIMT1 were cultured, respectively, in RPMI, McCoy’s 5A, and DMEM plus 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin. HC11 mammary epithelial cells were cultured in RPMI-1640 plus 10%FBS, EGF, and insulin; and 293T cells in low-antibiotic (0.1XPen/Strep) DMEM supplemented with 10% FBS.

Mouse short hairpin RNA kinase library (TRC1) and Tbk1 shRNA clones

A subset of the Broad Institute RNAi Consortium (TRC) plKO.1 short hairpin RNA (shRNA) library targeting 520 mouse kinase genes with 2,567 lentiviral clones (4–5 shRNAs/gene) were used for screens in a total of 28 × 96-well plates (21). Each plate included 3 to 5 wells with empty vector (Lenti-GFP) as negative controls. shRNA clones TRC1 00000081S2 and 3185 targeting human Tbk1 were purchased from Sigma-Aldrich; lentishRNA vector was used as negative control. For constructing inducible Tbk1 shRNA vector, Tbk1 shRNA primers: 5′-CGG CCT CGG AGG AAC AAA GAA GTA CTCGAG TAC TTC TCT TTG CCT CGG AGG TTGTG-3′ and 5′-AATTCAAAA CCT CGG AGG AAC AAA GAA GTA CTCGAG TTC TTT CTG CCT CGG AGG-3′ were annealed and ligated into pLKO-Tet-On vector (22), which was subsequently transformed into Stbl2, and confirmed by sequencing. Lentiviral particles were produced by transfection of 293T cells with pLKO.1 shRNA vector together with packaging plasmids using TransIT-LT1 (Mirus Bio; MIR2300).

Lentiviral shRNA screens

Briefly, on day 0, 500 Neu monolayer cells were seeded onto 96-well plates. On day 1, 100 μL fresh DMEM containing 8 μg/mL polybrene was added to each well. In parallel, 500 tumorsphere cells were seeded per well onto ultra-low attachment 96-well plates with 100 μL DMEM/F-12 HAM medium containing bFGF, EGF, B-27, and polybrene at 8 μg/mL. TRC1 shRNA library plates for each experiment were thawed at room temperature and aliquots of 10 μL lentivirus-shRNA were transferred to each well via Rapidplate 96/384 Station (Caliper Bioscience). Of note, 2 to 3 shRNA library plates were screened in each experiment (28 plates total). On day 3, medium was gently aspirated and replaced with 200 μL fresh medium. On day 7, tumorsphere counts and MTT assays were carried out (20).

Transplantation

Dissociated tumorsphere, monolayer, or HCC1954 cells were resuspended in 20 μL DMEM/F-12 HAM medium and Matrixel (BD Biosciences; 1:1 ratio) and injected into number 4 mammary glands of FvB or NOD/SCID mice.

Antibodies

We used Rabbit mAb (Cat.#04-856) for Tbk1 (Upstate; Millipore); mouse anti-Bcl-xl (Cat.# 610746) and mouse anti-CASK (Cat.# 610782) from BD Biosciences; mouse anti-p16ink4a (sc-6658), goat anti-Stk25 (sc-6658), and rabbit anti-Acyr2b (sc-25453) from Santa Cruz Biotechnology; rabbit mAb to NF-kB p65 (Cat.# 8242), rabbit mAb to phospho-Ser536-NF-kB p65 (Cat.# 3033), and mAb to p53 from Cell Signaling Technology; anti-Atg1/ULK1 antibody (A7481) and monoclonal anti-Camk2d (ab0081177) and rabbit anti-TGFβRI (SABI3001113) from Sigma.

TBK compound II and other drugs

Details about synthesis/purification of TBK1-II are available upon request. Inhibitors for Tbk1 (BX-795; Axon Medchem BV); CaM kinase II (KN-62; EMD Chemicals); TGFβRI (SB505124, SIS3), 3-Methyladenine (3-MA), chloroquine, and lapatinib were from Sigma. 3-Methyladenine (3-MA) and chloroquine were dissolved in water at room temperature and aliquots of 10 μL 3-MA/methanol or chloroquine/methanol were added to each well. For Tbk1 inhibition, 10 μL lentivirus-shRNA were added. For 3-MA treatment, cells were incubated with 10 μM 3-MA for 5 minutes before using.

Apoptosis, cell-cycle, and flow cytometry analysis

Apoptosis was detected using a PE-Annexin V Kit (BD Biosciences). Cell-cycle analysis was performed on propidium
iodide–stained cells (BD Pharmingen; Cat no. 550825; ref. 20). Anti-CD49f (allophycocyanin) and anti-CD24 (FITC) were from BD Biosciences. Flow cytometry analysis was performed on FACSCalibur (Becton Dickinson).

Senescence-associated β-galactosidase (X-gal) staining
After TBK1-II treatment or infection with lenti-shRNATbk1 for 72 hours, cells were washed twice with PBS, fixed in 3% formaldehyde (Sigma), and stained with X-gal solution (1 mg/mL X-gal, 40 mmol/L citric acid, 5 mmol/L potassium ferrocyanide, 150 mmol/L NaCl, and 2 mmol/L MgCl2) overnight in a CO2 incubator at 37°C. β-Galactosidase–positive cells were counted using a phase contrast microscope and were expressed as a percentage of total number of cells.

Microarray analysis
Microarray analysis was performed at the UHN Microarray Centre using Illumina HumanHT-12 v4 BeadChip with 500 ng of total RNA prepared by the RNeasy Mini Kit (Qiagen; Cat. no. 74104) from TBK1-II–treated versus untreated HCC1954 cells (GEO accession number GSE53658). Microarray data were processed and normalized by “lumi” package from BioConductor in R using the quantile method. Statistical differences between samples were calculated by Bayesian statistic using “limma” package from BioConductor in R to obtain moderated T value for subsequent pathway analysis.

Pharmacokinetic analysis of TBK1
TBK1-II (OICR7680) was administered at 3 mg/kg i.v. and 30 mg/kg i.p. to mice. Blood samples were collected into micro tubes (EDTA) from 3 mice each through the saphenous vein at the following time points for all three groups: 5, 15, and 30 minutes, 1, 2, 4, 6, 8, and 24 hours. Plasma was separated from blood by centrifugation at 3,000 rpm (1,620 × g) for 10 minutes at 4°C, transferred into Eppendorf tubes, immediately placed on dry ice and stored at −80°C pending ultra performance liquid chromatography (UPLC) and mass spectrometric analysis. A waters acquity UPLC coupled with Xevo QToF mass spectrometer was used, and data analyzed with WinNonlin software.

Statistical analysis
All data are presented as means ± SD. Two-tailed distribution Student t tests were carried out with P values of <0.05 considered statistically significant.

Results
p53 mutation and frequency of TICs in long-term Her2 Neu tumorsphere versus adherent cultures
To establish conditions for high-content lentivirus screens, primary MMTV-Her2/Neu tumors were dissociated and cultured in ultra-low attachment plates in minimal media containing EGF and bFGF (9). Under these conditions and at early passage, MMTV-Her2/Neu tumor cells formed tumorspheres that seemed encapsulated (Fig. 1A). After 3 to 4 passages, about 50% of sphere cultures became "immortalized" with spheres

![Figure 1](https://example.com/figure1.png)

Figure 1. Characterization of Her2/Neu tumorsphere and monolayer cells. A, images of primary and third passage Her2/Neu tumorspheres. B, images of primary and third passage Her2/Neu monolayer cells. C, immunohistochemistry of primary (left) and secondary (middle; right) Her2/Neu tumors induced after injection of third passage tumorsphere or monolayer cells into recipient mice, showing high expression of p53 in secondary tumors, indicating stabilizing mutations. D, representative CD24–CD49f–flow cytometry profiles of tumorsphere and monolayer Her2 tumors at indicated passage, E, frequency of TICs in tumorsphere versus monolayer cells.

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<th>4/12</th>
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becoming more retractile with uneven boundaries, resembling MMTV-Wnt1 tumorspheres (Fig. 1A; ref. 9). Similarly, early-passage MMTV-Her2/Neu-adherent cells, cultured in 10% serum, were enlarged and grew slowly; after 3 to 4 passages, about 50% of cultures became “immortalized” with epithelial-like cuboidal cells (Fig. 1B). Following transplantation of immortalized tumorsphere or monolayer cells into recipient mice, the resulting tumors exhibited robust nuclear staining of p53, indicative of stabilizing mutations (Fig. 1C; ref. 23). Loss of p53 is in accord with the observation that approximately 37% of MMTV-Her2/Neu tumors acquire missense mutations in p53 (24). Thus, culturing conditions in vitro likely select for survival of preexisting p53-mutant Her2/Neu tumor cells. As noted approximately 72% of HER2+ breast cancers contain mutations or deletions in TP53 (2), and therefore these “immortalized” mouse spheres and adherent cultures resemble human HER2+ breast cancer. Flow cytometry analysis revealed that the CD24+/CD49f+ fraction, which contains TICs (9, 19), was sustained in spheres cultures, being approximately 77%, 68%, and 46% after first, third, and seventh passages, respectively (Fig. 1D). In contrast, the TIC fraction rapidly diminished under adherent conditions, being approximately 80%, 36%, and 2.7% after first, third, and seventh passages. To determine TIC frequency, we transplanted 1,000, 250, or 50 cells from “immortalized” cultures into mammary glands of isogenic FvB recipient mice (n = 12 for each). TIC frequency was 1/213 (range, 1/122–1/372; P < 0.0001 by ANOVA) and 1/3,375 (range, 1/280–1/8,899) in spheres and monolayer cells, respectively (Fig. 1E). Together these results indicate that sphere conditions maintain TICs, whereas monolayer cells lose these tumorigenic cells after serial passages. All experiments described below were performed with these “immortalized” p53-mutant Her2+ spheres and monolayer cells from passage three to eight, isolated from 12 to 15 independent MMTV-Her2/Neu mammary tumors.

**An shRNA kinase screen identifies kinases required for p53-mutant Her2+ sphere and adherent growth**

We used a scheme presented in Fig. 2A to screen 520 kinases with approximately five shRNA per kinase (~2,567 independent shRNAs) from the TRC1 library (Fig. 2B; ref. 21). To minimize off-target effects, we followed hits in which at least two of the approximately five shRNAs per gene suppressed growth below a threshold, which we set at 3-fold inhibition (Fig. 2B). shRNAs for five genes (0.95%) preferentially suppressed sphere growth, 29 genes (5.5%) suppressed both spheres and monolayer, and nine genes (1.7%) only suppressed monolayer growth (Fig. 2C and Supplementary Fig. S1; Supplementary Tables S1–S3). The latter genes were not analyzed further. To validate our initial screen, we generated independent lentishRNAs for all hits that targeted spheres only or spheres plus monolayer, and restated their effects on adherent versus nonadherent growth. This secondary screen agreed in general with the initial screen (Supplementary Fig. S2A and S2B). In addition, we determined the effect of these shRNAs on growth of the immortalized mouse mammary epithelial cell line HC11. shRNAs against genes such as Lats2 and Durk1a that inhibited HC11 growth by more than 25% were excluded from further analysis (Supplementary Fig. S2C).

The remaining hits targeted the mitogen-activated protein kinase (MAPK) pathway (A-Raf, B-Raf, Mapk3k14, Mapk15, and mapkapk3) and TGFβR superfamily (Bmpr1b, Tgfbr1, Tgfbr2, Acrv2t, and Acrv2p), which were previously linked to HER2 signaling/cancer. Although inhibitors of the RAS/MAPK pathway such as MEK are promising, TGFβ family receptors have negative and positive effects on mammary tumor initiation and metastasis, respectively (25), suggesting that they may not represent good therapeutic targets. We also identified calcium/calmodulin-dependent protein kinases Camk2d and Cask, oxidant stress-activated serine/threonine kinase Stk25, proautophagy related factor/unc-51–like kinase (ATG1/ULK1; ref. 26), and the noncanonical IkB kinase (IKK), TBK1 (27), which have not been previously linked to HER2+ breast cancer.

**HER2+ breast cancer cells are highly sensitive to autophagy inhibitors and to shRNA-mediated knockdown of Atg1 or Tbkl**

Cells transformed by activated RAS are highly sensitive to autophagy and TBK1 inhibition (28, 29). However, the importance of Atg1 and TBK1 in HER2+ breast cancer in which RAS is activated at a significantly lower level is largely unknown. To investigate the role of these factors in HER2+ breast cancer, we first analyzed their expression in primary and secondary (transplanted) mammary Her2/Neu tumors (19). Both Atg1/Ulk1 and Tbkl were readily detected by RNA microarray (Supplementary Fig. S3) and immunoblotting (Fig. 2D). We next tested for efficient shRNA knockdown of Atg1/Ulk1 and Tbkl as well as several other kinases identified in our screen: Acrv2t, Tgfbr1, Camk2d, Cask, and Stk25. Expression of each protein, with the exception of Camk2d, which we were unable to detect at RNA or protein levels, was efficiently knocked down by the corresponding shRNA (Fig. 2D).

ATG1/ULK1 plays a critical role in autophagosome assembly; it recruits other ATG proteins and induces autophagosome formation by phosphorylating Beclin-1 and activating VPS34 lipid kinase (26). To determine the effect of inhibiting autophagy on HER2+ breast cancer cells, we used chloroquine, an inhibitor of autophagosome–lysosome fusion (30, 31). Treatment of Neu monolayer cells with this drug led to accumulation of fast migrating, lipidated LC3 (LC3-II), a component of the autophagosome (Supplementary Fig. S4A; ref. 26), indicating robust autophagy flux. Chloroquine readily inhibited growth of these cells (Supplementary Fig. S4B). 3-MA, an inhibitor of VPS34, also potently inhibited HER2+ tumor growth. Importantly, both chloroquine and 3-MA also inhibited growth of human HER2+ breast cancer cell lines HCC1954 and SKBR3 (Supplementary Fig. S4B). These autophagy inhibitors induced apoptosis in mouse Her2/Neu monolayer as well as human HCC1954 and SKBR3 cells as revealed by flow cytometry with Annexin V, which accumulates on the surface of apoptotic cells (Supplementary Fig. S4C and S4D). Thus, HER2+ breast cancer cells are highly sensitive to Atg1 and autophagy inhibitors.

TBK1 was the only kinase that had a stronger effect on tumorsphere than monolayer growth. TBK1 and the related...
TBK1/IKKε as Targets for HER2+ Breast Cancer

Figure 2. Parallel screens of tumorsphere and monolayer cells with shRNA kinase library. A, schematic diagram of parallel shRNA screens of tumorspheres and monolayer cells. B, scatter plots by MTT assays (top) or sphere formation counts (bottom) were carried out on monolayer cells or TFUs, respectively. Optical density values or sphere counts ≤3-fold relative to lenti-GFP control was chosen as cutoff. C, distribution of “hits” in sphere and/or Neu monolayer cells. D, Western blot analysis following knockdown by indicated shRNAs. E, Western blot analysis of indicated cultures after shRNA-mediated knockdown using tet-inducible mouse Tbk1 shRNA or two independent human Tbk1-shRNAs. F, inhibition of Neu monolayer proliferation and tumorsphere formation by doxycyclin, 1 week posttransduction. Results, mean ± SD. **P < 0.001; ***P < 0.001 by t test. G, human Tbk1-shRNAs suppress growth of HCC1954 and SKBR3 HER2+ breast cancer cells.

TBK1/IKKε inhibitor, TBK1-II, cooperates with lapatinib to suppress HER2+ tumor cell growth in vitro and in vivo

Next, we determined the effect of drug inhibitors for TBK1 and several other kinases identified in our screen. For TBK1, we used BX795, a TBK1/IKKε inhibitor, which also binds several other kinases (35). We also synthesized TBK1-II, a high-affinity/high-specificity TBK1 and IKKε inhibitor (36). At 10 μmol/L, Cask and TGFβR1 antagonists effectively but not completely abrogated sphere growth, whereas TBK1/IKKε inhibitors BX795 and TBK1-II completely suppressed sphere formation (Fig. 3A, bottom). This is contrasted with the more even effect of these drugs on monolayer growth (Fig. 3A, top), once again demonstrating the sensitivity of Her2+ tumor-spheres to TBK1 inhibition. Dose–response analysis revealed that TBK1-II was most potent with IC50 of 0.74 μmol/L for...
mouse Her2/Neu monolayer and 0.87, 0.88, and 2.78 μmol/L for the HER2⁺ breast cancer lines SKBR3 and HCC1954 and the trastuzumab-resistant line JIMT1 (Fig. 3B and Supplementary Fig. S6). In comparison, the EGFR1/HER2 inhibitor, lapatinib (37, 38), exhibited IC₅₀ of 0.75, 0.99, 1.73, and 3.38 μmol/L, respectively.

To test for cooperation between TBK1-II and lapatinib, we treated adherent or nonadherent HER2⁺ tumor cells with each drug alone or in combination. Reproducibly, combined treatments with 1 μmol/L of each inhibitor had a stronger effect than single-drug treatment of 2 μmol/L for each inhibitor alone (Fig. 3C), suggesting synergistic effect. Importantly, microscopic examination revealed complete loss of viable cells following combination, but not single treatments, in both mouse and human HER2⁺ breast cancer cultures (Fig. 3D).

To assess the effect of Tbk1 knockdown on growth in vivo, Her2/Neu tumorsphere cells were infected with lenti-shRNA directed against Tbk1. We also knocked down...
TBK1/IKKε as Targets for HER2⁺ Breast Cancer

Figure 4. Pathway analysis on TBK1-II–treated HER2⁺ breast cancer cells. GSEA pathway analysis showing signaling pathways that are upregulated in TBK1-II–treated cells (red) or –untreated cells (blue). Node color intensity is proportional to enrichment score as per scale. *P < 0.005; false discovery rate < 0.5.

Atg1, Cask, and Tgfr1, and used lenti-GFP as negative control. Six days after infection and puromycin selection, 100 live cells of each group were injected into mammary glands of recipient FvB mice (n = 6 each). Under these conditions, 3 of 6 mice injected with control GFP mice and 2 of 6 mice injected with Tgfr1-shRNA–infected cells developed tumors within 3 months. In contrast, no tumor (0/6) was detected in mice injected with Tbk1-, Ulk1- or Cask-shRNA–infected Her2/Neu tumor cells (P = 0.0455 by the χ² test; Fig. 3E).

To determine the combined effect of TBK1-II and lapatinib in vivo, we performed pharmacokinetic analysis of TBK1-II after intraperitoneal or subcutaneous injection of immuno-compromised NOD/SCID mice. This analysis revealed that the half-life of TBK1-II was approximately 2 hours with plasma concentration of approximately 1 μmol/L up to 3 hours after 30 mg/kg i.p. dose (Supplementary Fig. S7). To test for cooperation between TBK1-II and lapatinib, we used suboptimal concentrations of each drug. As the optimal dose of lapatinib is 100 mg/kg given twice daily by gavage (39), we used 50 mg/kg once a day. We also used a single, daily dose of 50 mg/kg TBK1-II (i.p.). Two million HCC1954 HER2⁺ tumor cells were injected into mammary glands of NOD/SCID mice. After 21 days, mice with palpable tumors were randomized and treated daily with vehicle (H2O), 50 mg/kg TBK1-II, 50 mg/kg lapatinib, or both drugs (Fig. 3F, top). After additional 21 days, tumors were dissected and weighed. Under these conditions, single treatments with TBK1-II or lapatinib alone failed to significantly suppress tumor growth. In contrast, combined TBK1-II plus lapatinib therapy blocked tumor expansion more than 2-fold (P = 0.023; Fig. 3F, bottom). Thus, TBK-II and lapatinib can cooperate to suppress HER2⁺ breast cancer growth both in vitro and in vivo.

TBK1-II induces cell-cycle arrest, not apoptosis

To determine the mechanisms by which TBK1 affects HER2⁺ breast cancer growth, we first tested the effect of TBK-II on signaling pathways. RNA extracted from TBK1-II–treated HCC1954 cells was subject to transcriptional profiling and global gene set enrichment analysis (GSEA; ref. 40), followed by "functional enrichment maps" to visualize the results (19, 41). Lists of genes/pathways that were highly up- or downregulated are shown in Supplementary Table S4A and S4B. In accordance with the biology of TBK1 and IKKe, pathways involved in "viral reproduction," "response to viral infection," and "response to interferon" were downregulated in TBK1-II–treated cells (Fig. 4). Also downregulated were pathways involved in "TCA cycle" and "oxidoreductase/glutathione" as well as "RNA metabolism" and "cell cycle/mitosis." The major upregulated pathways included "Toll" and "condensed chromosome."

Interestingly, cell death or apoptotic pathways were not upregulated in response to TBK1-II. Consistent with this, Annexin V–7AAD staining failed to detect apoptosis following lenti-shRNA–transduced or TBK1-II treatment (1–2 μmol/L; Fig. 5A). Some apoptosis was detected at 4 μmol/L.
Figure 5. TBK1-II induces large cell formation and polyploidy but not apoptosis in HER2⁺ breast cancer cells. A, left, representative flow cytometry profiles of Annexin V–stained Neu monolayer cells treated with indicated shRNA or drugs. Right, apoptotic response in Neu monolayer, HCC1954, and SKBR3 cells to indicated drug treatment for 4 days. Each point represents mean Annexin V staining of two independent experiments for each drug and concentration, relative to intrinsic apoptosis in DMSO or GFP-treated cells. Bars, SD. B, left, representative flow cytometry profiles of propidium iodide–stained Neu monolayer cells treated with indicated drugs. Right, histogram of cell-cycle distribution after treatment with shRNA or drugs for 4 days. Lanes 1, lenti-GFP; 2, lenti-shRNATbk1; 3, TBK1-II (2 μmol/L); and 4, lapatinib (2 μmol/L). Data, mean ± SD of two independent experiments. *P < 0.05; **P < 0.001 (t test). C, left, representative flow cytometry profiles showing cell size increase in Neu monolayer treated with TBK1-II or lapatinib. Right, large cell fraction in Neu monolayer, HCC1954, or SKBR3 cells treated with TBK1-II or lapatinib normalized to DMSO control.
(Fig. 5A, bottom). In contrast, lapatinib treatment readily induced apoptotic cell death at 1 to 2 μM/L.

As the pathway analysis revealed inhibition of the 'cell-cycle/mitosis' pathway, we next determined the effect of TBK1-II on the cell cycle by flow cytometric analysis of propidium iodide–stained cells. Lenti-shRNA

[cia]transduction inhibited cell growth as evident from increased percentage of cells in G0-G1 (M1 gate) with a corresponding reduction in S-G2-M (M2 gate; Fig. 5B). Lapatinib (2 μM/L) had only a mild effect on cell-cycle distribution. Strikingly, TBK1-II treatment (2 μM/L) virtually eliminated accumulation of cells in all phases and dramatically increased the percentage of cells with >4N chromosomes (M3 gate). This effect was also observed by forward- and side-scattering flow cytometry analysis (Fig. 5C). Thus, whereas lapatinib had no effect on tumor cell size, TBK1-II treatment increased the percentage of large cells, up to 8% to 12% of the culture, in a dose-dependent manner (Fig. 5C, left). This effect was observed in mouse Her2/Neu tumor cells as well as both human HER2+ breast cancer lines (Fig. 5C, right).

**TBK1-II induces cell senescence through inhibition of p65–NF-κB phosphorylation and activation of p16INK4a, and accelerates apoptosis together with lapatinib**

Our observation that TBK1-II treatment increased cell size, prompted us to determine whether it induced cellular senescence. Remarkably, large β-galactosidase-positive cells were observed in approximately 20% of lenti-shRNA

[cia]transduced, and TBK1-II–treated cultures (Fig. 6A). TBK1 was reported to control survival of several cell types by regulating Bcl-xL expression and Akt phosphorylation (29, 36, 42). However, in accordance with our observation that TBK1 inhibition did not compromise cell survival, we found only minor effects of genetic or pharmacologic inhibition of TBK1 or TBK1/IKKe on Bcl-xL, and phospho-Ser473-AKT levels (Fig. 6B). The NF-κB family proteins, RelA (p65), c-Rel, RelB, p50, and p52, bind to DNA as dimers, the most common being a p65-p50 heterodimer (43). p65–NF-κB activity is induced by phosphorylation of serine536 (44). In untreated HER2+ breast cancer cells, p65–NF-κB was highly phosphorylated on serine536 (Fig. 6C). Importantly, TBK1-II treatment of mouse and human HER2+ breast cancer cells dramatically suppressed serine536 phosphorylation, hence, activity of p65–NF-κB. In addition, expression of the prosenescence cyclin-dependent kinase (CDK) inhibitor p16INK4A (45) was dramatically induced in both mouse and human HER2+ breast cancer cells (Fig. 6C). These results suggest that TBK1/IKKe prevents cell senescence by activating NF-κB, and conversely, inhibition of these non-canonical IKK factors in HER2+ breast cancer cells suppresses NF-κB activity and induces p16INK4A expression, resulting in cellular senescence.

Finally, we investigated the combined effect of TBK1-II, which induces senescence, and lapatinib, which induces apoptosis, on these parameters. Lapatinib did not increase the level of cellular senescence in combination with TBK1-II (Fig. 6D, bottom). In contrast, cotreatment with TBK1-II increased the level of apoptotic cells relative to lapatinib alone (Fig. 6D, top). Thus, although TBK1-II inhibition promotes cell senescence, not apoptosis, it cooperates with lapatinib to accelerate apoptotic cell death in HER2+ breast cancer.

**Discussion**

We report on the identification of TBK1 and ATG1/ULK1 as novel therapeutic targets for HER2+ breast cancer. Consistent with the requirement for ATG1/ULK1, we showed that treatment with the autophagosome–lysosome fusion inhibitor chloroquine efficiently suppressed growth of mouse and human HER2+ breast cancer cells. Inhibition of autophagy has been shown to augment the efficacy of conventional chemotherapy in divergent tumor types, including sensitivity of HER2+ breast cancer to lapatinib and trastuzumab (46). Indeed, chloroquine is being evaluated in several clinical trials for the prevention of invasive breast cancer (e.g., 0811–0147). Given the central role of ATG1 in initiating autophagic vesicle formation (while chloroquine acts downstream), ATG1/ULK1 inhibitors may prove effective against HER2+ breast cancer in combination with conventional or anti-HER2 therapy.

TBK1 was the only validated target that was preferentially required for growth of mouse Her2/Neu tumorsphere versus monolayer cells. Its differential effect on sphere versus adherent cultures may explain why it has not been identified in other high-content screens of monolayer HER2+ cancer cells (e.g., ref. 47). Our shRNA library contained five shRNAs against IKKe, which invariably scored below the cutoff, suggesting that this gene is not essential for growth of mouse Her2/Neu tumor cells. In contrast, shRNA–mediated knockdown of TBK1 alone efficiently inhibited growth of both mouse and human HER2+ breast cancer cells. Thus, TBK1 is required to maintain growth of these cells, and IKKe does not seem to compensate for TBK1 when the latter is knocked down. However, IKKe is amplified in a third of human breast cancer. Coamplification of IKKe and HER2 is seen in approximately 17% of HER2+ breast cancer (cBioPortal for Cancer Genomics database). Thus, the contribution of IKKe to HER2+ breast cancer growth, hence, response to TBK1/IKKe inhibitors, likely varies depending on its amplification/expression levels in different tumors.

Contrary to recent reports on RAS-transformed lung cancer and other tumor types (29, 36, 42), we found that in HER2+ breast cancer cells, inhibition of TBK1 did not dramatically induce apoptosis or suppress Bcl-xL levels or AKT phosphorylation. Instead, TBK1 or TBK1/IKKe inhibition led to cellular senescence that was accompanied by suppression of p65–NF-κB phosphorylation/activity and induction of p16INK4A. Interestingly, in different contexts, NF-κB was shown to induce or suppress cellular senescence (44, 48). Our results suggest that in HER2+ breast cancer, NF-κB actively prevents senescence downstream of TBK1/IKKe. Zhang and colleagues have recently reported that the canonical NF-κB activator, IKKα, regulates subcellular localization, hence activity of the CDK inhibitor p27Kip1 (49). Whether TBK1 also regulates p27Kip1, and whether IKKe, like TBK1, affects p16INK4A expression remain to be seen. In addition, whether TBK1 inhibition suppresses autophagy-mediated cell death in HER2+ breast cancer cells, as was observed in other contexts (50), is yet to be determined. Finally, we showed that TBK1-II inhibited growth of a trastuzumab-
resistant line (JIMT1) better than lapatinib; its effect on large cohorts of HER2\(^{+}\) breast cancer lines as well as other cancer types driven by activation of HER2 signaling awaits further investigation.

Importantly, although TBK1-II treatment did not induce apoptosis, it cooperated with lapatinib to accelerate apoptosis, compared with lapatinib alone. Consistent with this, TBK1-II and lapatinib synergized to kill HER2\(^{+}\) breast cancer cells in vivo under conditions of low doses in which each drug alone had no significant effect. Our results should, therefore, encourage the assessment of TBK1/IKKe inhibitors in combination with anti-HER2 therapy as a potential new treatment for HER2\(^{+}\) breast cancer.

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

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Figure 6. TBK1/IKKe inhibition induces cellular senescence in HER2\(^{+}\) breast cancer cells through inhibition of p65–NF-κB and activation of p16\(^{ink4a}\), and cooperates with lapatinib to accelerate apoptosis. A, left, representative images of senescence-associated X-gal staining of HCC1954 and SKBR3 cells treated with TBK1-II (2 μmol/L) for 4 days (original magnification, ×400). Right, fraction of β-galactosidase-positive cells after Tbk1-shRNA knockdown or TBK1-II treatment for 4 days. Mean and SD of two independent experiments are shown. \(^{1}\), P < 0.001. B, Western blot analysis of TBK1, BCL-xL, and phospho-Ser473-ATK in Neu monolayer and HCC1954 cells after Tbk1 knockdown or TBK1-II drug treatment for 4 days, respectively. C, representative immunoblot analysis of p65–NF-κB, phospho-p65–NF-κB, p16\(^{ink4a}\), and cyclin D1 after TBK1-II drug treatment for 4 days of Neu monolayer or HCC1954 cells (n = 3). D, selective cooperation between TBK1-II and lapatinib in inducing apoptosis but not cellular senescence. HCC1954 cells were treated with TBK1-II and/or lapatinib and subjected to X-gal/senescence staining or Annexin V flow cytometry. Left, average of three experiments. Right, representative Annexin V staining.
TBK1/IKKr as Targets for HER2+ Breast Cancer

References


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