shRNA kinome screen identifies TBK1 as a therapeutic target for HER2+ breast cancer

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ABSTRACT

HER2⁺ breast cancer (BC) 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⁺ BC and potentiate the effect of anti-HER2 inhibitors, in particular those that can target tumor-initiating cells (TICs). Here we show that MMTV-Her2/Neu mammary tumor cells cultured as non-adherent 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⁺ tumorsphere versus monolayer cells with a lenti-virus shRNA kinome library. We identified kinases such as MAPK and TGFβR protein family, previously implicated in HER2⁺ BC, as well as autophagy factor Atg1/Ulk1 and the non-canonical IκB kinase, TBK1, which have not been previously linked to HER2⁺ BC. Knock-down of TBK1 or pharmacological inhibition of TBK1 and the related protein, IKKε, suppressed growth of both mouse and human HER2⁺ BC cells. TBK1/IKKε inhibition promoted cellular senescence by suppressing p65-NFκB and inducing p16INK4a. In addition, TBK1/IKKε inhibition cooperated with lapatinib, a HER2/EGFR1 targeted drug, to accelerate apoptosis and kill HER2⁺ BC cells both in culture and in xenografts. Our results suggest that patients with HER2⁺ BC 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⁺ BC.
INTRODUCTION

HER2+ breast cancer (BC) is caused by over-expression/amplification of the HER2/ERBB2/NEU receptor tyrosine kinase and represent ~20% of breast tumors (1). About 72% of HER2+ BCs contain mutations or deletions in the tumor suppressor p53 (2). HER2+ patients are treated with chemotherapy plus anti-HER2 inhibitors such as trastuzumab, a monoclonal antibody 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 effective kill HER2+ BC.

Many cancer types exhibit hierarchical organization, whereby only a subset of tumor cells, termed tumor-initiating cells (TICs), 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 non-adherent 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 BCs 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. Muller and colleagues have developed a mouse model for HER2+ BC, 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 (TFUs)(9, 18), and to generate a powerful prognostic signature for HER2+ BC (19), demonstrating its utility in modeling the human disease. Here, we show that following several passages of Her2/Neu tumor cells as adherent or non-adherent 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+ BC. The screen identified Tbkl as essential. Genetic and pharmacological inhibition of TBK1, and its close relative IKKe, either alone or together with the anti-EGFR/HER2 inhibitor, lapatinib, suppressed growth of human HER2+ BC cell in vitro and in vivo. Our results identify TBK1/IKKe as novel therapeutic target for HER2+ BC.
MATERIALS AND METHODS

Animals, tumorspheres and cell culture. MMTV-Her2/Neu mice on pure FvB background were maintained as per UHN Animal Care Committee Guideline. 0.5-1 cm mammary tumors were minced and digested with 100 units/ml collagenase/hyaluronidase (StemCell Technologies) for 60 min at 37°C. Following negative selection with EasySep Kit (StemCell Technologies), enriched Lin− mammary epithelial cells were plated onto ultra–low attachment 24-well plates (Corning, Costar) in DMEM/F-12 HAM medium containing bFGF, EGF and B-27 supplement as described (9, 20) or onto regular plates with DMEM plus 10%FBS for monolayer cell cultures. HER2+ BC cell lines - HCC1954 (ATCC® CRL-2338™), SKBR3 (ATCC® HTB-30™) and JIMT1 (DSMZ # ACC-589) - were obtained from Dr. Tak W Mak, University Health Network, 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, McCoys 5A and DMEM plus 10% FBS, 100-units/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 shRNA kinase library (TRC1) and Tbk1 shRNA clones. A subset of the Broad Institute RNAi Consortium (TRC) pLKO.1 shRNA library targeting 520 mouse kinase genes with 2567 lentiviral clones (4-5 shRNAs/gene) were used for screens in a total of 28x96-well plates (21). Each plate included 3-5 wells with empty vector (Lenti-GFP) as negative controls. shRNA clones TRCN 000003182 and 3185 targeting human Tbk1 were purchased from Sigma-Aldrich; lenti-Scrambled
shRNA was used as negative control. For constructing inducible Tbk1 shRNA vector, Tbk1 shRNA primers: 5’ CCGG CCT CGG AGG AAC AAA GAA GTA CTCGAG TAC TTC TTT GTT CCT CCG AGG TTTTTG 3’ and 5’ AATTCAAAAA CCT CGG AGG AAC AAA GAA GTA CTCGAG TAC TTC TTT GTT CCT CCG 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).

**Lenti-viral 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 (Caliperlifescience). 2-3 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 Matrigel (BD Bioscience; 1:1 ratio) and injected into no. 4 mammary glands of FvB or NOD/SCID mice.

**Antibodies.** We used Rabbit monoclonal antibody (Cat.#04-856) for Tbk1 (Upstate, Millipore); mouse anti-Bcl-xL (BD; Cat.# 610746) and mouse anti-CASK (Cat.# 610782) from BD Biosciences; mouse anti-p16ink4a (sc-1661), goat anti-Stk25 (sc-6865), rabbit anti-Acvr2b (sc-25453) from Santa Cruz.
Biotechnology; rabbit mAb to NF-kB p65 (Cat.# 8242), rabbit mAb to phospho-Ser536-NFκB p65 (Cat.# 3033), and mAb to p53 from Cell Signaling; anti-Atg1/ULK1 antibody (A7481) and monoclonal anti-Camk2d (WH00008177) and rabbit anti-TGFβR1 (SAB13001113) 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); TGFβR1 (SB505124, SIS3), 3-Methyladenine (3-MA), chloroquine (CQ) and lapatinib were from Sigma. 3-MA dissolved in water at room temperature, was placed at 65°C for 5 minutes before using. All other compounds were dissolved in DMSO and stored at -20°C at stock concentration of 1 mmol.

**Apoptosis, Cell cycle and Flow cytometry analysis.** Apoptosis was detected using a PE-AnnexinV kit (BD Biosciences, San Jose, CA). Cell cycle analysis was performed on propidium iodide stained cells (BD Pharmingen™, Cat No.550825)(20). Anti-CD49f (allophycocyanin, APC), and anti-CD24 (FITC) were from BD Biosciences. Flow cytometry analysis was performed on FACS Calibur (Becton Dickinson).

**Senescence-associated β-galactosidase (X-gal) staining.** After TBK1-II treatment or infection with lenti-shRNA^Tbk1^ for 72h, cells were washed twice with PBS, fixed in 3% formaldehyde (Sigma), and stained with X-gal solution (1mg/ml X-gal, 40mM citric acid, 5mM potassium ferrocyanide, 150mM NaCl, 2mM 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 (UHNMAC, Ontario, Canada) using Illumina HumanHT-12 v4 BeadChip with 500 ng of total RNA prepared by RNeasy mini kit (QIAGEN, Cat. No. 74104) from TBK1-II treated vs. 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 IV and 30 mg/kg IP to mice. Blood samples were collected into micro tubes (EDTA) from three mice each through the saphenous vein at the following time points for all three groups: 5, 15, 30 min, 1, 2, 4, 6, 8 and 24h. Plasma was separated from blood by centrifugation at 3000 rpm (1620g) for 10 min at 4°C, transferred into Eppendorf tubes, immediately placed on dry ice and stored at -80°C pending ultra performance liquid chromatography 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 ± standard deviation. Two-tailed distribution Student’s t tests were carried out with P values of ≤0.05 considered statistically significant.
RESULTS

p53 mutation and frequency of tumor initiating cells (TICs) in long-term Her2/Neu+ tumorsphere versus adherent cultures

To establish conditions for high content lenti-virus screens, primary MMTV-Her2/Neu tumors were dissociated and cultured in ultra-low attachment plates in minimal media containing EGF and basic-FGF (9). Under these conditions and at early passage, MMTV-Her2/Neu tumor cells formed tumorspheres that appeared encapsulated (Fig. 1A). After 3-4 passages, about 50% of sphere-cultures became “immortalized” with spheres becoming more retractile with uneven boundaries, resembling MMTV-Wnt1 tumorspheres (Fig. 1A)(9). Similarly, early passage MMTV-Her2/Neu adherent cells, cultured in 10% serum, were enlarged and grew slowly; after 3-4 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)(23). Loss of p53 is in accord with the observation that ~37% of MMTV-Her2/Neu tumors acquire missense mutations in p53 (24). Thus, culturing conditions in vitro likely select for survival of pre-existing p53 mutant Her2/Neu tumor cells. As noted ~72% of HER2+ BCs contain mutations or deletions in TP53 (2), and therefore these “immortalized” mouse spheres and adherent cultures resemble human HER2+ BC. Flow cytometry analysis revealed that the CD24+CD49f+ fraction, which contains TICs (9, 19), was sustained in sphere cultures, being ~77%, 68% and 46% after first, third and seventh passages, respectively (Fig. 1D). In contrast, the TIC fraction rapidly diminished under adherent conditions, being ~80%, 3.6%
and 2.7% after first, third and seventh passages. To determine TIC frequency, we transplanted 1000, 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 1/3375 (range 1/1280 - 1/8899) 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 12-15 independent MMTV-Her2/Neu mammary tumors.

An shRNA kinome 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 ~5 shRNA per kinase (~2567 independent shRNAs) from the TRC1 library (21)(Fig. 2B). To minimize off-target effects, we followed hits in which at least 2 of the ~5 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 9 genes (1.7%) only suppressed monolayer growth (Fig. 2C; supplementary Fig. S1, Tables S1-3). The latter genes were not analyzed further. To validate our initial screen, we generated independent lenti-shRNAs for all hits that targeted spheres only or spheres plus monolayer, and re-tested their effects on adherent vs. non-adherent growth. This secondary screen agreed in general with the initial screen (supplementary Fig. S2A-B). 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 over 25% were excluded from further analysis (supplementary Fig. S2C).
The remaining hits targeted the MAPK pathway (A-Raf, Raf1, Map3k14, Mapk15, mapkapk3) and TGFβ-receptor superfamily (Bmpr1β, Tgfβr1, TgfβR2, Acvr2α, Acvr2β), which were previously linked to HER2 signaling/cancer. While inhibitors of 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, pro-autophagy related factor/unc-51-like kinase (Atg1/Ulk1)(26), and the non-canonical IκB Kinase (IKK), TANK-binding kinase 1 (TBK1)(27), which have not been previously linked to HER2+ BC.

**HER2+ BC cells are highly sensitive to autophagy inhibitors and to shRNA-mediated knockdown of Atg1 or Tbk1**

Cells transformed by activated RAS are highly sensitive to autophagy and TBK1 inhibition (28, 29). However, the importance of Atg1 and TBK1 in HER2+ BC in which RAS is activated at a significantly lower level is largely unknown. To investigate the role of these factors in HER2+ BC, we first analyzed their expression in primary and secondary (transplanted) mammary Her2/Neu tumors (19). Both Atg1/Ulk1 and Tbk1 were readily detected by RNA microarray (supplementary Fig. S3) and immunoblotting (Fig. 2D). We next tested for efficient shRNA knockdown of Atg1/Ulk1 and Tbk1 as well as several other kinases identified in our screen: Acvr2α, Tgfβr1, 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.
TBK1/IKKe as targets for HER2+ BC
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To determine the effect of inhibiting autophagy on HER2+ BC cells, we used chloroquine (CQ), 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 (26) (supplementary Fig. S4A), indicating robust autophagy flux. CQ readily inhibited growth of these cells (supplementary Fig. S4B). 3-Methyladenine (3-MA), an inhibitor of VPS34, also potently inhibited HER2+ tumor growth. Importantly, both CQ and 3-MA also inhibited growth of human HER2+ BC 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-D). Thus, HER2+ BC 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 kinase IKKe play important roles as regulators of innate immunity by modulating interferon and NFκB signaling (27). In addition, TBK1 was identified in a synthetic lethal screen for genes that could kill non-small cell lung cancer cell lines driven by activated K-RAS (29), whereas IKKe was found to be amplified in 30% of BC and is able to transform breast epithelial cells through activation of NFκB (32-34). Using a doxycycline-inducible shRNA expression system, we independently confirmed that knock-down of mouse Tbk1 hindered the growth of mouse Her2/Neu spheres better than monolayer cells (Fig. 2E-F). Importance of this kinase in human HER2+ BC cells was confirmed using 2 shRNAs (TRCN3182 and TCRN3185), which suppressed expression 2.3 to 3.2 fold in HCC1954 and SKBR3 HER2+ BC cells, and inhibited adherent growth by 65-70% (Fig. 2E, G; supplementary Fig. S5).
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 μM, 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⁺ tumorspheres to TBK1 inhibition. Dose-response analysis revealed that TBK1-II was most potent with IC₅₀ of 0.74 μM for mouse Her2/Neu monolayer and 0.87, 0.88 and 2.78 μM for the HER2⁺ BC lines SKBR3 and HCC1954 and the trastuzumab-resistant line JIMT1 (Fig. 3B; supplementary Fig. S6). In comparison, the EGFR1/HER2 inhibitor, lapatinib (37, 38), exhibited IC₅₀ of 0.75, 0.99, 1.73 and 3.38 μM, respectively.

To test for cooperation between TBK1-II and lapatinib, we treated adherent or non-adherent HER2⁺ tumor cells with each drug alone or in combination. Reproducibly, combined treatments with 1 μM of each inhibitor had a stronger effect than single drug treatment of 2 μM 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⁺ BC 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 Atg1, Cask and Tgfβr1, and used lenti-GFP as negative control. Six days post 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, 3/6 mice injected with control GFP mice and 2/6 mice injected with Tgfβr1-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 Chi squared; Fig. 3E).

To determine the combined effect of TBK1-II and lapatinib in vivo, we performed pharmacokinetic analysis of TBK1-II after intraperitoneal (IP) or subcutaneous (sub. cu.) injection of immune-compromised NOD/SCID mice. This analysis revealed that the half-life of TBK1-II was ~2hrs with plasma concentration of ~1 µM up to 3 h after 30 mg/kg IP dose (supplementary Fig. S7). To test for cooperation between TBK1-II and lapatinib, we used sub-optimal concentrations of each drug. As the optimal dose of lapatinib is 100mg/kg given twice daily by gavage (39), we used 50mg/kg once a day. We also used a single, daily dose of 50mg/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), 50mg/kg TBK1-II, 50mg/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 over 2-fold (P=0.023; Fig. 3F, bottom). Thus, TBK-II and lapatinib can cooperate to suppress HER2+ BC growth both in vitro and in vivo.

TBK1-II induces cell cycle arrest, not apoptosis

To determine the mechanisms by which TBK1 affects HER2+ BC 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)(40), followed by “Functional Enrichment Maps” to visualize the results (19, 41). Lists of genes/pathways that were
highly up- or down-regulated are shown in Supporting Information Table S4A-B. In accordance with the biology of TBK1 and IKKε, pathways involved in “viral reproduction”, “response to viral infection” and “response to Interferon” were down-regulated in TBK1-II treated cells (Fig. 4). Also down-regulated were pathways involved in “TCA cycle” and “Oxidoreductase/GSH” as well as “RNA metabolism” and “cell cycle/mitosis”. The major up-regulated pathways included “Toll” and “condensed chromosome”.

Interestingly, cell death or apoptotic pathways were not up-regulated in response to TBK1-II. Consistent with this, AnnexinV-7AAD staining failed to detect apoptosis following lenti-shRNA$^{Tbk1}$ transduction or TBK1-II treatment (1-2 μM; Fig. 5A). Some apoptosis was detected at 4 μM (Fig. 5A, bottom). In contrast, lapatinib treatment readily induced apoptotic cell death at 1-2 μM.

As the pathway analysis revealed inhibition of “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$^{Tbk1}$ 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) had only a mild effect on cell cycle distribution. Strikingly, TBK1-II treatment (2 μM) 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 8-12% 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+ BC lines (Fig. 5C, right).
TBK1-II induces cell senescence through inhibition of p65-NFκB phosphorylation and activation of p16\textsuperscript{ink4a}, 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 ~20% of lenti-shRNA\textsuperscript{Tk1} 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 pharmacological inhibition of TBK1 or TBK1/IKK\textsubscript{ε} on Bcl-xL and phospho-Ser473-AKT levels (Fig. 6B). The NF-B 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-NFκB activity is induced by phosphorylation of serine536 (44). In untreated HER2\textsuperscript{+} BC cells, p65-NFκB was highly phosphorylated on serine536 (Fig. 6C). Importantly, TBK1-II treatment of mouse and human HER2\textsuperscript{+} BC cells dramatically suppressed serine536-phosphorylation, hence activity of p65-NFκB. In addition, expression of the pro-senescence cyclin-dependent kinase inhibitor p16\textsuperscript{INK4A} (45) was dramatically induced in both mouse and human HER2\textsuperscript{+} BC cells (Fig. 6C). These results suggest that TBK1/IKK\textsubscript{ε} prevents cell senescence by activating NFκB, and conversely, inhibition of these non-canonical IKK factors in HER2\textsuperscript{+} BC cells suppresses NFκB activity and induces p16\textsuperscript{INK4A} 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, co-treatment with TBK1-II increased the level of apoptotic cells relative to lapatinib alone (Fig. 6D, top). Thus, while TBK1-II
inhibition promotes cell senescence, not apoptosis, it cooperates with lapatinib to accelerate apoptotic cell death in HER2+ BC.

**DISCUSSION**

We report on the identification of TBK1 and ATG1/ULK1 as novel therapeutic targets for HER2+ BC. Consistent with the requirement for ATG1/ULK1, we showed that treatment with the autophagosome-lysosome fusion inhibitor CQ efficiently suppressed growth of mouse and human HER2+ BC cells. Inhibition of autophagy has been shown to augment the efficacy of conventional chemotherapy in divergent tumor types, including sensitivity of HER2+ BC to lapatinib and trastuzumab (46). Indeed, CQ is being evaluated in several clinical trials for the prevention of invasive BC (e.g. 0811-0147). Given the central role of ATG1 in initiating autophagic vesicle formation (while CQ acts downstream), ATG1/ULK1 inhibitors may prove effective against HER2+ BC 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 vs. monolayer cells. Its differential effect on sphere vs. adherent cultures may explain why it has not been identified in other high-content screens of monolayer HER2+ cancer cells (e.g. (47)). Our shRNA library contained 5 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+ BC 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 BC. Co-amplification of IKKe and HER2 is seen in ~17% of HER2+ BC (cBioPortal for Cancer
Genomics data base). Thus, the contribution of IKKε to HER2+ BC growth, hence response to TBK1/IKKε 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+ BC cells, inhibition of TBK1 did not dramatically induce apoptosis or suppress Bcl-xL levels or AKT phosphorylation. Instead, TBK1 or TBK1/IKKε inhibition led to cellular senescence that was accompanied by suppression of p65-NFκB phosphorylation/activity and induction of p16INK4A. Interestingly, in different contexts, NFκB was shown to induce or suppress cellular senescence (44, 48). Our results suggest that in HER2+ BC, NFκB actively prevents senescence downstream of TBK1/IKKε. The Karin group has recently reported that the canonical NFκB activator, IKKα, regulates subcellular localization, hence activity of the CDK inhibitor p27KIP1 (49). Whether TBK1 also regulates p27KIP1, and whether IKKα, like TBK1, affects p16INK4A expression remain to be seen. In addition, whether TBK1 inhibition suppresses autophagy-mediated cell death in HER2+ BC 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+ BC lines as well as other cancer types driven by activation of HER2-signaling awaits further investigation.

Importantly, while TBK1-II treatment did not induce apoptosis, it cooperated with lapatinib to accelerate apoptosis, compared to lapatinib alone. Consistent with this, TBK1-II and lapatinib synergized to kill HER2+ BC 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/ε inhibitors as potential new therapeutics for HER2+ BC in combination with anti-HER2 therapy.
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We thank Huiqin Li for help with some PCR analysis, Giovanna Pellecchia and Daniele Merico for pathway analysis, Bill Muller for discussion on p53 mutation in MMTV-Her2/Neu tumors, and Val Lapin and Tak W. Mak for HER2+ BC lines.

AUTHOR CONTRIBUTIONS

TD, JCL, PC, DU, ZJ, AA, BJ, NFS: performed experiments; TK, JM: provided lenti-shRNA kinome library, supervised lenti-shRNA screens; RR SEE, RAA: supervised, coordinated experiments; EZ: conceived, supervised, wrote manuscript.
REFERENCES


FIGURE LEGENDS

Figure 1. Characterization of Her2/Neu tumorsphere and monolayer cells. A, Images of primary and 3rd passage Her2/Neu tumorspheres. B, Images of primary and 3rd passage Her2/Neu monolayer cells. C, Immunohistochemistry of primary (left) and secondary (center, right) Her2/Neu+ tumors induced after injection of 3rd-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+ tumor cells at indicated passage. E, Frequency of TICs in tumorsphere vs. monolayer cells.

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 of OD value by MTT assays (top) or sphere formation counts (bottom) carried out on monolayer cells or tumorsphere-forming units (TFU), respectively. OD 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 knock-down 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 inducible pLKO-Tet-Tbk1 shRNA with indicated concentrations of doxycyclin, 1 week post transduction. Results are expressed as mean ± SD. * P<0.05 by t-test. G, Human Tbk1-shRNAs suppress growth of HCC1954 and SKBR3 HER2+ BC cells.
Figure 3. Growth suppression of HER2+ BC cells by TBK1/IKKe inhibitors in vitro and in vivo.

A, Effects of indicated inhibitors (10 μmol) on Her2/Neu-monolayer growth and tumorsphere formation. * P<0.05, ** P<0.001 (student t-test). B, Dose response curves of HCC1954 cells to indicated drugs (n=4). C, Effects of TBK1-II and lapatinib combination on indicated cells. D, Representative images of HCC1954 cells and Her2/Neu tumorspheres treated with indicated drugs (original magnification X100). E, shRNA-mediated knockdown of Tbk1, Atg1 or Cask suppressed Her2/Neu tumor growth in vivo. F, Cooperation of TBK1-II and lapatinib in inhibiting xenograft growth of HCC1954 cells in vivo. Only TBK1-II plus lapatinib combination arm showed a statistically significant inhibition of tumor growth (P=0.023). ns, not significant.

Figure 4. Pathway analysis on TBK1-II-treated HER2+ BC 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 (FDR) <0.5.

Figure 5. TBK1-II induces large cell formation and polyploidy but not apoptosis in HER2+ BC cells. A, Left, representative flow cytometry profiles of AnnexinV–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 AnnexinV 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 PI-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-shRNA_{Tbk1}; 3, TBK1-II (2μM); 4, lapatinib (2μM). Data are mean ± standard error of two independent experiments. *P<0.05, **<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.

**Figure 6. TBK1/IKKe inhibition induces cellular senescence in HER2+ BC cells through inhibition of p65-NFκB and activation of p16ink4a, 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) for 4 days (original magnification 400x). 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. ** P<0.001. B, Western blot analysis of TBK1, BCL-xL, and phospho-Ser473-AKT in Neu-monolayer and HCC1954 cells after Tbk1-knockdown or TBK1-II drug treatment for 4 days, respectively. C, Representative immunoblot analysis of p65-NFκB, phospho-p65 NFκB, p16ink4a 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 subject to X-gal/senescence staining or AnnexinV flow cytometry. Left, average of 3 experiments. Right, representative AnnexinV staining.
**Fig. 1**

**A**
Sphere

1st Sphere

3rd Sphere

**B**
Monolayer

1st Monolayer

3rd Monolayer

**C**
Primary
Secondary

**D**

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**E**

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**CD24-FITC**

**p53 IHC**

**Primary**

**Secondary**

**Monolayer**

**Sphere**
A

MMTV-Her2/Neu tumor

Collagenase/Single cell suspension

Ultralow attachment plates

Serum-free/EGF, bFGF

Primary spheres

Passage 3-8 - morphological alterations - p53 stabilization

Sphere conditions

Lenti-shRNA / Lenti-GFP

MTT assay

Sphere count

B

Viability

(% of GFP control)

Sphere count

(% of GFP control)

C

Hits on spheres 0.95%

Hits on monolayer & spheres 5.5%

Negative 92.5%

D

ULK1  TBK1  ACVR2α  TGFβR1  CASK  STK25  CAMK2D

Lenti-Ulk1  Lenti-Tbk1  Lenti-Acvr2

Lenti-Cask  Lenti-Stk25  Lenti-Camk2d

4.8  4.1  5.2  4.8  3.6  3.1

Tubulin

E

SKBR3  HCC1954  Neu-monolayer

Lenti-GFP  TRCN3182  TRCN3185

Lenti-SCR  Lenti-IndTbk

Tubulin

2.3  2.7  3.2  2.9  3.1

F

Cell Viability (% lenti-shRNA-GFP)

Sphere Formation (% lenti-shRNA-GFP)

G

Cell Viability (% lenti-GFP)

HCC1954  SKBR3

Doxcycline 10µg/ml

Doxcycline 20µg/ml

Fig. 2
A

Fig. 3

A

Cell Viability (% DMSO)

Sphere Formation (% DMSO)

BX795 TBK1- II SIS3 KN62 DMSO

B

Cell Viability (% DMSO)

HCC1954

TBK1 TGFβR1 CASK

D

HCC1954

Neu-spheres

DMSO TBK1-II Lap TBK1-II+Lap DMSO

E

Tumors/Injections

shRNA 1 Month 3 Months

GFP 2/6 3/6

TGFβR1 1/6 2/6

CASK 0/6 0/6

ATG1/ULK1 0/6 0/6

TBK1 0/6 0/6

F

Low drug doses

21 days 21 days

Inject tumor cells Start treatment Weigh tumors

HCC1954

Tumor weight

C T L T+L

ns ns P=0.023

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Fig. 4

Mitochondrial import
Peroxisome
Viral reproduction
Ribosome biogenesis
Response to viral infection
Splicing/mRNA stability
DNA repair
Telomere maintenance
TCA cycle
Oxidoreductase/ GSH

Response to unfolded proteins
Epidermal growth
Endosome
Response to interferon

Rheumatoid arthritis
Glutamate secretion
Gliogenesis
Calcium CD4+ Tcr
PcG
Voltage-gated potassium channel
Potassium channel
Actin
ABC transporter
Steroid biosynthesis
AP1
Condensed chromosome

Control - 2.56 NES
Treated + 2.04 NES

Amine metabolism
Base excision repair

Amine metabolism
Base excision repair

Fig. 4
Fig. 5
Fig. 6
shRNA kinome screen identifies TBK1 as a therapeutic target for HER2 + breast cancer

Tao Deng, Jeff C. Liu, Philip E.D. Chung, et al.

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