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Selective ERBB2 and BCL2 Inhibition is Synergistic for Mitochondrial-mediated Apoptosis in MDS and AML cells

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Abstract

The *ERBB2* proto-oncogene is associated with an aggressive phenotype in breast cancer. Its role in hematologic malignancies is incompletely defined, in part because *ERBB2* is not readily detected on the surface of cancer cells. We demonstrate that truncated *ERBB2*, which lacks the extracellular domain, is overexpressed on primary CD34+ myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML) cells compared to healthy hematopoietic cells. This overexpression of *ERBB2* is associated with aberrant, oncogenic signaling with autophosphorylation of multiple tyrosine sites. Like in breast cancers, *ERBB2* can exist as truncated isoforms p95^{ERBB2} and p110^{ERBB2} in MDS and AML. Neutralization of *ERBB2* signaling with *ERBB2* tyrosine kinase inhibitors (i.e., lapatinib, afatinib, and neratinib) increases apoptotic cell death and reduces human engraftment of MDS cells in mice at 21 weeks post-transplantation. Inhibition of *ERBB2* modulates the expression of multiple pro- and anti-apoptotic mitochondrial proteins, including B-cell lymphoma 2 (*BCL2*). Dual blockade with *ERBB2* and *BCL2* inhibitors triggers additional reductions of *BCL2* phosphorylation and myeloid cell leukemia-1 (*MCL1*) expression compared to single drug treatment. Dual therapy was synergistic at all tested doses, with a dose reduction index of up to 29 for lapatinib + venetoclax compared to venetoclax alone. Notably, these agents operated together and shifted cancer cells to a pro-

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apoptotic phenotype, resulting in increased mitochondrial cytochrome *c* release and activated caspase-3-mediated cell death.

Implications: These findings warrant study of ERBB2 and BCL2 combination therapy in patients with MDS and AML.

Keywords

ERBB2; BCL2; Lapatinib; Venetoclax; Acute myeloid leukemia

Introduction

Chemotherapy and hematopoietic stem cell transplantation for patients with acute myeloid leukemia (AML) and high-risk myelodysplastic syndrome (MDS) cause significant morbidity and mortality. Because of the limitations of current toxic treatments, there has been a recent shift in focus on developing more targeted cancer therapy. Effective targeted cancer therapies rely on selective inhibition of driver mutations that enhance cell proliferation and survival. Such therapies include tyrosine kinase inhibitors (TKIs) that could increase efficacy when used in combination with chemotherapies (1). Further study of combination chemotherapy with more targeted agents is required because many patients do not achieve a complete durable remission.

The epidermal growth factor receptor (EGFR) family consists of four members: *EGFR*/*ERBB1*, *ERBB2*, *ERBB3*, and *ERBB4*. The TKIs targeting *EGFR* and *ERBB2*, like erlotinib and lapatinib, were studied in AML in pre-clinical or early phase clinical studies (2-4), but the mechanisms of action for these agents in non-solid tumors remain unclear. This is because based on proteomic studies, the full length receptors or extracellular domains of these receptors, were not detected in leukemic cell lines, so it was thought that treatment of leukemic cells with TKIs exerted anti-leukemic properties through off-target effects (2,3). The finding that EGFR and ERBB2 are not expressed in AML has been challenged. Using messenger RNA expression analysis of over 143 patient samples and AML cell lines, about 33% were found to express *EGFR* (5). Based on protein and peptide phosphorylation arrays for EGFR detection, levels of EGFR expression and phosphorylation are about 15% for adult and pediatric AML patients (6). A recent deep sequencing analysis of 185 patients with hematologic malignancies indicates that two patients with AML harbor oncogenic point mutations in the *ERBB2* gene (4). These studies altogether suggest the presence of *EGFR* and *ERBB2* in a subset of patients with AML. However, little is known about the characterization of the receptors expressed in the leukemic cells.

In early phase studies of patients with intermediate or high-risk MDS, treatment with erlotinib, an EGFR TKI, demonstrated improved hematologic responses, including complete remissions in a subset of patients (7). When erlotinib and azacitidine were combined in cultures of AML cell lines, there was decreased cell survival with enhanced degradation of anti-apoptotic B cell lymphoma 2 (BCL2) proteins (8). BCL2 is an anti-apoptotic protein and promotes cell survival by sequestering pro-apoptotic proteins(9). Combination therapy with azacitidine and BCL2 inhibitors demonstrate synergistic activity with increased sensitization of AML and MDS cells in vitro compared to azacitidine alone (10). While

BCL2 inhibition has potent anti-leukemic properties (11), the median duration of response may be less than 12 months (12). Therefore, the therapeutic benefit of venetoclax could be further maximized through synergistic effects with other agents such as TKIs.

Based on both these findings and our prior work where we discovered EGFR signaling in murine hematopoietic stem cells (13,14), we sought to determine whether the EGFR family could be functional in blood cancers like AML and MDS. In a screen of AML cell lines for *EGFR*, *ERBB2*, *ERBB3*, and *ERBB4*, we discovered that *EGFR* was either not detected or detected at low levels, whereas *ERBB2* was present in all leukemic cells. Importantly, *ERBB2* is highly expressed in primary CD34+ MDS and AML compared to normal CD34+ cord blood cells. Here, we demonstrate that neutralization of ERBB2 signaling in CD34+ MDS and AML could impair cancer cell proliferation and survival via mitochondrial-mediated apoptosis, without increased toxicity to healthy marrow cells. Further, dual inhibition of ERBB2 and BCL2 was synergistic to promote mitochondrial apoptosis. We propose that dual inhibition of ERBB2 and BCL2 could offer improved tumor cell eradication in patients with MDS and AML.

Materials and Methods

Primary human samples

Bone marrow collection from healthy donors and patients with biopsy-proven MDS or AML was performed in accordance with the Declaration of Helsinki and approved by the Duke Institutional Review Board. Written, informed consent was obtained from all patients. CD34+ cells were isolated using a CD34 Microbead kit (Miltenyi Biotec, Somerville, MA) and cultured as described (15).

Cell lines and TKIs

MDS-L cells were a gift from Kaoru Tohyama, MD, PhD (Department of Laboratory Medicine, Kawasaki Medical School, Kurashiki, Okayama, Japan), and were cultured as described (1). Human myeloblastic leukemia K562, Kasumi1, KG-1 and acute lymphoblastic leukemia CEM were purchased from the Duke Cancer Institute Cell Culture Facility. Cells were thawed and used in experiments within 4 weeks of thawing. Human breast epithelial cells MCF-10A were transduced with either p185 kDa full-length ERBB2 receptor or p110 kDa truncated isoform of ERBB2 under a doxycycline inducible promoter as described (2). Cell lines were negative for mycoplasma. They were tested for mycoplasma by Mycoplasma PCR Detection kit (Sigma, St. Louis, MO) within 6 months of studies being performed.

Lapatinib was purchased from LC Laboratories (Woburn, MA). Afatinib, neratinib, venetoclax, maritoclax and AZD5991 were purchased from Selleckchem (Houston, TX).

ERBB2 expression

For flow cytometric analysis of the extracellular domain of ERBB2, cells were surface stained with mouse anti-human ERBB2 PE antibody (cat #340552, BD Biosciences, San Jose, CA) and anti-CD34 (BD Biosciences). For intracellular staining of total ERBB2 and

phospho-ERBB2 in flow cytometric studies, cells were labeled with anti-CD34, then fixed and permeabilized with Perm Buffer III (BD Biosciences). Cells were stained with antibodies against ERBB2, phosphorylated tyrosine Y877, Y1248 and Y1221/1222 (Abcam), and Alexa-Fluor 488 secondary antibody.

For immunofluorescence analysis of ERBB2, cells were spun onto Poly-L-lysine-coated slides, fixed in 4% paraformaldehyde, permeabilized in 0.3% Triton-X-100 in phosphate-buffered saline (PBS), and blocked with 3% goat serum. Cells were stained with mouse anti-ERBB2 antibody (Abcam, Cambridge, MA), Alexa Fluor 488-conjugated goat anti-mouse antibody (Thermo Fisher Scientific, Waltham, MA), and 4',6-diamidino-2-phenylindole (DAPI). Images were captured on Zeiss Axio Imager Z2.

Cell Death and Cell Cycle Analysis

Flow cytometric analysis of cytochrome *c* release was performed as described (3). Cytochrome *c* was labeled with FITC conjugated anti-Cytochrome *c* antibody (Biolegend, San Diego, CA). For activated caspase 3, cells were permeabilized with 0.5% Tween 20 in 1x PBS, and stained with AlexaFluor 488 conjugated anti-activated caspase 3 antibody (Cell Signaling Technology). For cell death analysis, cells were labeled with anti-CD34 antibody, Annexin V, and 7-Aminoactinomycin D (7-AAD) according to manufacturer's specifications (BD Biosciences). For cell cycle analysis, cells were stained with Ki-67 antibody (BD Biosciences) and 7-AAD after fixation and permeabilization.

Functional Analysis of ERBB2

K562 cells were cultured in Accell media and either nontargeting small interfering RNA (SiNontarget) or 4 pooled small interfering RNA for *ERBB2* (siERBB2) for 7 days according to manufacturer's specifications (Horizon Discovery, Cambridge, United Kingdom). Viable cells were quantified using trypan blue exclusion (Lonza, Durham, NC). For cultures of colony-forming cells (CFCs), cells were plated in MethoCult H4434 (STEMCELL Technologies, Vancouver, Canada) and scored between days 10–14 by two independent investigators.

Cell Viability and Synergy Calculations

Cell viability was also measured using 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) assays as described (4). The absorbance was measured at a wavelength of 490 nm using ELx808™ Microplate Reader (Biotek, Winooski, VT). Results were expressed as viability compared to control conditions.

The Combination index (CI) was calculated according to Chou-Talalay's method with CompuSyn software(5). The Combination Index (CI) is determined by the following equation:

$$CI = D_{comb1} / D_1 + D_{comb2} / D_2$$

where D_1 and D_2 = dose of drug 1 and drug 2 alone to inhibit a given level of viability, and D_{comb1} and D_{comb2} = doses of drug 1 and drug 2 in combination necessary to induce the

same effect. The CI value quantitatively defines synergism ($CI < 1$), additive effect ($CI = 1$) and antagonism ($CI > 1$). The Dose Reduction Index (DRI) is a ratio between the dose of the drug alone, at a given level of viability, and the dose of a drug in combination necessary to give the same effect. DRI is calculated as below:

$$DRI = D_1 / D_{comb1}$$

The value of DRI indicates no dose-reduction ($DRI = 1$), unfavorable ($D < 1$) or favorable drug reduction ($D > 1$).

Real-time quantitative PCR

RNA was extracted with RNeasy Mini or MicroKit (Qiagen, Waltham, MA). Complementary DNA was synthesized using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Waltham, MA). Gene expression assays were performed using TaqMan primers (Thermo Fisher Scientific, Waltham, MA), quantified on a QuantStudio™ 6 Flex Real-Time PCR System (Applied Biosystems), and analyzed as described (1).

Immunoprecipitation and Western blot

MDS-L, K562 and primary AML cells were lysed in Pierce™ IP lysis buffer (Thermo Fisher Scientific) containing protease inhibitors (cOmplete™, Sigma-Aldrich), and incubated with 1 µg of anti-ERBB2 antibody. ERBB2 was precipitated with Dynabeads Protein G (Thermo Fisher Scientific) and eluted in 2x Laemmli sample buffer. Whole cell extracts were obtained by lysing MCF10A-p185 or -p110 ERBB2, MDS-L and primary AML cells in RIPA buffer (Thermo Fisher Scientific).

Immunoprecipitated elution and lysates were resolved by SDS-PAGE gels, transferred to polyvinylidene difluoride membranes, and probed with specified antibodies: anti-ERBB2 (Abcam), anti-phospho-ERK1/2 (Thr202/Tyr204) and anti-ERK1/2, anti-BBC3, anti-MCL1, anti-BCL2, anti-phosphoBCL2 (Ser 70), anti-BAX, and anti-actin (all from Cell Signaling Technology). Secondary antibodies are conjugated with horseradish peroxidase, IRdye 700, or IRdye 800. Quantification of signals was performed using ImageJ software.

Long-term engraftment studies with human MDS cells

Animal studies were approved by the Duke Institutional Animal Care and Use Committee. Male and female Nod-SCID-IL2Rγ^{-/-} (NSG) mice (Jackson Laboratory, Bar Harbor, ME) were used in studies between ages 8-12 weeks. Transplantation of MDS-L cells was performed 24 hours (h) after 2.5 Gy total body irradiation as described (17). At 21 weeks post-transplantation, femur and spleen cells were collected for engraftment analyses by flow cytometry as described (17).

Statistical analyses

Data are shown as means ± S.E.M. or S.D. as specified in figure legends. Student two-tailed, unpaired *t* test and Dunnett's or Sidak multiple comparisons test after one-way ANOVA were performed using GraphPad Prism (v8.0).

Results

ERBB2 is constitutively overexpressed and truncated in MDS and AML cells

While *ERBB2* is a known oncogene in breast cancer and other solid malignancies, its role in hematologic malignancies is incompletely defined. We sought to first quantify *ERBB2* expression and other EGFR family of receptors (*ERBB1*, *ERBB3*, and *ERBB4*) in MDS and AML cells. *ERBB1* (also known as epidermal growth factor receptor, *EGFR*), and *ERBB4* were either not detected or detected at low levels compared to the human acute lymphocytic leukemia cell line CEM (18) (Figure 1A). Of these cell lines, *ERBB3* was more highly expressed in K562 compared to CEM. *ERBB2* messenger RNA (mRNA) was expressed 32- and 19-fold higher in MDS-L cells, a patient-derived cell line that closely recapitulates clinical features of myelodysplastic syndrome (19), and leukemic cell lines (i.e., K562, Kasumi1, and KG-1 leukemic cells), respectively (Figure 1A). This increase in *ERBB2* mRNA expression is also detected at higher levels in primary CD34+ MDS and AML cells compared to either CD34+ healthy, non-malignant hematopoietic cells (Tables S1, Table S2, Figure 1B). By flow cytometric analysis for surface expression of ERBB2, the ERBB2 extracellular domain was abundantly expressed on BT474 breast cancer cells, which is known to have high levels of ERBB2. Surprisingly, very little of the ERBB2 extracellular domain was expressed on MDS-L, K562 and primary CD34+ AML cells (Figure 1C).

We asked whether truncated ERBB2 could explain the lack of the ERBB2 extracellular domain in MDS-L and AML cells. This is because in a subset of therapy-resistant breast cancers, truncated ERBB2 is generated by metalloproteinase cleavage and alternative mRNA translation forming p95^{ERBB2} and p110^{ERBB2} (20,21). When we performed flow cytometric analysis to detect an intracellular, cytoplasmic epitope of ERBB2, we found a significant amount of ERBB2 in MDS-L and K562 (Figure 1D). Further, primary CD34+ MDS and CD34+ AML cells express up to 5-fold greater ERBB2 protein compared to healthy marrow (Figure 1E, S1A). Consistent with these findings, immunofluorescence staining shows that ERBB2 is localized in perinuclear, nuclear, and cytoplasmic regions of MDS and AML cells, rather than at the cellular membrane (Figure 1F, G). These findings are in contrast to previously described ERBB2 expression in solid cancers where ERBB2 is generally localized to the cellular membrane (21). To confirm that ERBB2 could exist as truncated isoforms in MDS and AML cells, we performed immunoprecipitation of the receptor using a specific antibody against ERBB2 at the amino acid residues 1242-1255, which are located at the C-terminus. Using control human mammary cells MCF10A that express either full-length p185^{ERBB2} or the truncated isoform p110^{ERBB2} after induction with doxycycline, Western blot analysis in MDS-L and K562 cells reveal ERBB2 at both 95 and 110 kilodaltons (kDa), which corresponds to truncated ERBB2 isoforms described previously in breast cancer (Figure S1B) (20). No full-length ERBB2 receptor (p185^{ERBB2}) was detected in MDS-L or K562 cells (Figure S1B). Like MDS-L and K562 cells, Western blots of CD34+ AML cells show ERBB2 isoforms at 95 and 110 kDa to the exclusion of the full-length receptor at 185 kDa, which was abundantly detected in the breast cancer cell line BT474 that serves as a ERBB2 positive control (Figure 1H). Taken together, these findings indicate that truncated ERBB2 is expressed at high levels in MDS-L and primary AML cells.

Constitutive phosphorylation of ERBB2 in MDS-L, K562 and primary AML is decreased with Lapatinib

Since the activated (phosphorylated) receptor may indicate a more biologically relevant marker of aggressive disease than ERBB2 receptor expression alone (22), we measured directly the phosphorylation of multiple ERBB2 tyrosine sites. We discovered that ERBB2 displayed constitutive activation of several phosphorylation sites (phospho-tyrosine 877, 1248, 1221 and 1222; Figures 2A, B) in MDS-L and K562 cells, which is characteristic of oncogenic signaling. Like MDS-L and K562 cells, CD34+ AML cells demonstrate autophosphorylation of Y877 and Y1248 ERBB2 (Figure 2C).

Next, we sought to determine whether ERBB2 inhibitors could downregulate autophosphorylation of ERBB2. Since ERBB2 in MDS and AML cells appear to be truncated, without detection of the full-length ERBB2 receptor or the extracellular domain, using trastuzumab and other anti-ERBB2 antibodies that require an extracellular domain to neutralize ERBB2 signaling would likely be ineffective (23). Therefore, we chose to use lapatinib (GW572016, GW2974, Tykerb®), which is a reversible dual EGFR and ERBB2 tyrosine kinase inhibitor (21) and directly competes with ATP at the ERBB2 catalytic kinase domain. Further, lapatinib could inhibit transactivation of EGFR and ERBB2, which is critical for blocking downstream Akt activation and cancer cell proliferation, whereas trastuzumab does not (24). The concentrations of lapatinib chosen for our studies was based on both prior viability studies (25) and our own viability data showing that half maximum inhibitory concentration (IC₅₀) and IC₉₀ of lapatinib is 5 µM and 10 µM, respectively (Figure S2A). Of note, this concentration range of lapatinib is comparable to the level of the drug in cancer tissues necessary to deactivate ERBB2 when oral lapatinib is administered at the maximum tolerated doses (26). Cultures of MDS-L cells with 5 µM and 10 µM lapatinib decreased phosphorylation of tyrosine 1248-ERBB2 by 35% and 71% compared to control cultures, respectively (Figure 2D). Likewise, lapatinib inhibited phosphorylation of tyrosine 1221 and Y1222-ERBB2 by 62% and 69% compared to control cultures (Figure S2B). Following 24 h cultures with K562 cells, lapatinib also decreased phosphorylation of tyrosines Y1248-ERBB2 and Y877-ERBB2 by similar levels compared to control cultures (Figure 1D and S2C). Following 24 h culture with lapatinib, there was up to a 2-fold reduction of phosphorylation of mitogen-activated protein kinase pathway, ERK1/2 (Thr202/Tyr204), compared to control cultures (Figure 1E). This neutralization of ERK1/2 is associated with halting cell cycle progression, cell proliferation, and correlates with inhibitory effects of drugs in cancers (27). These data show that lapatinib can effectively inhibit phosphorylation of ERBB2 and downstream ERBB2 signaling.

Constitutively activated ERBB2 promotes survival and proliferation of MDS and AML cells

We utilized small interfering RNAs (siRNAs) to neutralize ERBB2 signaling. Cultures of K562 cells with a pool of siERBB2 for 7 days resulted in a 75% reduction of *ERBB2* mRNA compared to cultures with a pool of 4 non-targeting control siRNAs (Figure 3A). Reduction of *ERBB2* expression was sufficient to increase the number of trypan blue+ cells and annexin V+ cells (Figure 3B, C). To complement these studies with siRNA to neutralize *ERBB2*, we used lapatinib to pharmacologically block ERBB2 signaling. Compared to control cultures, cultures of CD34+ AML cells with 5 µM lapatinib impaired cell

proliferation by up to 5-fold and increased annexin V+ cells by a 4.2-fold at 72 h and a 2.7 fold at 7 days (Figure 3D, 3F-H, S3D). Even with a lower dose of 1 μ M lapatinib, CD34+ AML cells displayed significantly increased cell death compared to control cultures (Figure S3A-B). Treatment of CD34+ AML with 5 μ M lapatinib either for 72 h or 7 days decreased committed progenitor cells in methylcellulose assays (CFCs) (Figure 3E, S3E). Cultures of K562 cells with lapatinib also showed up to an 85-fold decrease in total cell proliferation at 72 h, while cultures of MDS-L cells with 10 μ M lapatinib reduced cell proliferation by 3.2- and 6.5-fold at 48 h and 72 h, respectively (Figure 3D). This inhibition of MDS-L cell proliferation with lapatinib is comparable to that observed with clinically relevant doses of azacitidine, a chemotherapy commonly used in MDS and a subset of AML patients (17). Lapatinib can suppress cell proliferation of both MDS-L and K562 cells for at least 7 days (Figure S3C). *ERBB2* inhibition in MDS-L and K562 also significantly decreased committed progenitor cells in methylcellulose assays (Figure 3E) and increased annexin V+ cells compared to control cultures (Figure 3G, H). Importantly, cultures of CD34+ healthy marrow with lapatinib displayed no depletion of cells, colony forming cells, or decreased cell survival (Figures 3D-F, H), indicating that hematopoietic toxicity of *ERBB2* inhibition may be limited to cancer cells alone. These data show that inhibition of *ERBB2* signaling with lapatinib impairs cell proliferation and survival in MDS and AML cells.

To determine whether the inhibition of *ERBB2* and increased cancer cell death were drug effects specific to lapatinib, we repeated these assays using afatinib, an irreversible *EGFR*, *ERBB2*, and *ERBB4* kinase inhibitor (28), and neratinib, a pan-*ERBB* receptor TKI (29). Based on an IC_{50} of 2 μ M afatinib (Figure S4A), cultures with afatinib display a reduction of cell proliferation, CFCs, and increased annexin V+ cells compared to control cultures with either MDS-L cells (Figure S4B-D) or K562 cells (Figure S4E-H). Moreover, MDS-L cell cultures with neratinib reduced total cell proliferation by 2.6- and 3.4-fold compared to control cultures at 72 h and 7 days, respectively (Figure S4I-J). Like afatinib, neratinib reduced CFCs and promoted increased annexin V+ cells (Figure S4K-L). In cultures of primary CD34+ MDS cells, inhibition of *ERBB2* displayed up to a 5.3-fold reduction in cell proliferation and a 3.6-fold increase in annexin V+ cells from cultures with afatinib (Figure S4M-N). These data indicate that inhibition of *ERBB2* and subsequent increased cell death could be a general effect of TKIs for *EGFR/ERBB2* and is not a drug-specific effect of lapatinib alone.

Neutralization of *ERBB2* impairs long-term human MDS cell engraftment

We generated a human-murine xenograft model for MDS (19,30) to determine the effect of *ERBB2* inhibition on long-term MDS-L cell engraftment. Following 24 h exposure to 10 μ M lapatinib or DMSO, equal numbers of viable MDS-L cells were transplanted via intrafemoral injection into Nod-SCID-IL2R $\gamma^{-/-}$ (NSG) mice, which are permissive for human engraftment (31) (Figure 4A). Immediately before injection of these cells, we found that lapatinib caused no differences in apoptotic cell death (Figure S5A) and a modest block in cell cycle progression with increased cells in G₀ compared to control cultures at 24 h (Figure S5B). The recipients of lapatinib-treated cells displayed the preservation of marrow architecture and a 5.5-fold reduction in mean cancer engraftment at 21 weeks posttransplantation compared to control mice (Figure 4B-D). Moreover, the levels of

engraftment for CD13, CD33, and CD38 were 4.4- to 6.8- fold decreased in recipients of DMSO-treated cells compared to recipients of lapatinib-treated cells (Figure S5C). Recipients of lapatinib-treated cells displayed a significant decrease in splenic infiltration with MDS cells compared to control mice (Figure 4E, F). These findings indicate that ERBB2 inhibition could prevent long-term engraftment of MDS-L cells.

ERBB2 inhibition modulates the family of BCL2 proteins in MDS and AML cells

The BCL2 family of proteins regulates mitochondrial-mediated apoptosis by regulating mitochondrial outer membrane permeabilization (9). Increased levels of BCL2 pro-survival proteins are associated with ERBB2 overexpression to suppress apoptosis in breast cancer (32). However, we found that ERBB2 inhibition by lapatinib induces *BCL2* mRNA and protein levels in MDS-L and CD34+ AML cells (Figure 5B-D). A similar effect of lapatinib on BCL2 is also observed in breast cancer (33). This suggests that cancer cells develop BCL2 dependence for survival following lapatinib treatment.

ERBB2 can bind directly to and phosphorylate BCL2 binding component-3 (BBC3), a pro-apoptotic member of the BCL2 family of proteins (34). There exists an inverse relationship between phosphorylation of ERBB2 and BBC3, such that suppressed levels of ERBB2 phosphorylation increases BBC3 and drives breast cancer toward cell death (34). Following ERBB2 inhibition with lapatinib, MDS-L and CD34+ AML cells displayed a pro-apoptotic shift with a 2-fold increase in *BBC3* mRNA and protein (Figure 5A, C, D). Despite this increase in *BBC3* following lapatinib treatment, we detected no differences in the level of pro-apoptotic BCL2-associated X (*BAX*) (Figure 5C, D). In response to stress stimuli, cytosolic BAX integrates into the outer mitochondrial membrane, forms death pores, and facilitates the release of cytochrome c, triggering the intrinsic pathway of apoptosis (9). When cells are exposed to cytotoxic drugs, full-length BAX (p21 BAX) is cleaved to yield a p18 BAX fragment. Increased levels of p18 BAX accelerates apoptosis. While lapatinib treatment did not cause BAX cleavage in MDS-L cells, p18 BAX was increased 6.8-fold following lapatinib in cultures of primary AML cells compared to control cultures (Figure 5C, D). Finally, the pro-survival protein myeloid cell leukemia-1 (MCL1), which is essential for normal hematopoiesis (35) and is highly expressed in leukemia stem cells (36), was strikingly reduced by nearly 60% in MDS-L and primary AML cells following lapatinib treatment (Figure 5C, D). To determine whether reduced MCL1 expression could promote cell death, we used a MCL1 inhibitor maritoclax to trigger protein degradation of MCL1 in MDS-L cells (Figure 5E). This reduction in MCL1 protein corresponds to a 3.5-fold decrease in cell expansion and a 2.9-fold increase in annexin V+ cells (Figure 5F, G). Since maritoclax could exhibit non-specific inhibition of MCL1 (37), we also examined whether the specific MCL1 inhibitor AZD5991 could replicate these results (38). Following AZD5991 treatment, MDS-L cells displayed increased cell death compared to control cultures at levels that were comparable to maritoclax treatment (Figure S6A-B). These results indicate that reduced MCL1 level or MCL1 inhibition is sufficient to cause cell death in MDS and AML cells. Moreover, dual treatment with AZD5991 and lapatinib was not additive to increase annexin V+ cells compared to AZD5991 alone (Figure S6C). These findings suggest that both lapatinib via ERBB2 inhibition and AZD5991 promote mitochondrial-mediated apoptosis at least partially through MCL1 inhibition.

Dual ERBB2 and BCL2 blockade synergistically promotes apoptotic death of MDS-L and AML cells

Since lapatinib increased levels of BCL2, we hypothesized that dual therapy of lapatinib with a BH3 mimetic like venetoclax could be synergistic to impair cell viability. Cell viability by methyl thiazole tetrazolium (MTT) analysis for dual therapy with lapatinib + venetoclax was synergistic at all 49-treatment combinations tested at both 24 h and 72 h in culture, with greater sensitivity at 72 h (Figure 6A,B and Figure S7A,B). At no concentrations tested did dual therapy exert antagonistic drug effects or promote cancer cell growth. Dual therapy yielded 29-fold and 18-fold in the dose-reduction index compared to venetoclax monotherapy at 72h and 24h, respectively (Figure 6C and Figure S7C). Moreover, dual therapy could decrease cell proliferation by up to 5.1-fold or 6-fold compared to monotherapy with either lapatinib or venetoclax in MDS-L cells and CD34+ AML cells, respectively (Figure 6D). The reduction in cell proliferation is correlated with reduced CFCs with dual therapy compared to monotherapy and control cultures (Figure S7D). Flow cytometric analysis following 72 h in cultures with CD34+ AML and MDS-L cells display increased apoptotic cells (annexin V+, 7-AAD) and necrotic cells (annexin V+, 7-AAD+), with up to a 6.8-fold increase in cultures with dual therapy compared to either lapatinib or venetoclax alone (Figure 6E, F). These data demonstrate that dual therapy with lapatinib + venetoclax synergistically promotes cancer cell death compared to venetoclax monotherapy.

Synergy of mitochondrial-mediated death signals in primary AML cells by ERBB2 and BCL2 inhibition

Next, we sought to define the mechanisms by which dual therapy with lapatinib and venetoclax accelerates apoptosis by measuring levels of several BCL2 family members compared to monotherapy with either drug alone. There were no differences in total BCL2 compared to control cultures following 24 h treatment of MDS-L cells with either venetoclax or lapatinib + venetoclax (Figure 7A). Phosphorylated BCL2, rather than total BCL2 levels, could predict IC₅₀ to drugs and sensitize cancer cells to BCL2 inhibitors (39,40). Levels of BCL2 phosphorylated at serine 70 (pBCL2) were decreased by up to 2.5-fold and 3-fold in MDS-L cells and primary AML cells, respectively, following treatment with lapatinib + venetoclax compared to cultures with venetoclax alone (Figure 7A-C). Dual therapy suppressed MCL1 by 5- to 8- fold in MDS-L and 1.7-fold in primary AML cells compared to venetoclax monotherapy (Figure 7A-C). For pro-apoptotic proteins, BCL2 inhibition with venetoclax or lapatinib + venetoclax in MDS-L and primary AML maintained a significantly low level of BBC3 (Figure 7A-C), suggesting a BBC3-independent cell death induced by dual treatment. Additionally, venetoclax increases p18 BAX by up to 21-fold in MDS-L and 10-fold in primary AML cells compared to control cultures (Figure 7A-C). Addition of lapatinib to cultures with venetoclax further increased p18 BAX by 2-fold in MDS-L and 1.5-fold in primary AML compared to venetoclax monotherapy (Figure 7A-C). Following dual therapy with lapatinib + venetoclax, both pBCL2 and MCL1 were decreased and p18 BAX was increased compared to venetoclax monotherapy. These findings suggest a mechanism for the observed synergy of these drugs in MDS cells and primary AML cells.

Since ERBB2 inhibition could modulate the BCL2 family of proteins, we sought to determine whether ERBB2 inhibition could facilitate mitochondrial apoptosis via the opening of a permeability transition pore and release cytochrome *c* (9). Following either ERBB2 or BCL2 inhibition, release of cytochrome *c* was elevated compared to control cultures (Figure 7D). Dual therapy further increased the release of cytochrome *c* by 12-fold and 6-fold compared to lapatinib or venetoclax alone, respectively (Figure 7D). Released cytochrome *c* activates downstream caspase 3, which ultimately leads to cell death (9). Activated caspase 3 was increased with dual therapy compared to monotherapy and control cultures alone (Figure 7E). The levels of activated caspase 3 directly correspond to both cytochrome *c* release and percentage annexin V+ cells, indicating that ERBB2 and BCL2 inhibition could coordinate to facilitate mitochondrial-mediated apoptosis.

Discussion

The dependence on a single critical oncogene to determine cell fates of proliferation, survival, or death is a well-established concept of oncogene addiction (41). An example of oncogene addiction is *ERBB2* overexpression in breast cancer, in which enhanced downstream activation of pro-survival signaling is associated with a more aggressive cancer phenotype, shortened time to disease relapse, and decreased overall survival (42). Using deep sequencing, Joshi et al. detected *ERBB2* point mutations in 3 of 185 patients with hematologic malignancies, two of which found in AML patients were in the extracellular domain and one near the C-terminus (4). Here, we show that ERBB2 is overexpressed and constitutively activated in MDS and AML cells compared to healthy CD34+ cells. In elegant studies by Baccelli *et al.* (3), ERBB2 was not detected in AML cell lines or specimens using flow cytometry analysis with surface marker staining, which is consistent with our findings here. Only with intracellular ERBB2 staining and Western blot analysis was ERBB2 detected at strong levels. Quenching ERBB2 signaling is sufficient to promote mitochondrial-mediated apoptosis, suggesting that ERBB2 overexpression could represent oncogene addiction in MDS and AML.

Truncated isoforms of ERBB2 like p95^{ERBB2} and p110^{ERBB2} have been described in breast cancer (20). ERBB2 isoforms are thought to be generated from post-translational proteolysis of the receptor, and alternative initiation of translation (43,44). The localization of ERBB2 in MDS and AML cells is distributed in the nucleus or cytoplasm and to a lesser degree at the cellular membrane where the full-length ERBB2 receptor is detected (44,45). From immunoblot analysis, both truncated ERBB2 isoforms p95^{ERBB2} and p110^{ERBB2} were detected in MDS and AML cells, while the full-length receptor p185^{ERBB2} was not detected. Importantly, truncated isoforms of ERBB2 participate in crucial pathological functions. These isoforms are found to have higher kinase activity than full length receptors, thereby increasing multiple downstream signaling pathways and malignant transformation (46). Consistent with these findings, ERBB2 isoforms in MDS and AML cells also display constitutive phosphorylation or activation, which is hypothesized to be a driving factor for AML oncogenicity in this study. Although our screen of primary AML cells displayed low levels of the extracellular domain of ERBB2, we do not exclude the possibility that some MDS and AML could express a full-length ERBB2 receptor at higher levels and be

responsive to anti-ERBB2 antibodies like trastuzumab or ERBB2-CD3 biphenotypic antibodies.

To pharmacologically neutralize ERBB2 phosphorylation, lapatinib was selected over anti-ERBB2 monoclonal antibodies (i.e., trastuzumab, pertuzumab) since it is effective to neutralize truncated ERBB2 signaling in breast cancer (20,21). Here we show that lapatinib effectively neutralizes autophosphorylation of ERBB2 and the downstream target phospho-ERK1/2, resulting in potent anti-leukemic effects. These anti-leukemic effects from ERBB2 inhibition were not specific to lapatinib. We demonstrate comparable efficacy of decreased MDS and AML cell proliferation, increased cellular apoptosis and decreased ability to generate colony forming cells following culture with afatinib and neratinib. Importantly, the levels of decreased cell viability and function following culture with these TKIs were comparable to cultures with hypomethylating chemotherapies (i.e., azacitidine and decitabine) for MDS (17). Based on our data, the advantages of utilizing anti-ERBB2 TKIs is that there were no detectable negative impacts on healthy hematopoietic cells. These findings are consistent with both pre-clinical and clinical studies where no increased hematologic toxicities were detected with ERBB2 inhibition (3,47).

Pro-survival proteins within the BCL2 family are commonly upregulated in cancers, as a means to both escape mitochondrial-mediated apoptosis and sustain cancer growth. Increased MCL1 protein expression is essential for the growth of AML (48). Chemotherapy like anthracyclines, which are used to treat a subset of patients with AML, are thought to have anti-leukemic effects in part by downregulating MCL1 (49). Because of the crucial role of MCL1 in promoting a variety of cancer growth, small molecule inhibitors for MCL1 such as S63845 and AZD5991 are in development and early clinical studies. Here, we demonstrate that either ERBB2 or BCL2 inhibition was sufficient to decrease MCL1 protein expression. This reduction of MCL1 protein sensitizes ERBB2 overexpressing cells to mitochondrial-mediated apoptosis, as was reported in models of murine fibroblasts (50). When both ERBB2 and BCL2 are inhibited, MCL1 protein levels are further suppressed beyond either monotherapy alone. Since elevated MCL1 protein is a mechanism for resistance with treatment with BCL2 inhibitors, it is possible that adding lapatinib to BCL2 inhibitors could restore therapeutic efficacy to patients who have developed resistance to BCL2 inhibitors.

Venetoclax has demonstrated efficacy in MDS/AML and could be synergistic in vitro and additive in vivo when used in combination with hypomethylating chemotherapies (12). When venetoclax is combined with lapatinib, we observe increased levels of annexin V+ cells, the release of cytochrome *c*, and downstream activation of caspase 3 compared to treatments with either drug alone. Based on our findings, dual therapy with ERBB2 and BCL2 inhibition could improve response rates for patients with MDS and AML and warrants rapid translation into clinical investigations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. ERBB2 is constitutively overexpressed and truncated in MDS and AML cells.

(A) Real-time PCR analysis of *ERBB1-ERBB4* mRNA expression of human acute lymphocytic leukemia cell line CEM, myelodysplastic syndrome cell line MDS-L, leukemic cell lines K562, Kasumi1, and KG-1. Each specimen is normalized to *GADPH* gene and shown relative to CEM for each primer. $n=3-6$ /group with three technical replicates. (B) Comparison of relative levels of *ERBB1-ERBB4* mRNA by real-time PCR of primary human cells: cord blood CD34+ cells (CB CD34+), peripheral blood CD34+ cells from healthy donors (Healthy Ctl), CD34+ myelodysplastic syndrome (MDS), and CD34+ acute myeloid leukemia (AML) cells. Each specimen is normalized to *GADPH* gene and were

shown relative to CB CD34+ for primers *ERBB2-4*. As *ERBB1* is not detected (N.D.) in CB CD34+, the expression of *ERBB1* was shown relative to Healthy Ctl. *ERBB1* ($n=1-5$), *ERBB2* ($n=4-9$), *ERBB3* ($n=3-5$), and *ERBB4* ($n=3-5$) biologic replicates per group. * $p=0.02$ for MDS or AML compared to normal hematopoietic cells. (C) Flow cytometric analysis for ERBB2 using an anti-ERBB2 antibody that detects the extracellular domain (ECD). ND, not detected. (D) Flow cytometric analysis and quantification for total ERBB2 in MDS-L (blue) and K562 (green) cells. The breast cancer cell line BT474 (red) is a positive control. Isotype control is shown in gray. $n=5$ /group. (E). Levels of total ERBB2 in healthy controls ($n=4$), CD34+ MDS ($n=7$) and AML cells ($n=8$) by flow cytometric analysis. * $p=0.02$ and $=0.0002$ for healthy ctl compared to MDS and AML, respectively. (F) Immunofluorescence for ERBB2 protein (green) in MDS-L and K562. Nuclear material was stained with DAPI (blue). Staining of BT474 cells is a positive control. Isotype antibody was used as a negative control. Scale bar, 20 μm . (G) Immunofluorescence for ERBB2 protein (green) and DAPI in CD34+ marrow cells from a healthy donor and AML patients (AML458 and AML477). Scale bar, 20 μm . (H) Western blot analysis for ERBB2. Immunoprecipitation with anti-ERBB2 from lysates of CD34+ AML cells (200 μg), BT474 (50 μg) and MCF10A-p110 (50 μg). MCF10A-p110 express the isoform p110^{ERBB2} after treatment with doxycycline. IgG isotype was used as a negative control. Actin was used as a loading control in the input (10% of the lysate) for immunoprecipitation.

Figure 2. Constitutive phosphorylation of ERBB2 in MDS-L and primary AML is decreased with Lapatinib.

(A) Flow cytometric analysis and (B) quantification of basal levels of phospho-ERBB2 at tyrosine 877 (pY877), tyrosine 1248 (pY1248), and tyrosines 1221 and 1222 (pY1221+1222) in MDS-L and K562 cells. Isotype controls are shown to the left of each plot. $n=2-5$ /group. (C) Flow cytometric analysis and quantification of basal levels of phospho-ERBB2 Y877 and Y1248 in primary CD34+ AML cells. $n=6$ /group. For (D-F), MDS-L and K562 cells were incubated with increasing doses of lapatinib (Lap) for 24 h. (D) Flow cytometric analysis and quantification of phospho-ERBB2 Y1248 in MDS-L and K562

cells. $n=2-3/\text{group}$. $*p = 0.002$ and 0.0007 for dimethyl sulfoxide (DMSO) compared to Lap in MDS-L and K562 cells, respectively. (E) Immunoblots of phosphorylated ERK and total ERK from 24 h culture of MDS-L cells. Quantification of immunoblots is shown to the right. $n=3-4/\text{group}$. $*p = 0.01$ for Lap compared to DMSO.

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Figure 3. Constitutively activated ERBB2 promotes survival and proliferation of MDS and AML cells.

(A) Levels of ERBB2 mRNA expression following 7-day culture with small interfering non-targeting RNA (siCtl) or with pooled small interfering ERBB2 RNA (siERBB2). (B) Quantification of trypan blue+ cells in specified culture conditions. * $p=0.003$. (C) Left, representative flow cytometric analysis of annexin V+ cells in cultures with siCtl (dotted line) or siERBB2 (solid line). Negative control is shown in gray. Right, quantification of annexin V+ cells. $n=3-4$ /group. * $p=0.0006$. Data are shown as means $\pm$ S.E.M. (D) Total cell expansion of healthy CD34+ marrow, CD34+ AML, MDS-L or K562 cells following

DMSO, 5 μ M or 10 μ M Lapatinib (Lap). $n=7$ healthy controls and 3-7 technical replicates per group for primary CD34+ cells. $n=5-24$ technical replicates/group for MDS-L and K562 cells. $*p<0.0001$, 0.002 and <0.0001 , <0.0001 , <0.0001 for Lap compared to DMSO in AML451, 458 and 477, MDS-L, and K562 cells respectively. **(E)** CFCs for 72 h culture with 5 μ M or 10 μ M Lap or DMSO. For primary cells, number of input cells per dish =1000. $n=2$ biologic samples for healthy control and 2 for AML, 3 technical replicates/group. $*p<0.04$ for Lap compared with DMSO. For MDS-L and K562 cells, number of input cells per dish = 1000 and 500, respectively. $n=3-6$ technical replicates/group. $*p=0.006$ for Lap compared with DMSO. **(F)** Flow cytometric analysis of annexin V+ and 7AAD+ cells from CD34+ healthy marrow and CD34+ AML and **(G)** in MDS-L and K562 cells following incubation with DMSO or Lap for 72 h. **(H)** Quantification of % annexin V+ cells. $n=5$ biologic samples/group for primary cells, analyzed as 4-5 technical replicates per sample. $n=4-6$ technical replicates for MDS-L and K562 cells. $*p<0.0001$ for Lap compared with DMSO. Data are shown as means $\pm$ SEM. Student two-tailed, unpaired t tests were used in these analyses.

Figure 4. Neutralization of ERBB2 impairs long-term human MDS cell engraftment.

(A) Schematic of study design. Twenty-four hours after irradiation with 2.5 Gy, NSG mice were transplanted by intrafemoral injection with 10^7 MDS-L cells that had been treated with 10 μ M Lapatinib (Lap) or DMSO. Femurs and spleens were collected at 21 weeks post-transplantation. (B) Hematoxylin and eosin (H&E)-stained femur sections (top) and Wright-stained bone marrow aspirates from recipients of lapatinib- or DMSO-treated MDS-L cells. Scale bars, 50 μ m in H&E and 20 μ m in Wright-stained images. (C) Flow cytometric analysis of engraftment of human CD45⁺ MDS-L cells at 21-weeks post-transplantation. murine CD45 (mCD45), human CD45 (hCD45). (D) Quantification of bone marrow engraftment of MDS-L cells and human lineages. $n = 4-5$ mice per group. * $p < 0.05$ for lapatinib compared to DMSO. (E) Quantification of spleen weight and a spleen image at 21 weeks post-transplantation. Scale bar, 1 cm. $n = 4-5$ /group. * $p = 0.004$. (F) H&E-stained splenic sections. Scale bars, 100 μ m top and 20 μ m bottom. Data are shown as means $\pm$ SD. Student two-tailed, unpaired t tests were used in these analyses.

Figure 5. ERBB2 inhibition modulates the family of BCL2 proteins in MDS and AML cells. (A, B) mRNA expression of *BBC3* and *BCL2* in MDS-L cells and primary CD34+ AML cells treated with either dimethyl sulfoxide (DMSO) or 5 μ M Lapatinib (Lap) for the indicated time. For MDS-L cells, 3 independent experiments were performed with three technical replicates/group. * p 0.02 for lapatinib compared with DMSO. For primary AML cells, $n=3$ biologic samples with three technical replicates. * $p<0.001$ for Lap compared to DMSO. (C) Immunoblots of BCL2, MCL1, BBC3, and BAX in MDS-L cells treated with indicated doses of Lap for 24 h. Actin was used as a loading control. Right, quantification of

immunoblots. Data were presented as protein expression level normalized to actin and relative to the DMSO group. $n=8$, $n=6$, and $n=5$, and $n=6$ per group for BCL2, MCL1, BBC3 and BAX, respectively. $*p=0.007$ for Lap compared to control. **(D)** Immunoblots of BCL2 family protein expression in primary CD34+ AML cells 24 h following incubation with either DMSO or 5 μ M Lap. Actin was used as a loading control. Right, quantification of immunoblots. $n=4$ biologic samples. $*p=0.04$ for Lap compared with DMSO. **(E)** Representative immunoblot of MCL1 expression following 24 h cultures of MDS-L cells with DMSO or 2 μ M maritoclax. **(F)** Total cell number for treatment described in **(E)**. $n=4-5$ technical replicates/group. $*p<0.0001$ for MCL1 inhibitor compared to DMSO control. **(G)** Representative flow cytometric analysis and quantification of annexin V+ cells from 24 h cultures of MDS-L with DMSO or maritoclax. $n=5$ technical replicates/group. $*p<0.0001$ for maritoclax compared to DMSO control. Data are shown as means $\pm$ SD. Student two-tailed, unpaired t tests were used in these analyses.

Figure 6. Dual ERBB2 and BCL2 blockade synergistically promotes apoptotic death of MDS-L and AML cells.

For (A-C), MDS-L cells were incubated with venetoclax (Venet) or Lapatinib (Lap), alone or in combination for 72h, and cell viability was determined by MTT. (A) Synergy and antagonism matrices were analyzed with Combenefit software for drug combinations. Peak synergy occurs with combination of 2.5-5 μ M Lap and 0.1-0.2 μ M Venet (Bliss model). No combinations were antagonistic or promoted cancer growth. (B) Values of Combination Index (CI) and Fraction affected (Fa, fraction affected by indicated dose) for cultures of MDS-L cells treated with 5 μ M Lap and different doses of Venet. Values were calculated by

CompuSyn based on Chou-Talalay's method. (C) Viability of MDS-L cells following incubation with 5 μ M Lap+Venet (blue line) compared to Venet alone (black line). Data are shown relative to the corresponding controls (i.e., DMSO or Lap alone). $n=7$ /group. Dose reduction index for venetoclax (DRIvenet) at IC50 is 29-fold in combination with Lap compared to Venet alone. (D) Cell expansion in MDS-L and primary CD34+ AML cells after 72 h culture with DMSO, 5 μ M Lap, 0.2 μ M Venet or Lap+Venet. For MDS-L, $n=8-9$ technical replicates/group. For primary AML, $n=3$ (AML452, 458, and 464), analyzed as 4-6 technical replicates/group. $*p<0.0001$ for Lap, Venet and Lap+Venet compared to DMSO. $\#p=0.04$ for Lap+Venet compared to Lap or Venet alone. (E-F) Flow cytometric analysis of annexin V+ and 7AAD+ cells of 72 h cultures following indicated treatments. Quantification of annexin V+ cells is shown to the right. $n=3-6$ /group. $*p=0.02$ for Lap, Venet and Lap+Venet compared to DMSO. $\#p<0.0001$ for Lap+Venet compared to either Lap or Venet alone. Data are shown as means $\pm$ SD. Sidak multiple comparison tests after one-way ANOVA were used in these analyses.

Figure 7. Synergy of mitochondrial-mediated death signals in primary AML cells by ERBB2 and BCL2 inhibition.

(A) Western blot analysis of BCL2 family proteins from 24 h cultures of MDS-L treated with increasing doses of Venet without/with combination of 5 μ M Lap (- Lap and + Lap). Protein expressions of BCL2, MCL1, BBC3, and BAX are normalized to actin and relative to DMSO control. The phosphorylated level of BCL2 (pBCL2) is normalized to total BCL2 and relative to DMSO. $n=3-8$ /group, from at least three independent experiments. * $p < 0.01$ for Venet or Lap alone compared to DMSO. # $p < 0.03$ for Lap+Venet compared to Venet alone or Lap alone. (B) Representative western blots of BCL2 family expression from 24 h

cultures of primary AML treated with DMSO, 5 μ M Lap, 0.2 μ M Venet or Lap+Venet. (C) Quantification of immunoblots in (B). $n=2$ biologic samples/group. * $p < 0.02$ for Lap, Venet, and Lap+Venet compared to DMSO. # $p < 0.05$ for Lap+Venet compared to either Lap or Venet. (D, E) Flow cytometric analysis of released cytochrome *c* and activated caspase 3 at 24 h in MDS-L cells with indicated treatments. $n=6-10$ /group. * $p < 0.0001$ for Lap, Venet, and Lap+Venet compared to DMSO. # $p < 0.0001$ for either Lap or Venet compared to Lap + Venet. Data are shown as means $\pm$ SD. Sidak multiple comparison tests after one-way or two-way ANOVA were used in these analyses.