Cancer Research

Synthetic Lethality through Combined Notch–Epidermal Growth Factor Receptor Pathway Inhibition in Basal-Like Breast Cancer

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Abstract

Basal-like breast cancers (BLBC) are highly aggressive, yet selective therapies targeting the specific oncoproteins driving these tumors have not been developed. These cancers frequently express epidermal growth factor receptor (EGFR), with resistance to its inhibition being well documented, albeit poorly understood. Notch pathway activation is also common in this breast cancer subtype and can be suppressed by γ-secretase inhibitors, which effectively block receptor cleavage and activation. Herein, we show that although inhibition of either EGFR or Notch signaling alone is insufficient to suppress basal-like breast tumor cell survival and proliferation, simultaneous inhibition uncovers a synthetic lethal relationship between these two oncogenic pathways. This lethality is due in part to significant decreases in AKT activation caused by combined EGFR and Notch inhibition. Expression of the activated form of Notch1 restores AKT activity and enables cells to overcome cell death after dual-pathway blockade. Combined pathway inhibition is also dramatically more effective at suppressing tumor growth in mice than blocking EGFR or Notch signaling alone. Thus, we show that Notch pathway activation contributes to resistance to EGFR inhibition, and provide a novel treatment strategy for BLBCs. *Cancer Res; 70(13); 5465–74. ©2010 AACR.*

Introduction

The identification of breast cancer subtypes has resulted in a refinement in prognostic capability and promises to result in the development of more selective, molecularly targeted therapies that possess improved efficacy (1, 2). The basal-like breast cancer (BLBC) subtype constitutes ~15% of tumors and is characterized by the absence of expression of estrogen receptor (ER) and progesterone receptor (PR) and *Her2/neu* amplification (and is therefore also referred to as "triple negative"). Unlike ER- and PR-positive or *Her2/neu*-amplified cancers, no targeted therapies exist for these tumors. In general, they are highly aggressive relative to other subtypes (2). Metastases tend to arise in the visceral organs as opposed to bone, and a rapid progression from recurrence to death is common (3, 4). This poor prognosis is due in part to the lack of available therapeutics targeting the specific pathways responsible for their aggressive behavior. In contrast, targeted therapeutics have had a profound effect on survival in other subtypes (5).

BLBCs, unlike other subtypes, commonly show expression of the epidermal growth factor receptor (EGFR; HER1), one of the four HER kinase family of receptors (6). Unrestrained activation of EGFR leads to dysregulated proliferation and transformation in experimental systems, and activating mutations have been observed in human brain (7) and lung (8) tumors. Ligand engagement of EGFR results in homodimerization and heterodimerization with other members of the HER family, resulting in intrinsic kinase activation. This activation leads to stimulation of the Ras–Raf–mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) kinase–MAPK pathway, promoting multiple cellular processes, including proliferation, invasion, and suppression of apoptosis (9). Because of the compelling genetics and biochemistry suggesting an essential role for EGFR signaling in some cancers, highly specific small-molecule inhibitors and antibodies have been developed against EGFR and have shown efficacy against lung, colon, and head and neck cancer (10). Unfortunately, these inhibitors have shown only limited clinical activity against breast cancers, even when inhibition of EGFR phosphorylation has been documented *in vivo* (11). One possible explanation for these observations is that complementary pathways exist that confer intrinsic resistance to EGFR inhibition in breast cancer.

The Notch pathway has also been shown to be hyperactivated in BLBCs (12, 13), and Notch receptors are oncogenic in the mammary gland (14, 15). During development, Notch receptors function in a tissue-specific manner to regulate cell

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fate, proliferation, survival, and cell death (16). The four Notch receptors are activated by two intramembranous cleavage events, the first being dependent on ADAM metalloproteinase and the second dependent on the γ-secretase holoenzyme consisting of presenilin/nicastrin/APH-1/PEN-2 subunits (17). After the second cleavage event, the intracellular domain of the receptor (NICD) translocates to the nucleus and interacts with RBP-Jκ and Mastermind to generate a large transcriptional complex (18). Notch1 promotes an aggressive phenotype in breast cancer cells through several mechanisms. It has been implicated in survival signaling by activation of AKT (19, 20) and survivin (21), resulting in chemoresistance. Notch signaling is involved in resistance to Herceptin in *Her2/neu*-amplified breast cancer (22). Notch can provoke an epithelial-to-mesenchymal transition in carcinoma cells (23), which recently has been shown to drive the intrinsically chemoresistant breast cancer stem cell phenotype. Because of the hyperactivation of the Notch pathway and its relationship to cell survival in breast and other tumors, highly specific γ-secretase inhibitors (GSI) are in clinical development, but whether these are cytotoxic or not in breast cancer is unclear (24).

Cross talk between the EGFR and Notch pathways has been identified in lung (25), skin cancers (26), and gliomas (27). Because BLBCs frequently show constitutive hyperactivation of both pathways, we sought to determine how signaling from these pathways might converge by assessing the effects of dual-pathway inhibition in this subtype of cancer. We found that whereas neither pathway alone is essential for proliferation or cell survival, dual inhibition using γ-secretase and EGFR inhibitors results in a marked decrease in proliferation, a dramatic increase in cell death, and significantly reduced tumor growth in a xenograft model of BLBC. This loss of viability is due to a role for Notch1 in sustaining AKT activity, whose activation is an established mechanism of resistance to treatment with EGFR inhibitors (28). Expression of an activated Notch1 intracellular domain (NICD1) in the presence of both inhibitors restored AKT activity and cell viability. These data show that Notch signaling contributes to the observed resistance to EGFR inhibition in breast cancer and that combined Notch-EGFR pathway inhibition is a rational treatment strategy for BLBCs.

Materials and Methods

Cell culture and reagents

The HCC1806 and HCC1937 cells were provided by Dr. M. Ellis (Washington University), and MDA-MB-468 and MDA-MB-231 cells were purchased from the American Type Culture Collection. These cell lines have been reported to be triple negative (29), and we confirmed that their *Her2/neu* status is negative (Supplementary Fig. S1A). HCC1806 and HCC1937 were grown in RPMI 1640 containing 10% fetal bovine serum with antibiotics and supplements (10 mmol/L HEPES, 4.5 g/L glucose, 2 mmol/L L-glutamine, and 1 mmol/L sodium pyruvate). MDA-MB-468 and MDA-MB-231 cells were maintained in DMEM supplemented with 10% fetal bovine serum and 1% penicillin. All cell lines were incubated at 37°C with 5% CO_2 in a humidified incubator. Drugs were purchased from Calbiochem (DAPT, used at 10 μmol/L; ref. 30), Alexis [compound E, used at 10 μmol/L (31) or 3 mg/kg *in vivo* (32)], and Axon Medchem [gefitinib, used at 3 μmol/L or 150 mg/kg *in vivo* (28), and erlotinib, used at 5 μmol/L (28)].

Constructs and transfections

NICD1 was excised from pYY161, a gift from J. Aster (Harvard University), and cloned into pBabe-puro vector by *Eco*RI and *Sal*I. pBabe-Puro-Myr-Flag-AKT1 was purchased from Addgene. Short hairpins targeting human nicastrin with the sequences 5′-CCCATCTTTCTTCTTGAAGAT-3′ and 5′-GCTCTACTGAATACTCTACAT-3′ were cloned into the lentiviral vector pLKO-puro (provided by Sheila Stewart, Washington University). Retroviral and lentiviral infections were carried out as previously described using FuGENE 6 transfection reagent (Roche) according to the manufacturer's instructions (33). Infected cells were selected in 2 μg/mL puromycin.

Immunoblot analysis and antibodies

Total cell protein was extracted with 1× NP40 buffer, which includes 0.5% NP40 supplemented with protease inhibitor cocktail (Roche). Lysates were cleared by centrifugation and protein concentration was tested by the Bradford assay (Bio-Rad). Lysates were boiled with SDS sample buffer, separated by SDS-PAGE, and transferred to polyvinylidene difluoride membrane (Millipore). Western blots were blocked in 5% nonfat dry milk TBS-T [10 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 0.1% Tween 20] buffer and incubated in primary antibodies diluted in blocking buffer at 4°C overnight. Blots were washed with TBS-T buffer and incubated with horseradish peroxidase–conjugated secondary antibodies (1:10,000; GE Healthcare) in blocking buffer at room temperature. Immunocomplexes were visualized with an enhanced chemiluminescence kit (GE Healthcare). Primary antibodies for immunodetection were sourced as follows: cleaved Notch1 (Val1744), nicastrin, phospho-AKT (Ser^{473}), AKT, MAPK (ERK1/2), phospho-MAPK (phospho-ERK1/2), cleaved caspase-3, Her2/neu, EGFR, and phospho-EGFR (Cell Signaling Technology) and actin (Sigma).

Proliferation assay

Cells were seeded at 20% to 30% confluence in 6-cm dishes. Twenty-four hours later, cells were placed in fresh medium containing indicated concentration of drugs and allowed to grow for 6 days. Cells were harvested by trypsinization, and the viable cell number was counted with the trypan blue exclusion method at indicated time points.

DNA replication assay

Measurement of bromodeoxyuridine (BrdUrd) incorporation was performed according to the manufacturer's recommendations. Briefly, cells were grown on coverslip and treated with indicated drugs for 4 days. Then, BrdUrd (Sigma) was added to cells with a final concentration of 20 μmol/L and incubated for 2 hours at 37°C. Cells on coverslip

were fixed with 4% paraformaldehyde for 30 minutes at room temperature, washed three times with PBS, and permeabilized with 0.25% Triton X-100. Subsequently, cells were incubated in 1.5 N HCl at room temperature for 10 minutes. After HCl incubation, the slide was washed with PBS and blocked with blocking buffer (2% goat serum in PBS-T) at 37°C for 30 minutes. Monoclonal mouse anti-BrdUrd (BD Pharmingen) was applied to the slide in blocking buffer for 30 minutes at 37°C. Slides were then washed with PBS three times and incubated in blocking buffer with secondary antibody, Alexa Fluor 568–labeled goat anti-mouse IgG (Invitrogen), for 30 minutes at 37°C. 4′,6-Diamidino-2-phenylindole (DAPI) was used to counter stain nuclei. A fluorescence microscope with appropriate filter sets was used to detect BrdUrd and DAPI fluorescence. The software ImageJ was used to count the BrdUrd-stained cells.

Cell death assay

Cell death was assessed by TUNEL assay with an *In Situ* Cell Death Detection Kit, TMR Red (Roche) and Annexin V assay with the Vybrant Apoptosis Assay Kit #3 (Invitrogen). Briefly, after drug treatment, cells were fixed in 4% paraformaldehyde. Cell labeling was performed according to the manufacturer's instructions. DAPI was used to counterstain nuclei. Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL)– or Annexin V–positive cells were detected with a fluorescence microscope, and cell number was counted by the ImageJ software. At least 200 cells were counted for each experiment.

Clonogenic assay

HCC1806 cells (1×10^3) were seeded in 10-cm tissue culture dishes and grown for 24 hours at 37°C, at which time DMSO, DAPT, gefitinib, or combinations were added daily for 4 days. Then, 1×10^3 cells were seeded in the absence of drugs in 10-cm dishes. Colonies were allowed to grow for 14 days in the absence of drug, washed with PBS, and fixed in a mixture of 6.0% glutaraldehyde and 0.5% crystal violet for 30 minutes. Tissue culture dishes were rinsed with tap water and air dried overnight, and colonies >1 mm were scored as positive.

Animal studies

Animals were handled according to protocols approved by the Washington University Animal Studies Committee. HCC1806 cells (5×10^5) were mixed 1:1 with Matrigel (BD Biosciences) and injected s.c. into the flank of 6-week-old female nude mice. When tumors reached 50 to 100 mm^3 in volume, mice were randomized and treated with vehicle and drugs. Compound E (3 mg/kg) was injected i.p. 5 days a week. Gefitinib was dissolved in DMSO [10% (v/v) final concentration] and diluted in carboxymethylcellulose (0.25, w/v) to a final concentration of 20 mg/mL. Gefitinib was administered by oral gavage (150 mg/kg) 5 days a week. Mice were weighed and tumors were measured with caliper twice weekly. Tumor volumes were calculated with the following formula: $0.5 \times LW^2$ (with L and W representing the length and width of tumors, respectively).

Results

Combined γ-secretase and EGFR inhibition results in decreased proliferation and cell death in BLBC cells

Both the Notch and EGFR pathways have been implicated in proliferation and cell survival in some cancers. In breast cancer, pharmacologic inhibition of γ-secretase has previously been considered cytotoxic (21, 34), but this contention has recently been disputed because more selective GSIs administered at doses that do not inhibit proteasome function failed to induce a cytotoxic effect in a wide panel of breast cancer cell lines (24).

We treated the BLBC cell line HCC1806 with the specific GSI, DAPT, at a dose (10 μmol/L) that suppressed γ-secretase activity as judged by the inability to detect Notch cleavage using a cleavage-specific Notch1 antibody, Val1744, after treatment (Fig. 1A). Within 4 days of treatment, cleaved Notch was no longer detectable by immunoblot analysis. In agreement with the recent report in which selective GSIs were shown to lack cytotoxicity against breast cancer cells (24), enumeration of cell numbers revealed only a modest decrease in cell growth even after 6 days of treatment (Fig. 1B). In addition, in accord with previously published reports (35), 3 μmol/L, a dose of gefitinib capable of inhibition of phospho-EGFR, had only a modest effect on growth. In contrast, simultaneous treatment with both drugs caused a dramatic decrease in growth at doses that had negligible effects when used as single agents (Fig. 1B). Colony-forming assays confirmed the decrease of longer-term growth potential (Supplementary Fig. S2), consistent with the loss of proliferative capacity measured in short-term culture. To extend this finding and determine whether the observed synergistic loss of growth potential after combined pathway inhibition is common to other BLBC cell lines, we subjected MDA-MB-468 and HCC1937 BLBC cells to the same treatment regimens and confirmed the ability of DAPT to block Notch cleavage in these cells (Fig. 1A). After single treatment with DAPT or gefitinib, we observed only modest reductions in growth similar to what has previously been reported (35). Notch inhibition did not change total EGFR or phospho-EGFR protein levels, and gefitinib treatment did not reduce total Notch protein levels (Supplementary Fig. S1B). Importantly, we confirmed the dramatic suppression of cell growth after combined drug treatment in these two additional cell lines (Fig. 1B), indicating that the combined inhibition of Notch and EGFR pathways results in significant reductions in cell proliferation.

Mutations in *Ras*, which signals downstream of EGFR, have recently been shown to confer resistance to EGFR inhibitors in colon cancer (36). MDA-MB-231 BLBC cells harbor Ras pathway mutations (37). Based on this mutational status, MDA-MB-231 cells should be refractory to dual-pathway inhibition because Ras signaling downstream of EGFR would contribute to cell survival even in the presence of Notch and EGFR inhibition. Testing this possibility, we found that these cells were indeed refractory to dual-pathway inhibition, showing no growth or proliferative defects after combination treatment (Fig. 1A and B).

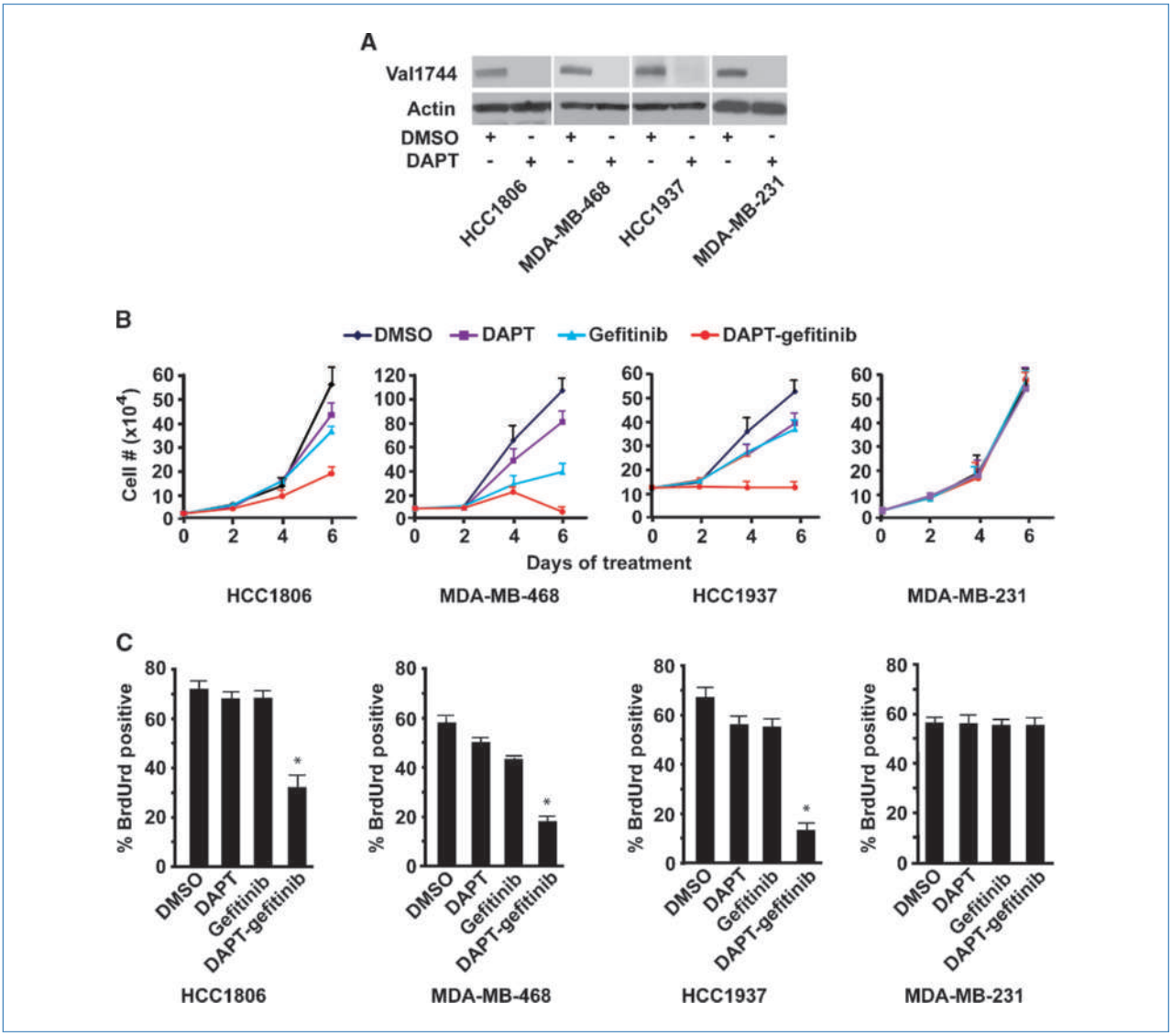

Figure 1. Effects of combined γ-secretase–EGFR inhibition on growth of BLBC cells. A, immunoblot analysis using an antibody specific for the cleaved form (Val1744) of Notch1 shows near-complete inhibition of Notch cleavage after 4 d of 10 μmol/L DAPT treatment. B, enumeration of cell numbers after the indicated drug exposures shows that DAPT-gefitinib treatment significantly decreased growth compared with no or single-drug treatment. MDA-MB-231 cells harbor *Ras* pathway mutations and are resistant to dual treatment. C, quantification of BrdUrd-positive cells reveals decreased proliferation as a contributor to reduced numbers after dual treatment. All experiments were performed in triplicate, and at least 100 cells were counted for each replicate. *, $P < 0.0001$, Fisher's exact test. A dose of 3 μmol/L gefitinib was used in all experiments. Columns, mean; bars, SE.

Next, we determined whether the effect of combined pathway inhibition on cell proliferation was due to a requirement for concurrent Notch and EGFR signaling in S-phase entry and/or cell death. Cells were pulsed with BrdUrd to allow for its incorporation into replicating DNA and stained with antibodies recognizing BrdUrd to assess the S-phase fraction in response to GSI, gefitinib, or combination treatment. No significant changes in S phase were observed after 4 days of treatment with either drug alone (Fig. 1C). However, in all three sensitive cell lines, combination treatment caused a dramatic reduction in the number of cells entering S phase, whereas it showed no effect on the resistant MDA-MB-231 cell line (Fig. 1C). To further analyze the mechanism underlying the observed loss of cell growth on dual Notch-EGFR pathway inhibition, we sought to determine whether combined treatment could cause cell death. After combination treatment for 4 days, a significant increase in cell death was observed and quantified by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL), whereas single-drug treatment had no measurable effect (Fig. 2). Increased apoptosis was also measurable as early as 2 days after dual-pathway inhibition using Annexin V staining (Supplementary Fig. S3). By immunoblot analysis of protein lysates harvested 2 days after the various drug exposures

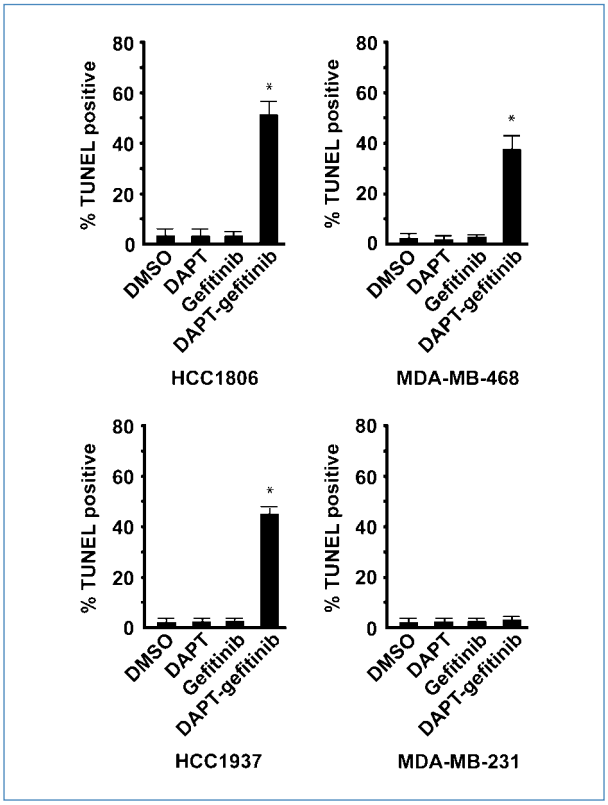

Figure 2. Effects of combined γ-secretase–EGFR inhibition on cell death of BLBC cells. TUNEL assays on drug-exposed cells performed after 4 d show a dramatic increase in cell death only after combination treatment. All experiments were performed in triplicate, and at least 100 cells were counted for each replicate. *, $P < 0.0001$, Fisher's exact test. Columns, mean; bars, SE.

with an antibody specific for cleaved caspase-3, we detected evidence of caspase cleavage only in MDA-MB-468 cells treated with dual-pathway inhibition, but not in HCC1806 or HCC1937 cells (data not shown), despite evidence of apoptosis as determined by TUNEL and Annexin V staining in these cell lines. This result suggests that blocking the Notch-EGFR pathways provokes cell death through multiple mechanisms, a finding that will warrant further investigation. More importantly, decreased proliferation and loss of viability were also observed when cells were treated with a different highly specific GSI, compound E, as well as another EGFR inhibitor, erlotinib, ruling out the specific chemistries of DAPT and gefitinib as causal (data not shown). Thus, simultaneous inhibition of Notch and EGFR uncovers a synthetic lethal relationship between these two pathways in BLBC cells.

γ-Secretase activity is required for sustained AKT activation in the presence of EGFR inhibition

Notch activity has been shown to inhibit apoptosis through several pathways (38–44), although in breast cancer phosphatidylinositol 3-kinase (PI3K)–AKT signaling is the best-characterized mechanism of Notch-dependent resistance to cell death (19, 20). Because AKT activity has been found to play a central role in mediating resistance to EGFR inhibitors in several contexts (28, 45, 46), we determined whether survival after dual-pathway inhibition involved loss of AKT signaling by assessing levels of phospho-AKT following single or combination drug treatment. Although dysregulation of either Notch or EGFR signaling alone had only minor effects on AKT activation, simultaneous pathway blockade dramatically reduced AKT phosphorylation in HCC1806 and MDA-MB-468 cells (Fig. 3). MAPK kinase signaling was not affected by single- or dual-pathway treatment (Supplementary Fig. S4) unlike that which is observed in lung cancer (25). Therefore, in BLBC cells, concerted EGFR and Notch signals are required to sustain the AKT activation, and loss of viability after combined inhibition is likely due to this requirement.

The γ-secretase holoenzyme is not essential for breast cancer cell proliferation and survival

The γ-secretase complex consists of presenilin/nicastrin/APH-1/PEN-2 and is essential for murine embryogenesis (17). The degree of cytotoxicity caused by inhibiting this complex in breast cancer has recently been disputed due to a

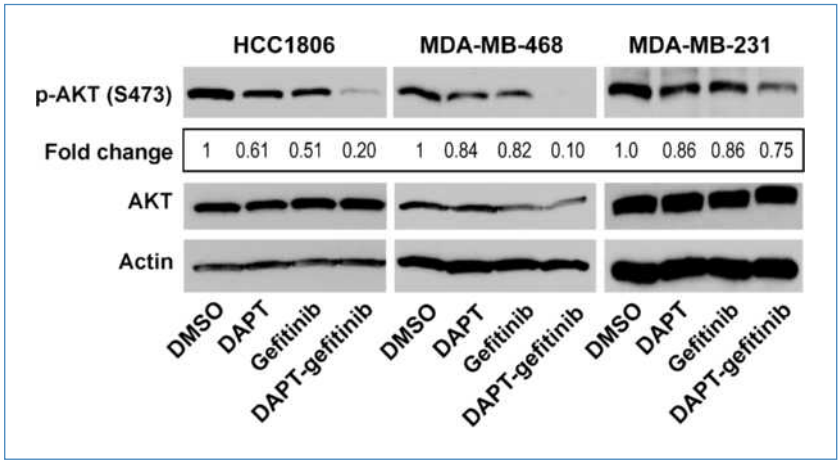

Figure 3. Effects of combined γ-secretase–EGFR inhibition on AKT phosphorylation of BLBC cells. Immunoblot analysis after 4 d of the indicated drug exposures reveals significantly decreased phospho-AKT (p-AKT) in cells sensitive to dual treatment. MDA-MB-231 cells sustain AKT activity in the presence of dual-pathway inhibition.

careful analysis of the more specific GSIs such as those used herein, which failed to show toxicity in breast cancer cells (24). To rule out the possibility that off-target effects of GSIs contributed to the synthetic lethal effects that we observed after dual-pathway inhibition, we generated a stable cell line lacking γ-secretase activity by knocking down nicastrin, an essential component of the holoenzyme (47). We used two short hairpins, each targeting a distinct region of the nicastrin coding sequence, to lentivirally infect HCC1806 cells. Control cells were generated by lentiviral transduction of a short hairpin targeting the luciferase gene, which contains coding sequences not present in the human genome. Stable, polyclonal knockdown cell populations were established after infection and drug selection. Cells harboring the nicastrin short hairpins showed a significant decrease of nicastrin protein levels and Notch cleavage, approximating that produced by pharmacologic γ-secretase inhibition (Fig. 4A). These cells were expanded for further analysis. As assessed by staining for BrdUrd incorporation, these cells contained equal S-phase fractions compared with control cells (Fig. 4B), and no loss of viability was observed after trypan blue staining (data not shown). Therefore, the intact γ-secretase holoenzyme is not required for the survival or proliferation of BLBC cells.

Having established that nicastrin-dependent γ-secretase activity is not essential in BLBC cells, we next asked whether nicastrin knockdown cells would exhibit a similar proliferative and survival defect after exposure to gefitinib as that observed on pharmacologic inhibition of the enzyme with GSIs. Control or nicastrin knockdown cells were exposed to gefitinib for 4 days, after which time S-phase entry was measured by BrdUrd incorporation. Similar to dual drug treatment, the addition of gefitinib to nicastrin knockdown cells caused a significant growth arrest, whereas cells harboring the control short hairpin proliferated normally (Fig. 4B). We then determined whether cells lacking nicastrin would undergo apoptosis as observed with acute dual pharmacologic treatment. By counting TUNEL-positive cells 4 days after gefitinib treatment, we found that cells engineered to lack nicastrin undergo high levels of cell death only on EGFR inhibition (Fig. 4C). Therefore, pharmacologic and RNA interference (RNAi)–mediated inhibition of γ-secretase activity causes a similar loss of both proliferative capacity and viability after EGFR inhibition.

A Notch1-AKT pathway can suppress synthetic lethality after combined pathway inhibition

Notch1 has been shown to be specifically hyperactivated in BLBC (12). Therefore, to further elucidate the role of Notch1 in the synthetic lethality induced by combined GSI-EGFR inhibition, we investigated the contribution of the cleaved form of Notch1 to this phenotype. Transduction of a retroviral vector carrying the NICD1 cDNA (cleaved, active form of Notch1, described in ref. 48) into HCC1806 cells was followed by drug selection with puromycin to establish stable HCC1806-NICD1–overexpressing cells. Control cells were established by transduction of an empty vector into HCC1806 cells. Immunoblot analysis confirmed an increase in a truncated Notch1 protein in cells infected with NICD1 containing vector (Fig. 5A). Cells were then exposed to GSI and gefitinib or DMSO, and the ability of NICD1 to suppress cell death after dual inhibition was measured by TUNEL. Strikingly, NICD1 expression dramatically inhibited apoptosis after drug treatment (Fig. 5B). Additionally, this NICD1-dependent resistance was associated with sustained AKT phosphorylation even in the presence of dual-pathway inhibition (Fig. 5A). To further assess the role of AKT in the observed loss of viability incurred after combined Notch-EGFR pathway inhibition, we expressed a constitutively active, myristoylated form of AKT (myr-AKT) through retroviral transduction into

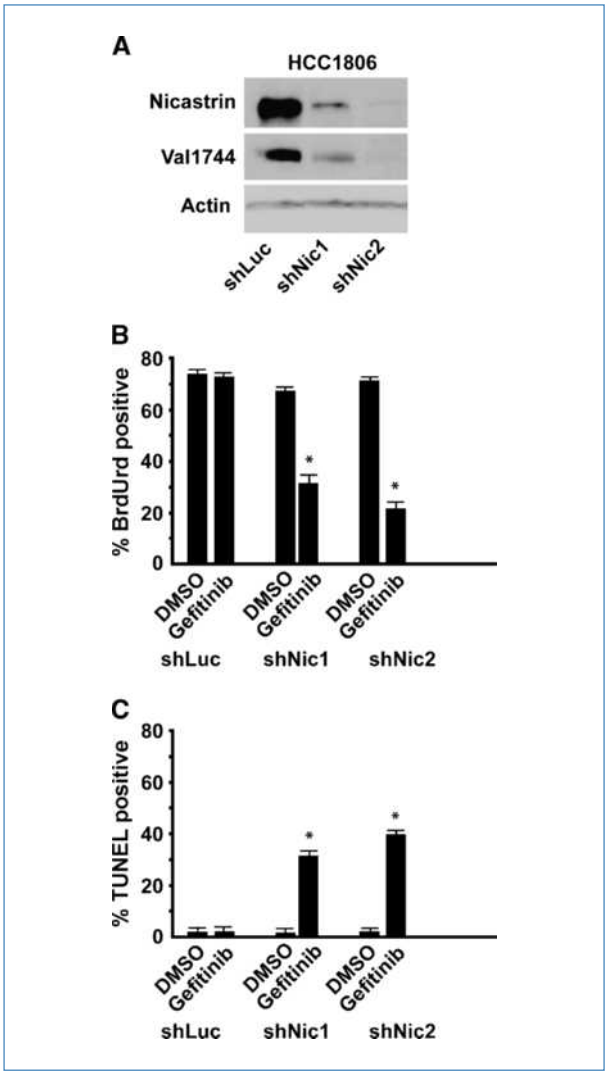

Figure 4. Role of nicastrin in BLBC cells. A, immunoblot analysis shows the reduction in nicastrin protein levels after short hairpin–mediated knockdown using two hairpins targeting distinct regions of the coding sequence. The corresponding decrease in Notch1 cleavage is shown using the cleavage-specific Val1744 antibody in nicastrin knockdown cells. Assessment of BrdUrd-positive (B) and TUNEL-positive (C) cells shows that decreased proliferation and increased cell death in nicastrin knockdown cells compared with the control luciferase vector are only observed after gefitinib exposure. Enumeration was performed after 4 d of drug treatment. All experiments were performed in triplicate, and at least 100 cells were counted for each replicate. *, $P < 0.0001$, Fisher's exact test. Columns, mean; bars, SE.

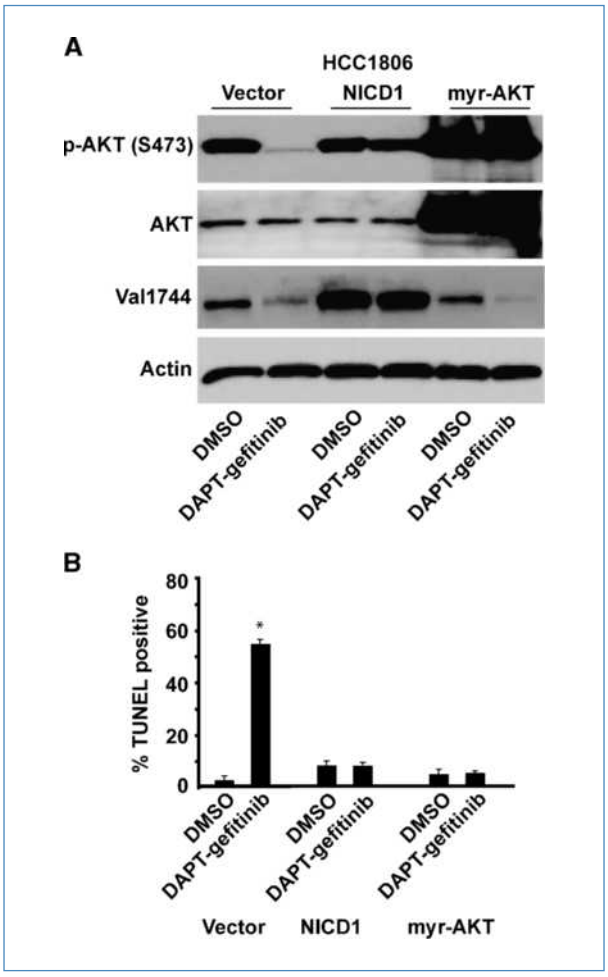

Figure 5. Loss of NICD1-dependent AKT activity is required for synthetic lethality after combined γ-secretase–EGFR inhibition. A, retroviral constructs carrying either NICD1 (the active cytoplasmic tail of Notch1) or constitutively active myr-AKT are sufficient to restore AKT activity after combined drug treatment when stably expressed in HCC1806 cells. An empty vector was used as a negative control and fails to rescue the loss of phospho-AKT after combined inhibition. B, expression of NICD1 or myr-AKT can inhibit cell death after combined drug treatment. All experiments were performed in triplicate, and at least 100 cells were counted for each replicate. *, $P < 0.0001$, Fisher's exact test. Columns, mean; bars, SE.

HCC1806 cells and exposed these and control, empty vector–containing cells to combined drug treatment (Fig. 5A). Cells harboring myr-AKT were also resistant to combined pathway inhibition (Fig. 5B). Therefore, these data indicate that a Notch1-AKT pathway imparts resistance to combined γ-secretase–EGFR inhibition in BLBC cells.

Dual-pathway blockade causes dramatic growth inhibition of BLBCs *in vivo*

To further investigate the potential efficacy of combining Notch and EGFR pathway inhibition, we performed a preclinical therapeutic drug trial *in vivo*. HCC1806 cells were xenografted into nude mice and allowed to grow to 50 mm^3, at which time mice were treated with the gefitinib and/or compound E, whose *in vivo* pharmacodynamics have been well characterized (32). In accord with the *in vitro* data, Notch or EGFR inhibition had only a modest effect on tumor growth. In contrast and also in agreement with the *in vitro* data, dual-pathway inhibition had a profound affect on tumor growth (Fig. 6). Collectively, the results of our *in vitro* and *vivo* experiments indicate that combining Notch and EGFR pathway inhibition is a rational treatment strategy for BLBC.

Discussion

EGFR inhibition has been regarded as an ineffective therapeutic strategy in breast cancer and only modestly so in others. Consequently, identifying mechanisms of resistance is an area of active investigation. The limited understanding of resistance in BLBC motivated us to ask whether additional prosurvival pathways could be identified that contribute to the poor efficacy of EGFR inhibition in this cancer subtype.

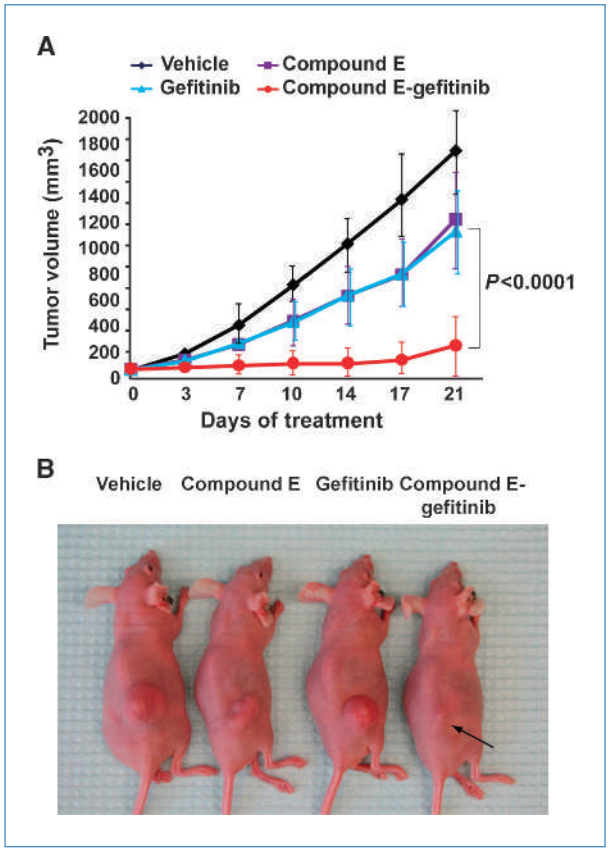

Figure 6. Therapeutic efficacy of dual-pathway inhibition *in vivo*. A, mice (10 per group) injected with HCC1806 cells were treated with DMSO or gefitinib (150 mg/kg) 5 d a week by oral gavage and/or compound E (3 mg/kg) i.p. 5 d a week once tumors reached 50 mm^3. Graphs show tumor volumes over a 21-d treatment period. ANOVA was used to calculate significance. B, photographs of representative mice treated with the respective drugs. Arrow points to a tiny tumor in a mouse treated with compound E and gefitinib.

The Notch pathway, which can promote cell survival, is often activated in BLBC. As such, we chose to test its role as a resistance factor and have identified a synthetic lethal relationship between the EGFR and Notch pathways. Whereas inhibition of either pathway alone failed to cause cell death, dual blockade induced a dramatic decrease in proliferation and massive apoptosis. These data provide a new perspective on the role of EGFR in BLBC. They indicate that the EGFR pathway is not simply dispensable for these tumors but rather signaling through the Notch pathway compensates for the loss of EGFR activity and becomes a requirement for cell proliferation and survival in the absence of EGFR-dependent signals. Therefore, Notch signaling is a potential cause of resistance to EGFR inhibition in BLBC.

The mechanism(s) of resistance to EGFR inhibition remains poorly understood in basal-like breast tumors despite significant advances in other tumor types. In colon cancer, mutations in Ras, which lies downstream of EGFR signaling, have recently been shown to be a major cause of resistance to EGFR inhibitors (36). In agreement with these data, MDA-MB-231 cells, which harbor Ras mutations (37), were resistant to EGFR inhibition (as has been previously reported in ref. 49) and remained so even when treated with GSIs. However, in breast cancer, *Ras* mutations are relatively uncommon and other mutations lying further downstream of the EGFR receptor have not been reported that would explain resistance to EGFR inhibition. In *Her2/neu*-amplified breast cancers, the mechanism of resistance to EGFR inhibitors has been shown to be due to *Her2/neu*-dependent activation of AKT (28). BLBCs express little or no *Her2/neu*, and therefore, this is an unlikely paradigm for understanding resistance in these tumors. For all of these reasons, we hypothesized that compensatory signaling originating outside of the EGFR pathway must exist to impart resistance in this subtype and reasoned that the Notch pathway was one possible source. Its activity is increased in BLBC and has been tied to the PI3K-AKT pathway in other contexts (39).

AKT is a recognized mediator of resistance to EGFR inhibition (28, 45, 46). On treatment with GSIs alone, we did not observe significant reductions in the levels of phospho-AKT. However, we did observe significant decreases after combined pathway inhibition, indicating that the Notch pathway does contribute to AKT activity in BLBC. Cell death after combined pathway inhibition was almost completely eliminated by expression of a constitutively active form of AKT, showing that decreased AKT activity was responsible for the loss of viability observed on combined drug treatment. In acute lymphocytic leukemia, Notch controls AKT activity through its ability to suppress PTEN expression (50). However, Notch does not regulate PTEN levels in breast cancer cells (20), and our data agree with this conclusion. MDA-MB-468 cells, which do not express PTEN, still lose AKT activity after combined treatment, implying that Notch maintains a PTEN-PI3K–independent mechanism for activating AKT in BLBC cells. It is interesting to note that we only observed a significant decrease in this activity after combined pathway inhibition. In contrast, it has been reported that treatment with GSIs is sufficient to reduce AKT activation in ER-positive breast cancer cells (20). This discrepancy may be explained by an established feature of Notch signaling and its cell type or context dependency (51). The different effects observed on γ-secretase inhibition between ER-positive and BLBC cells is consistent with this feature, particularly when considering the fact that the various breast cancer subtypes are thought to arise from distinct cellular origins within the mammary gland (52). Nevertheless, these data show a role for Notch signaling in sustaining AKT activity and cell survival after EGFR inhibition, thereby contributing to the EGFR resistance observed in BLBC cells.

Experiments examining the effects of γ-secretase inhibition on cell proliferation or cell survival in basal-like and other breast cancer subtypes have yielded conflicting results. Initially, GSIs were thought to possess cytotoxic and antiproliferative activity in breast cancer (21, 34). However, recently, the contribution of γ-secretase inhibition to the cytotoxic potential of commonly used inhibitors such as GSI I (z-Leu-Leu-Nle-CHO), which can inhibit the proteasome, has been disputed. More specific inhibitors devoid of antiproteasomal activity were shown to lack cellular toxicity, suggesting that some nonspecific inhibitors kill cells through their ability to inhibit the proteasome rather than their ability to block γ-secretase activity (24). We treated BLBC cells with two highly specific GSIs and did not observe cytotoxic activity after treatment. However, we chose to finally resolve this issue by asking whether the intact γ-secretase complex is essential for the proliferation and survival of BLBC cells by using RNAi to ablate an essential component of the enzyme, nicastrin (47). By eliminating the problems caused by chemical differences intrinsic to the inhibitors, we showed that γ-secretase is not essential for the proliferation and survival of these cells. Furthermore, nicastrin knockdown cells, like their γ-secretase–treated counterparts, were sensitized to EGFR inhibition, supporting the conclusion that we have identified a mechanism of resistance to pharmacologic inhibitors of this pathway. Moreover, specific pharmacologic γ-secretase inhibition phenocopies ablation of an essential component of the γ-secretase enzyme. This ability to selectively and simultaneously inhibit the enzyme is crucial for therapeutic targeting of the Notch pathway. Breast cancers frequently express more than one Notch receptor, and the relative contributions of each receptor to cell growth and survival remain to be fully elucidated (34, 53). In the case of BLBC, Notch1 seems to be an important receptor and its activity can be effectively inhibited pharmacologically.

Our data have important preclinical and clinical implications. They suggest an approach for the development of targeted therapeutics based on the need to consider the existence of compensatory pathways and their roles in cell survival after a given pathway stress. Here, we have identified the Notch pathway as one compensatory mechanism leading to resistance to EGFR inhibition in BLBC. Notably, Notch functions as a transcriptional

activator, and therefore, its contribution to resistance to EGFR inhibition should require activation of downstream targets, the identification of which will provide additional insights and potential strategies to overcome resistance. It is also probable that Notch-independent mechanisms of resistance to EGFR inhibition exist. Most importantly, clinical-grade inhibitors of the EGFR and Notch pathways are available, rendering dual-pathway inhibition a viable clinical strategy that can be tested in the near term for BLBCs.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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