PIK3CA and *PIK3CB* Inhibition Produce Synthetic Lethality when Combined with Estrogen Deprivation in Estrogen Receptor–Positive Breast Cancer

Robert J. Crowder,[1] Chanpheng Phommaly,[1] Yu Tao,[2] Jeremy Hoog,[1] Jingqin Luo,[2] Charles M. Perou,[5] Joel S. Parker,[5] Melinda A. Miller,[6] David G. Huntsman,[6] Li Lin,[1] Jacqueline Snider,[1] Sherri R. Davies,[1] John A. Olson, Jr.,[7] Mark A. Watson,[3,4] Anthony Saporita,[1] Jason D. Weber,[1,4] and Matthew J. Ellis[1,4]

Division of Oncology, Departments of [1]Medicine, [2]Biostatistics, and [3]Pathology and Immunology, Washington University School of Medicine; [4]Siteman Comprehensive Cancer Center, St. Louis, Missouri; [5]Lineberger Comprehensive Cancer Center, University of North Carolina, Chapel Hill, North Carolina; [6]Center for Translational and Applied Genomics and British Columbia Cancer Agency, Vancouver, British Columbia, Canada; and [7]Duke Comprehensive Cancer Center, Durham, North Carolina

Abstract

Several phosphoinositide 3-kinase (PI3K) catalytic subunit inhibitors are currently in clinical trial. We therefore sought to examine relationships between pharmacologic inhibition and somatic mutations in PI3K catalytic subunits in estrogen receptor (ER)–positive breast cancer, in which these mutations are particularly common. RNA interference (RNAi) was used to determine the effect of selective inhibition of PI3K catalytic subunits, p110α and p110β, in ER$^+$ breast cancer cells harboring either mutation (*PIK3CA*) or gene amplification (*PIK3CB*). p110α RNAi inhibited growth and promoted apoptosis in all tested ER$^+$ breast cancer cells under estrogen deprived-conditions, whereas p110β RNAi only affected cells harboring *PIK3CB* amplification. Moreover, dual p110α/p110β inhibition potentiated these effects. In addition, treatment with the clinical-grade PI3K catalytic subunit inhibitor BEZ235 also promoted apoptosis in ER$^+$ breast cancer cells. Importantly, estradiol suppressed apoptosis induced by both gene knockdowns and BEZ235 treatment. Our results suggest that PI3K inhibitors should target both p110α and p110β catalytic subunits, whether wild-type or mutant, and be combined with endocrine therapy for maximal efficacy when treating ER$^+$ breast cancer. [Cancer Res 2009;69(9):3955–62]

Introduction

Despite the use of adjuvant endocrine treatment, prognosis remains poor for a significant population of patients with estrogen receptor (ER)–positive breast cancer (1). The cellular basis for the efficacy of endocrine therapy treatment is principally through inhibitory effects on the tumor cell cycle (2) because, unlike cytotoxic chemotherapy, it has never been clearly shown that endocrine therapy promotes cell death through apoptosis (3). A logical approach to improving ER$^+$ breast cancer treatment is, therefore, to inhibit gene activities that promote survival in the presence of ER-targeting agents. To address this hypothesis, we focused on combining endocrine agents with inhibitors of phosphoinositide 3-kinase (PI3K) because this pathway promotes cell survival in several tumor types (4).

Aberrant activation of the PI3K pathway through mutation and epigenetic silencing of genes within the PI3K signaling cascade frequently occurs in breast cancer. Gain-of-function mutations in the PI3Kα catalytic subunit (*PIK3CA*) occur in ~30% of ER$^+$ breast cancer and, much less commonly, activating *AKT1* mutations. Loss-of-function mutations affect the PI3K negative regulator *PTEN* and gene amplification in S6 protein kinase 1 (*RPS6KB1*) and *AKT2* have also been reported (5–8). Whereas the precise consequences of these aberrations on the clinical outcome of ER$^+$ disease remain to be fully defined, *RPS6KB1* amplification and *PTEN* loss are both associated with poor prognosis and *PTEN* loss may correlate with endocrine therapy resistance in ER$^+$ tumors (9–15). In contrast, *PIK3CA* presents a more complex picture and mutations may differentially affect prognosis depending on the affected *PIK3CA* functional domain (15). Finally, a role for *PIK3CB*, the gene encoding the PI3Kβ catalytic subunit, has also recently been postulated in breast cancer, although mutations in this gene have not been detected (16, 17).

Several PI3K catalytic subunit inhibitors are advancing toward phase II clinical testing (18). The targets for these agents are the products of the class 1A PI3K catalytic subunit genes (*PIK3CA, PIK3CB*, and *PIK3CD*). *PIK3CA* and *PIK3CB* are believed to be broadly expressed in breast cancer, whereas *PIK3CD* gene expression is more limited (19). We sought to address several issues related to the clinical development of these compounds. First, it is not clear if *PIK3CA* mutation status restricts the efficacy of PI3K inhibitors. Second, catalytic subunit targeting strategies for achieving maximum therapeutic effect have not been developed. Finally, a rationale for the combination of a PI3K inhibitor and endocrine therapy in ER$^+$ breast cancer has not been established.

Materials and Methods

Human tumor samples. Fresh-frozen and formalin-fixed, paraffin-embedded human breast tumor biopsies for paired array comparative genomic hybridization (aCGH) and *PIK3CB* fluorescence *in situ* hybridization (FISH) were obtained from ER$^+$ breast cancer patients undergoing preoperative letrozole treatment (20). RNA for transcriptional profiling and cDNA synthesis (samples ≥50% tumor) and DNA for aCGH (samples ≥70% tumor cellularity) were prepared from sectioned fresh-frozen samples using RNeasy Mini and QIAamp DNA Micro kits (Qiagen) for RNA and DNA extractions. Tumor enrichment was done using macrodissection or an

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

Requests for reprints: Matthew J. Ellis, Department of Medicine, Washington University School of Medicine, 660 South Euclid Avenue, Box 8069, St. Louis, MO 63110. Phone: 314-747-7502; Fax: 3147479320; E-mail: mellis@dom.wustl.edu.

doi:10.1158/0008-5472.CAN-08-4450

Arcturus Veritas laser capture microdissection instrument (Arcturus Bioscience). A human breast tissue microarray obtained at the Siteman Cancer Center Tissue Core Facility and used for *PIK3CB* FISH was described previously (21).

aCGH. Details are provided in Supplementary Materials and Methods.

Transcriptional profiling. Details are provided in Supplementary Materials and Methods.

Gene resequencing. Details for *PIK3CA* and *PIK3CB* resequencing are provided in Supplementary Materials and Methods.

Cell culture. The HCC712 cell line (22) was provided by Dr. Adi Gazdar. Other cell lines were obtained from the American Type Culture Collection. Cell lines were propagated in RPM1 1640 containing 10% fetal bovine serum with antibiotics and supplements (50 μg/mL gentamicin, pyruvate, 10 mmol/L HEPES, and glucose to 4.5 g/L) in a humidified 37°C incubator containing 5% CO_2. To test the effects of estradiol (Sigma-Aldrich) treatment and withdrawal, cells were maintained in phenol red-free RPM1 1640 containing 5% charcoal-stripped serum (CSS; Invitrogen; CSS medium) for at least 7 days before small interfering RNA (siRNA) transfection or drug treatments.

Protein extracts. Details are provided in Supplementary Materials and Methods.

RNA interference transfection. Nuclease-resistant Stealth duplex siRNAs (Invitrogen) were used for RNA interference (RNAi) experiments. The following siRNAs were used: Universal Low GC Negative Control, *PIK3CA* siRNAs (target sequence 5′-GGUGGUGCGAAAUUCUCACACUAUU-3′ for primary siRNA duplex and 5′-CCCAAGAAUCCUAGUAGAAUGUUUA-3′ for alternative siRNA duplex), and *PIK3CB* siRNAs (target sequence 5′-GCUGUCAAUCAAGUGGAAUAAACUU-3′ for primary siRNA duplex and 5′-GCGCUUGAUGGAUUUACUCUGGAAA-3′ for alternative siRNA duplex). *PIK3CA* and *PIK3CB* siRNA knockdown efficiencies were determined by reverse transfection of siRNAs into cells and immunoblotting cell lysates prepared 3 days after transfection. Maximal knockdown efficiency (>70%) was achieved with 10 nmol/L *PIK3CA* or *PIK3CB* siRNA. Transfection efficiency assessed by the BLOCK-IT fluorescent oligo (Invitrogen) was >90% in all cell lines.

Immunoblotting. Details are provided in Supplementary Materials and Methods.

Cell growth assay. Details are provided in Supplementary Materials and Methods.

Cell death assay. Details are provided in Supplementary Materials and Methods.

***PIK3CB* FISH.** Details are provided in Supplementary Materials and Methods.

Statistical analysis. Unless indicated otherwise, quantitative data are presented as mean ± SE. The effect of siRNA knockdowns and pharmacologic treatments on cell growth and apoptosis was analyzed using ANOVA. If within-group comparisons reached statistical significance ($P < 0.05$), comparisons between specific treatments were made with Student's *t* test. Interactions between PAM50 subtypes or *PIK3CA* mutation status and *PIK3CA, PIK3CB*, and *PIK3CD* expression were analyzed by *t* tests using the SAS version 9.1 Statistical Package (SAS Institute).

Results

p110α and p110β expression in breast cancer cells. The expression of p110α and p110β was examined in breast cancer cell lines by Western blot analysis (Fig. 1*A*). The panel included ER^+ breast cancer cells with activating *PIK3CA* mutations (MCF-7 and T47D) or wild-type *PIK3CA* (HCC712; ref. 11). Both PI3K catalytic subunit isoforms were ubiquitously present; however, p110α and p110β expression varied widely. Notably, p110β protein expression was higher in ER^+ cells, with the HCC712 cell line (22) expressing the most. To determine if increased expression was associated with gene copy gain, high-resolution aCGH was done. This analysis revealed *PIK3CB* copy gain at 3q22.3 in the HCC712 cell line but not in other cell lines (Fig. 1*B*). *PIK3CB* gene copy number in HCC712 cells was confirmed by FISH (gene-to-centromere ratio ~2.5).

***PIK3CB* amplification in primary breast cancer.** aCGH analysis on ER^+ primary breast tumors revealed *PIK3CB* copy gain in at least one tumor examined (1 of 35), which was confirmed by FISH (amplification ratio, 2.3; Fig. 1*C*). In another series of primary breast cancer samples from a breast tissue microarray, low-level *PIK3CB* amplification or copy number gain was observed by FISH in different breast cancer subtypes (1 ER^-, 1 ER^+, and 2 ER^+/$HER\text{-}2^+$), suggesting that *PIK3CB* copy number gain occurs with an incidence of ~5% (Supplementary Table S1). To determine if *PIK3CB* was mutant, the *PIK3CB* helical and kinase domains in 22 primary ER^+ breast tumors were sequenced, including the *PIK3CB* amplified breast tumor illustrated in Fig. 1*C* and three breast cancer cell lines (HCC712, MCF-7, and T47D). No sequence anomalies were detected.

***PIK3CA* and *PIK3CB* are expressed at higher levels in Luminal B breast cancer in comparison with Luminal A breast cancer.** The variation in *PIK3CA* and *PIK3CB* expression observed in breast cancer cell lines led to an expression analysis in a series of ER^+ primary breast cancers. First, microarray studies were examined using the PAM50 model (23) to subtype cases into Luminal A (good prognosis - 31 cases) or Luminal B (poor prognosis - 44 cases; Table 1). There was strong evidence for higher expression of both *PIK3CA* and *PIK3CB* in poor prognosis Luminal B tumors when compared with Luminal A tumors. Higher expression of *PIK3CD* was also observed in Luminal B tumors, but the result was less striking. The presence of a *PIK3CA* mutation was associated with higher levels of *PIK3CA* mRNA but not *PIK3CB* or *PIK3CD*.

p110α is the predominant mediator of PI3K signaling in breast cancer cells, but p110β contributes in a cell line–restricted manner. To determine the individual effects of *PIK3CA* and *PIK3CB* on PI3K signaling, siRNAs were used to selectively knock down p110α and p110β expression (Fig. 2*A*). An analysis of signal transduction showed that *PIK3CB* RNAi had no effect on serum-stimulated Akt phosphorylation in MCF-7, T47D, and MDA-MB-231 cells but partially inhibited Akt phosphorylation in HCC712 cells (Fig. 2*A*). Knockdown of p110β had no clear effect on serum-stimulated phosphorylation of S6 protein in any of the cell lines tested. In contrast, *PIK3CA* RNAi suppressed serum-stimulated Akt phosphorylation in all cell lines tested. S6 phosphorylation was also significantly inhibited in MCF-7 and T47D cells but not in the HCC712 and MDA-MB-231 cell lines. Consistent with previous studies (24), we observed reductions in Akt and S6 protein levels in some experiments, particularly for MCF-7 cells. To test whether the lack of inhibition of serum-stimulated S6 phosphorylation by p110α knockdown in MDA-MB-231 and HCC712 cells was due to compensatory signaling through p110β, dual p110α/p110β knockdowns were done (Fig. 2*B*). Combined p110α/p110β knockdown had no clear effect on S6 phosphorylation in MDA-MB-231 cells. However, the combination partially inhibited S6 phosphorylation in HCC712 cells, indicating that both *PIK3CA* and *PIK3CB* must be inhibited to affect S6 kinase activation in this cell line. Overall, this analysis indicated that p110α is the major catalytic subunit that transduces PI3K pathway signals in ER^+ breast cancer cells, but p110β significantly contributes to pathway activation, particularly in cells containing higher levels of p110β expression.

***PIK3CA* and *PIK3CB* RNAi inhibit ER^+ breast cancer cell growth and survival.** To more precisely determine the cellular

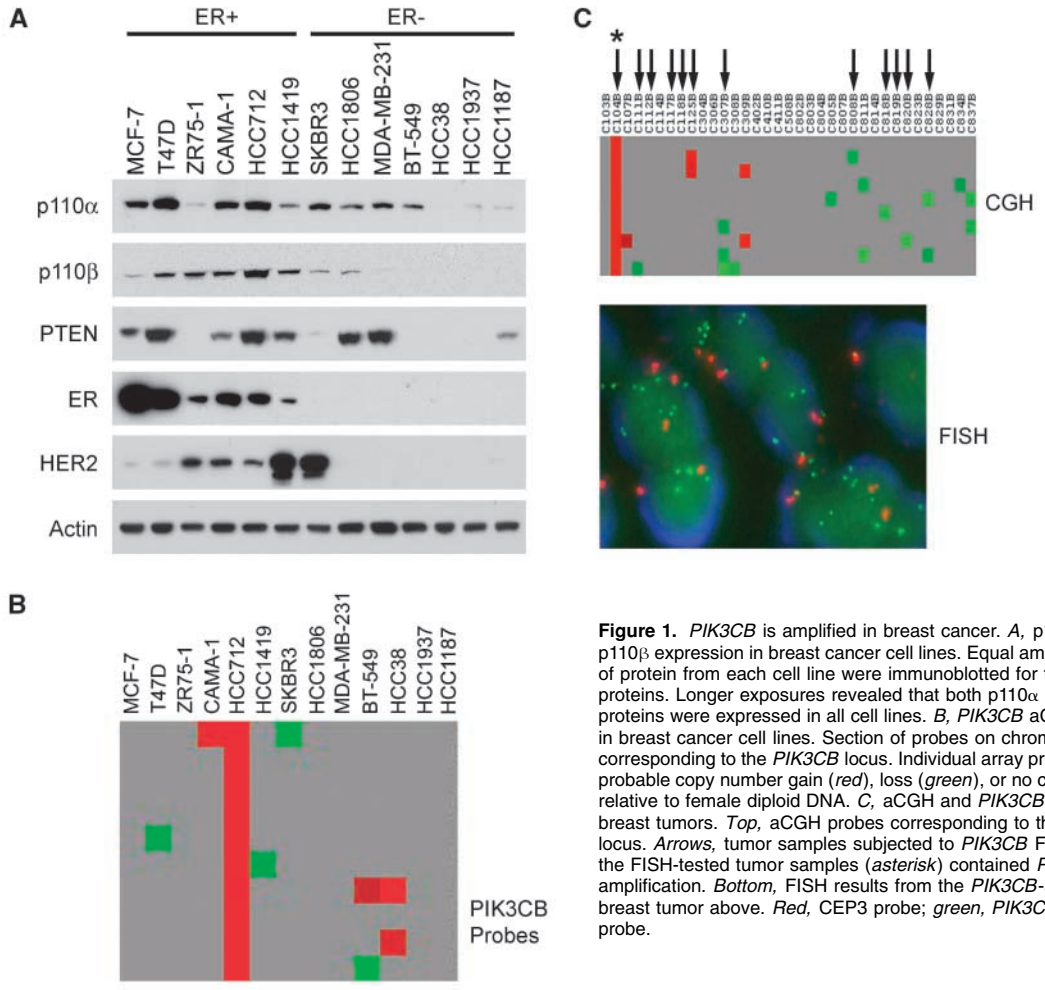

Figure 1. *PIK3CB* is amplified in breast cancer. *A,* p110α and p110β expression in breast cancer cell lines. Equal amounts (25 μg) of protein from each cell line were immunoblotted for the indicated proteins. Longer exposures revealed that both p110α and p110β proteins were expressed in all cell lines. *B, PIK3CB* aCGH analysis in breast cancer cell lines. Section of probes on chromosome 3 corresponding to the *PIK3CB* locus. Individual array probes indicate probable copy number gain (*red*), loss (*green*), or no change (*gray*) relative to female diploid DNA. *C,* aCGH and *PIK3CB* FISH in ER+ breast tumors. *Top,* aCGH probes corresponding to the *PIK3CB* locus. *Arrows,* tumor samples subjected to *PIK3CB* FISH. One of the FISH-tested tumor samples (*asterisk*) contained *PIK3CB* amplification. *Bottom,* FISH results from the *PIK3CB*-amplified breast tumor above. *Red,* CEP3 probe; *green, PIK3CB*-specific probe.

response of ER+ cells, the effects of RNAi-mediated p110α and p110β inhibition were examined under estrogen-dependent growth conditions (Fig. 3*A*). *PIK3CA* RNAi inhibited growth in all cell lines, ranging from a modest reduction in growth in the MDA-MB-231 cell line to a >90% reduction in MCF-7 cells. In contrast, *PIK3CB* RNAi inhibited growth only in HCC712 cells. To determine if *PIK3CA* and *PIK3CB* RNAi promoted cell death, apoptosis was quantified in the presence and absence of estradiol (Fig. 3*B*). Estrogen deprivation alone resulted in no significant increase in cell death in HCC712 and T47D cells but a modest (significant) increase in cell death in MCF-7 cells. However, *PIK3CA* RNAi resulted in significant activation of apoptosis in estrogen-deprived MCF-7, T47D, and HCC712 cells. In particular, p110α knockdown dramatically induced cell death in estrogen-deprived MCF-7 cells, with ~50% of cells dying via apoptosis 7 days after transfection (Fig. 3*B*). Consistent with data on cell growth, *PIK3CB* RNAi promoted apoptosis in HCC712 cells but not in the other cell lines examined. In contrast to the effects on ER+ cells, neither *PIK3CA* nor *PIK3CB* RNAi affected the survival of ER- MDA-MB-231 cells. Importantly, estradiol treatment suppressed the induction of apoptosis by *PIK3CB* RNAi in HCC712 cells and *PIK3CA* RNAi in all three ER+ cell lines, indicating that the combination of estrogen deprivation with specific PI3K inhibition caused synthetic lethality.

Combined *PIK3CA/PIK3CB* RNAi enhances apoptosis in estrogen-deprived ER+ breast cancer cells compared with either single-gene knockdown. Next, we examined the effects of simultaneous inhibition of *PIK3CA* and *PIK3CB* on cell growth and survival using RNAi (Fig. 4*A* and *B*). Dual knockdown of p110α and p110β reduced cell growth by ~90% in estrogen-deprived MCF-7 cells, similar to the inhibition of cell growth caused by p110α knockdown alone (Fig. 4*A*). In contrast, dual p110α/p110β knockdowns produced a greater reduction in cell growth in estrogen-deprived T47D cells (90% inhibition of cell growth) in comparison with the single-subunit knockdowns (65% growth inhibition for *PIK3CA* RNAi and no significant growth inhibition for *PIK3CB* RNAi). Combined RNAi was also effective in inhibiting HCC712 cell growth; however, the growth of ER- MDA-MB-231 cells was unaffected. Whereas dual p110α/p110β knockdown did not enhance cell death in estrogen-deprived MCF-7 cells compared with the marked effect already achieved by p110α knockdown alone, combined 110α/p110β knockdown resulted in ~4-fold higher levels of apoptosis in T47D and HCC712, similar to that achieved in MCF-7 cells with single *PIK3CA* knockdown. In contrast, the survival of MDA-MB-231 cells was unaffected by dual p110α/p110β knockdown (Fig. 4*B*). Importantly, estradiol treatment significantly rescued all three ER+ cell lines from cell death

Table 1. *PIK3CA, PIK3CB*, and *PIK3CD* expression in relation to ER$^+$ breast cancer subtype and *PIK3CA* mutation status

	Expression, mean (SD)	95% Confidence interval for mean difference	*P*
PIK3CA expression			
Luminal B	0.41 (0.42)	0.11-0.54	0.003
Luminal A	0.08 (0.50)		
PIK3CA mutant	0.45 (0.47)	0.04-0.49	0.02
PIK3CA wild-type	0.18 (0.47)		
PIK3CB expression			
Luminal B	−0.31 (0.46)	0.14-0.66	0.005
Luminal A	−0.72 (0.66)		
PIK3CA mutant	−0.41 (0.59)	−0.16 to 0.41	0.37
PIK3CA wild-type	−0.53 (0.58)		
PIK3CD expression			
Luminal B	−0.1 (0.16)	−0.16 to −0.01	0.03
Luminal A	−0.01 (0.16)		
PIK3CA mutant	−0.09 (0.16)	−0.13-0.03	0.25
PIK3CA wild-type	−0.05 (0.17)		

NOTE: Gene expression in primary breast tumors was measured by whole-genome expression arrays and ER$^+$ tumors were subtyped by PAM50 subclassification to Luminal A and Luminal B, and *PIK3CA* mutation status was determined. Mean (SD) was calculated for subtypes and mutation status. The 95% confidence intervals were calculated for the mean difference of Luminal B to Luminal A and *PIK3CA* mutant to wild-type. Two-sample *t* tests were used to determine differences in the expression of *PIK3CA, PIK3CB*, and *PIK3CD* based on Luminal B versus Luminal A subtypes and *PIK3CA* mutant versus *PIK3CA* wild-type tumors.

caused by dual p110α/p110β knockdown. It remained possible that the induction of apoptosis observed with RNAi knockdown was caused by off-target siRNA effects and that rescue of apoptosis with estradiol is not ER-dependent. However, dual p110α/p110β knockdowns in T47D cells with different siRNA than those used in Figs. 3*A* and *B* and 4*A* and *B* also induced apoptosis in estrogen-deprived cells. In addition, treatment with the ER-specific inhibitor fulvestrant abrogated rescue by estradiol, indicating that estradiol rescue was mediated by the ER (Fig. 4C).

BEZ235 induces apoptosis in estrogen-deprived ER$^+$ breast cancer cells. The *PIK3CA* and *PIK3CB* RNAi experiments in the cell line panel provide a defined system for examining the potential of pharmacologic PI3K inhibitors in breast cancer cells. The effects of BEZ235 (a dual PI3K class 1 catalytic subunit/mTOR inhibitor) was therefore investigated. BEZ235 has been shown to potently inhibit wild-type and mutant p110α at low nanomolar concentrations (IC_{50} ~5 nmol/L) and p110β at significantly higher concentrations (IC_{50} ~75 nmol/L; ref. 25). Signaling effects in the cell line panel are consistent with a selective p110α inhibitor (summarized in Fig. 5A). Low concentrations (5 nmol/L) of BEZ235 significantly inhibited the growth of all three ER$^+$ breast cancer cell lines both in the presence and absence of estrogen (Fig. 5B). In contrast, only high concentrations (50 nmol/L) of BEZ235 inhibited MDA-MB-231 cell growth. When the effect of BEZ235 on cell survival in the presence and absence of estrogen was examined, marked differences between the three ER$^+$ cell lines emerged (Fig. 5C). Treatment of estrogen-deprived MCF-7 and T47D cells with concentrations of BEZ235 as low as 5 nmol/L promoted cell death. However, effect of BEZ235 on survival was maximal using 5 nmol/L BEZ235 in T47D cells. In contrast, the level of apoptosis in MCF-7 cells increased with BEZ235 concentration and approached the levels observed with *PIK3CA* RNAi at 50 nmol/L. In estradiol rescue experiments, induction of cell death by 5 nmol/L BEZ235 in MCF-7 cells was completely blocked by estradiol, and in the presence of estradiol, 4-fold higher doses ≥20 nmol/L were required to induce cell death. Remarkably, estradiol abrogated the BEZ235-induced cell death in T47D cells at all doses tested. HCC712 cells were the least sensitive to BEZ235 treatment and required higher concentrations (≥20 nmol/L) to promote cell death under estrogen-deprived conditions. However, estrogen did suppress BEZ235-induced apoptosis in HCC712 cells such that cell death only occurred in estradiol treated cells at the highest concentration tested (50 nmol/L). Consistent with the *PIK3CA* and *PIK3CB* single and combination knockdown results, BEZ235 treatment did not induce apoptosis in MDA-MB-231 cells.

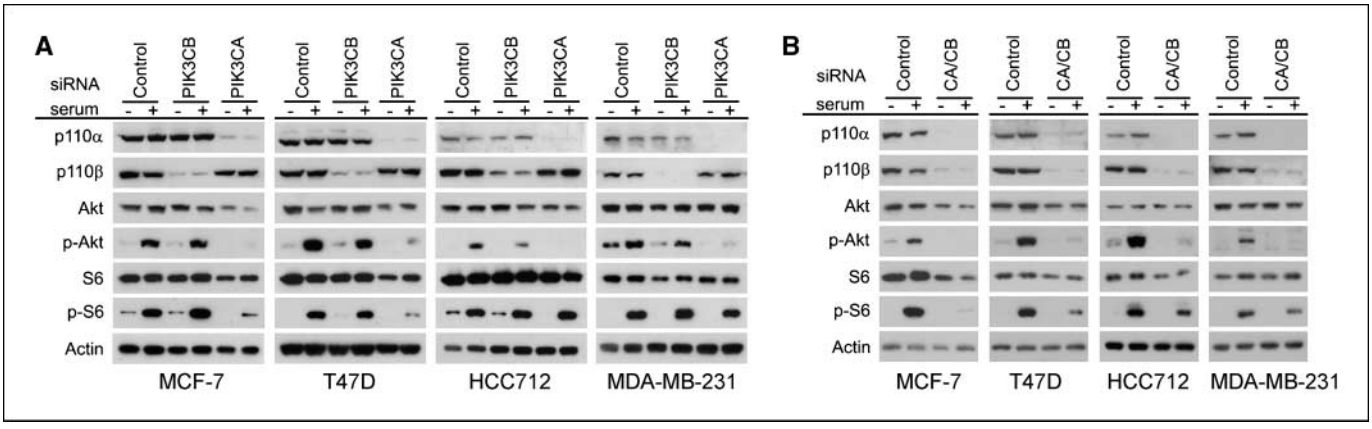

Figure 2. p110α is the predominant mediator of PI3K signaling in breast cancer cells. *A,* effect of p110α and p110β knockdown on PI3K signaling. Cells were transfected with control siRNAs (*Control*) or siRNAs against *PIK3CB* (*PIK3CB*) or *PIK3CA* (*PIK3CA*). Three days after transfection, serum-deprived cells were stimulated with 20% fetal bovine serum (final concentration) and lysates were analyzed for effects on PI3K pathway signaling through phospho-Akt (*p-Akt*) and phospho-S6 (*p-S6*) immunoblotting. Representative immunoblots obtained from at least three experiments per cell line. *B,* effect of p110α/p110β dual knockdown on PI3K signaling. Cells were transfected with control siRNAs or a mixture of *PIK3CA* and *PIK3CB* siRNAs (*CA/CB*), treated as above, and subjected to immunoblot analysis. Representative results obtained in at least two experiments per cell line.

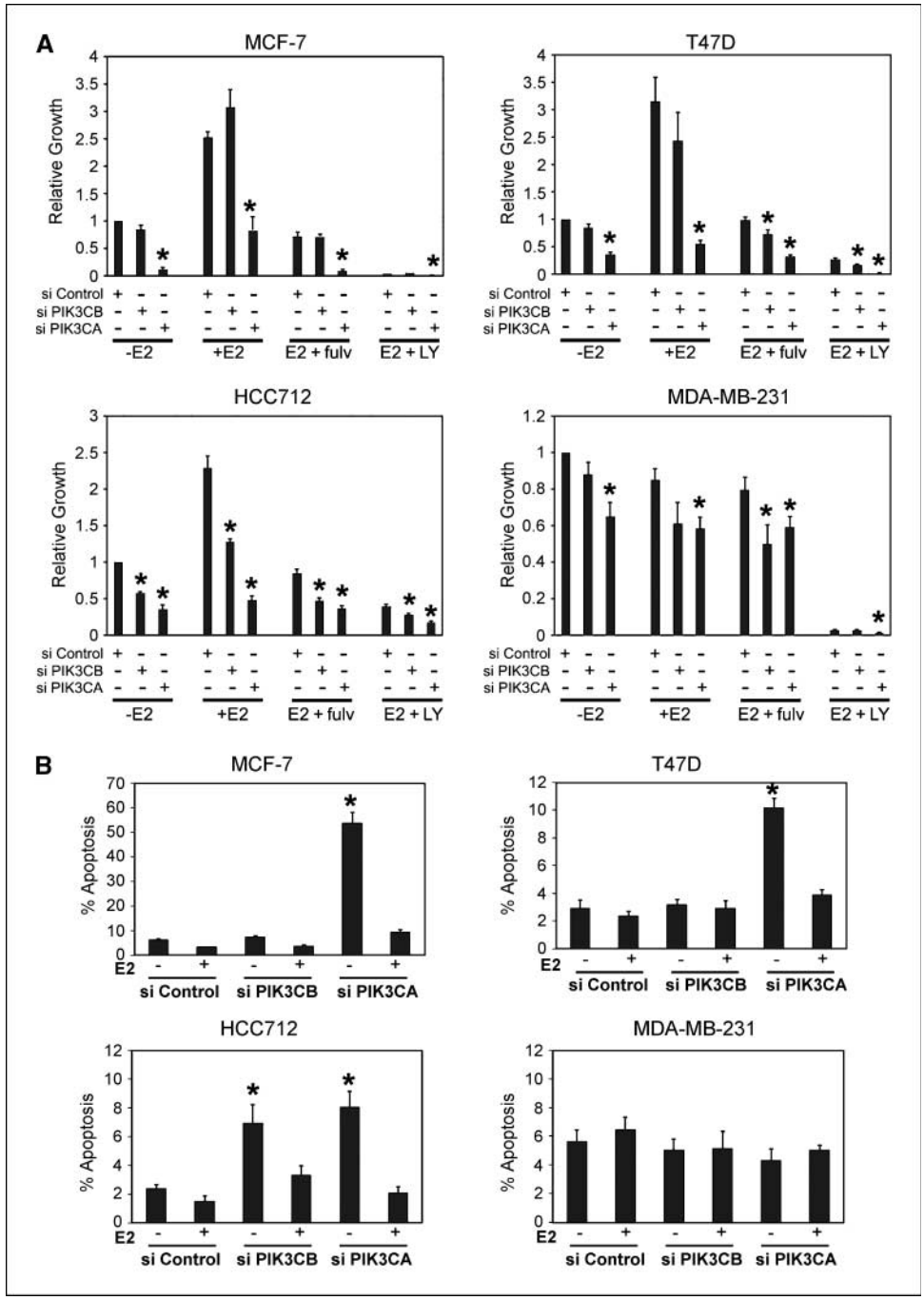

Figure 3. *PIK3CA* and *PIK3CB* RNAi cause synthetic lethality in estrogen-deprived ER+ breast cancer cells. *A, PIK3CA* and *PIK3CB* RNAi inhibit growth of ER+ breast cancer cells. Cells in CSS medium were transfected with 10 nmol/L control (*si Control*), *PIK3CB* (*si PIK3CB*), or *PIK3CA* (*si PIK3CA*) siRNAs. Cells were treated without (*-E2*) or with 10 nmol/L estradiol (*+E2*) in the absence or presence of 300 nmol/L fulvestrant (*fulv*) or 20 μmol/L LY294002 (*LY*). Growth was assessed after 10 days of treatment and is expressed relative to untreated (*-E2*) control siRNA-transfected cells. Results from five experiments per cell line. *, $P < 0.05$, significant differences between treatments in *PIK3CB* or *PIK3CA* siRNA-transfected cells and identical treatments in control siRNA-transfected cells. *B, PIK3CA* and *PIK3CB* RNAi promote apoptosis in estrogen-deprived ER+ cells. Cells growing in CSS medium were transfected with siRNAs as in *A* and treated without or with 10 nmol/L estradiol for 7 days. Apoptosis was assessed by counting TUNEL-positive or pyknotic Hoechst-stained nuclei. Results from four experiments per cell line. *, $P < 0.05$, significant differences between estrogen-deprived control siRNA and estrogen-deprived *PIK3CB* or *PIK3CA* siRNA-transfected cells. Estrogen suppression of *PIK3CB* RNAi-induced apoptosis in HCC712 cells was not statistically significant ($P = 0.06$).

Discussion

The role of estrogen in the proliferation of ER+ breast tumors is well established. However, the role of estrogen as a survival factor is less clear. Preclinical studies with the MCF-7 cell line showed that treatment with antiestrogens or estrogen deprivation increases apoptosis, as we can confirm (26–28). However, MCF-7 cells are unusual in this regard because we did not observe estrogen deprivation-induced apoptosis in the HCC712 or T47D cell lines, and in the neoadjuvant endocrine therapy setting, treatment did not increase apoptosis (3, 29). Our data indicate that signaling through the PI3K pathway may explain these observations because estradiol promotes survival only when PI3K is inhibited. The presence of two apparently independent cell survival mechanisms, one PI3K-dependent and one estradiol-dependent, creates an opportunity for synthetic lethality. At the current time, it is not known whether estrogen promotes survival through ER-dependent transcription or by rapid, nongenomic activation of signal transduction pathways (30, 31). Nevertheless, our data strongly suggest that the effectiveness of PI3K catalytic subunit inhibitors in treating ER+ breast cancer will be greatest when combined with endocrine therapy.

In vitro studies have shown that activated forms of p110α or p110β transform mammary epithelium (32, 33). Knock-in and knockout transgenic mouse studies confirmed these findings and p110β appears to be particularly important for *ERBB2*-driven

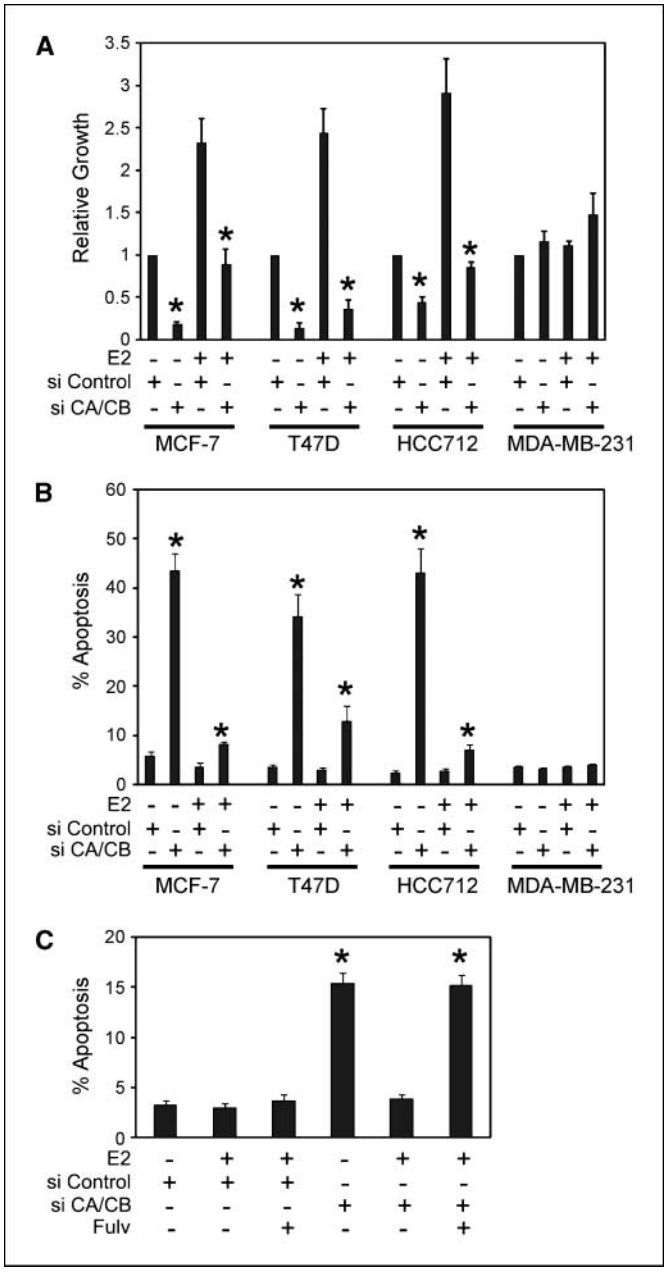

Figure 4. Dual *PIK3CA/PIK3CB* RNAi enhances apoptosis in estrogen-deprived ER$^+$ cells. *A,* dual *PIK3CA/PIK3CB* RNAi inhibits growth of ER$^+$ cells. Cells in CSS medium were transfected with 20 nmol/L control (*si Control*) or 10 nmol/L each (20 nmol/L final) of *PIK3CA* and *PIK3CB* siRNAs (*si CA/CB*). Cells were left untreated or treated with 10 nmol/L estradiol and growth was assessed after 10 days. Growth is expressed relative to untreated control siRNA-transfected cells. Results from four to six experiments per cell line. *, $P < 0.05$, significant differences between control siRNA and *PIK3CA/PIK3CB* siRNA-transfected cells in the presence or absence of estradiol. *B,* dual p110α/p110β knockdown enhances apoptosis in ER$^+$ cells. Cells were transfected with 20 nmol/L control or 10 nmol/L each of *PIK3CA* and *PIK3CB* siRNAs. Cells were left untreated or treated with 10 nmol/L estradiol for 7 days and apoptosis was assessed by counting Hoechst-stained nuclei. Results from four experiments per cell line. *, $P < 0.05$, significant differences between control siRNA and dual *PIK3CA/PIK3CA* siRNA-transfected cells in either the presence or the absence of estradiol. Estradiol significantly suppressed *PIK3CA/PIK3CB* RNAi-induced apoptosis in MCF-7, T47D, and HCC712 cells. *C,* dual p110α/p110β knockdown was done in T47D cells with alternative *PIK3CA* and *PIK3CB* siRNAs. Cells were left untreated, treated with 10 nmol/L estradiol, or treated with estradiol + 300 nmol/L fulvestrant (*Fulv*) for 7 days. Apoptosis was assessed by counting Hoechst-stained nuclei. Results from four experiments. *, $P < 0.05$, significant differences between control and *PIK3CA/PIK3CA* siRNA-transfected cells in either the presence or the absence of estradiol.

breast cancer (16, 34) and in the promotion of proliferation, survival, and invasiveness in a variety of cancer types (35–37). The data presented in this study are the first to show *PIK3CB* amplification in primary breast cancer and suggest that this amplification event may promote oncogenesis. Our initial screen indicates that *PIK3CB* copy number gain occurs at a low frequency (~5%) in tumors of breast cancer patients; however, this may be clinically significant, because breast cancer is common (38). We also find that *PIK3CB* is preferentially expressed in Luminal B breast cancer regardless of gene copy number, indicating that this isoform is a potentially important therapeutic target, perhaps as a conduit for the effects of other somatic mutations that activate the PI3K pathway such as *PTEN* loss (17). Interestingly, our *in vitro* data indicate that *PIK3CB* supports cell survival in HCC712 cells under estrogen-deprived conditions, implying that targeted p110β inhibition could be effective in treating *PIK3CB*-amplified breast tumors. However, our data also suggest that both *PIK3CA* and *PIK3CB* may have to be inhibited under these circumstances because high-level apoptosis only occurred when both catalytic subunits were targeted. Because *PIK3CA* is wild-type in HCC712 cells, we also conclude that *PIK3CA* gain-of-function mutations are not a prerequisite for the synthetic lethal effect when combining estrogen deprivation and PI3K inhibition. Additionally, inhibition of p110β also appears relevant in the presence of a *PIK3CA* mutation. T47D cells express modest levels of wild-type p110β as well as a mutant *PIK3CA*, raising the question of whether *PIK3CB* provides an escape from mutant p110α inhibition. A comparison between single and combined knockdowns suggests that this is the case, because both isoforms must be inhibited for maximal synthetic lethality. MCF-7 cells appear unusual in their extreme sensitivity to p110α inhibition alone; however, this may possibly reflect the low levels of *PIK3CB* expression in this cell line.

BEZ235 is an example of a new generation of PI3K inhibitors to enter clinical investigation in breast cancer (25). A comparison between the effects of BEZ235 and RNAi against *PIK3CA* and *PIK3CB* supports the conclusion that BEZ235 functions as a selective p110α inhibitor at low nanomolar concentrations (25). However, the apoptotic effect remained very modest in T47D cells, even at higher doses, consistent with lack of *PIK3CB* inhibition, which, based on the RNAi experiments, is necessary for the full synthetic lethal effect. Estradiol suppresses BEZ235-induced apoptosis in the three ER$^+$ cell lines, but estrogen rescue was not as dramatic as that observed in the *PIK3CA* and *PIK3CB* RNAi experiments. The reduced sensitivity to estradiol rescue likely reflects inhibition of other kinases by BEZ235.

We conclude that there is a strong rationale for the combination of endocrine therapy and PI3K inhibitors. In terms of the population of patients suitable for a clinical trial of a PI3K inhibitor combined with endocrine treatment, the data suggest that eligibility should not be restricted by *PIK3CA* mutation status. Furthermore, the association between *PIK3CA* and *PIK3CB* expression and Luminal B status indicates that the addition of a PI3K inhibitor may be particularly important in more aggressive forms of ER$^+$ disease. Finally, *PIK3CB* is emerging as an important therapeutic target, whose inhibition is important not only to increase the efficacy of *PIK3CA* inhibition but as a target in its own right, perhaps particularly in the setting of gene amplification. A combined inhibitor with low nanomolar inhibitory properties for both *PIK3CA* and *PIK3CB* may be necessary for maximal clinical efficacy in combination with an endocrine agent in the treatment of ER$^+$ breast cancer. However, this clinical

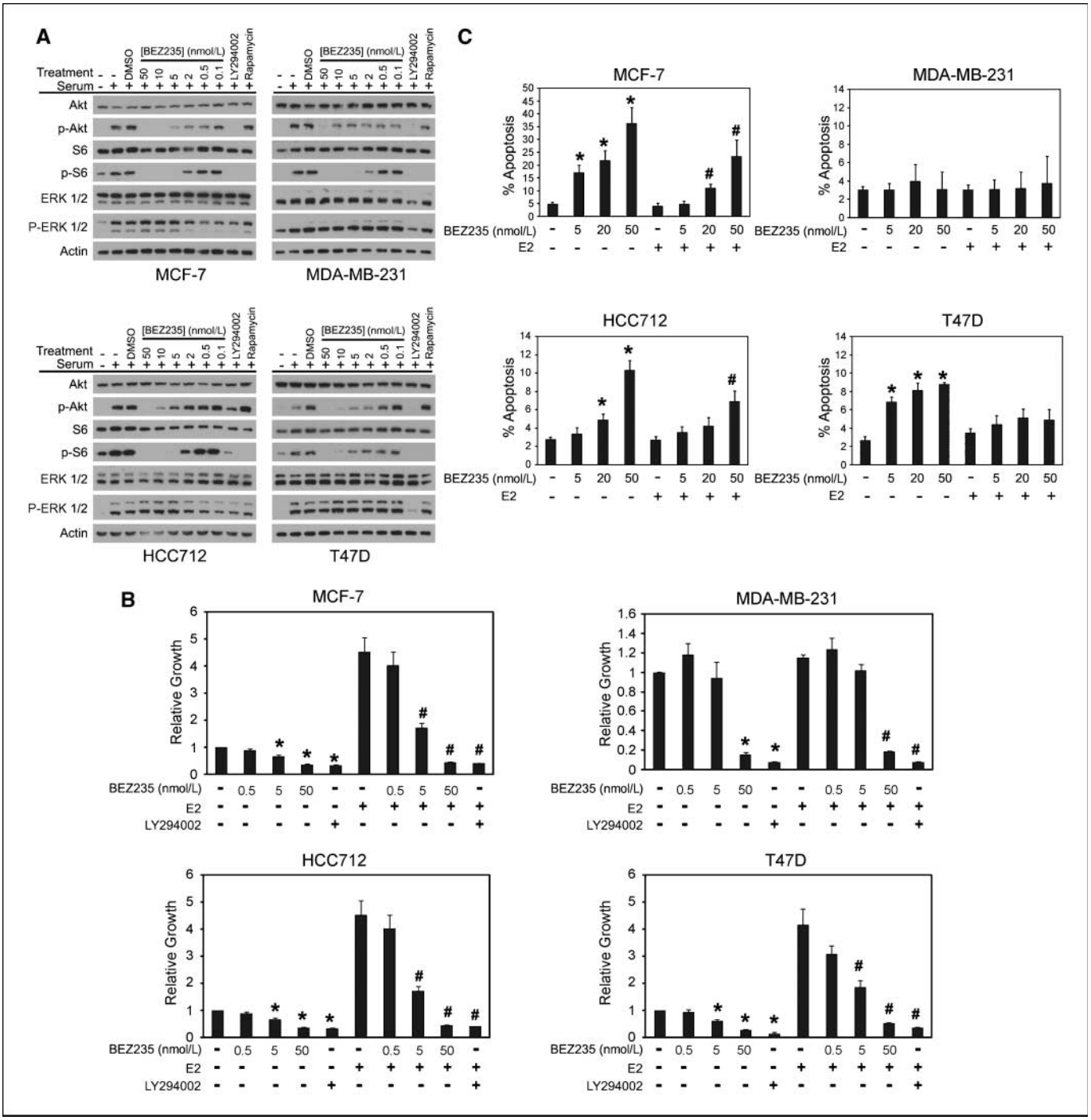

Figure 5. BEZ235 causes synthetic lethality in estrogen-deprived ER+ breast cancer cells. *A,* BEZ235 treatment inhibits PI3K pathway signaling in breast cancer cells. Serum-starved cells were treated with vehicle (DMSO) and the indicated concentrations of BEZ235, LY294002 (20 μmol/L), or rapamycin (100 nmol/L) and then stimulated with 20% fetal bovine serum. Cell lysates were immunoblotted to determine effects on PI3K signaling by phospho-Akt and phospho-S6 antibodies and on mitogen-activated protein kinase signaling via phospho-ERK (*p-ERK 1/2*) antibodies. Representative results from at least two experiments per cell line. *B,* BEZ235 inhibits growth of breast cancer cells. Cells in CSS medium were treated without or with BEZ235 in the absence or presence of 10 nmol/L estradiol. Cell growth was measured after 10 days and is calculated relative to growth in untreated cells. Results from five experiments per cell line. *, $P < 0.05$, significant differences in growth between estrogen-deprived and estrogen-deprived drug-treated cells; #, $P < 0.05$, significant differences in growth between estrogen-stimulated and estrogen-stimulated, drug-treated cells. *C,* BEZ235 promotes apoptosis in ER+ cells. Cells growing in CSS medium were treated with the indicated concentrations of BEZ235 without or with 10 nmol/L estradiol for 7 days. Apoptosis was assessed by counting Hoechst-stained nuclei. Results from four to six experiments per cell line. Significant induction of cell death in estrogen-deprived BEZ235-treated cells (*, $P < 0.05$) and cells treated with BEZ235 in the presence of estradiol (#, $P < 0.05$).

treatment strategy may be problematic because systemic inhibition of both catalytic subunits will cause derangements in insulin signaling and glucose homeostasis (16, 17, 39, 40), and metabolic toxicity could be further enhanced by estrogen deprivation. Nonetheless, it may be possible to pursue this strategy clinically because endocrine therapy in combination with a PI3K inhibitor is cytotoxic. Therefore, short-course high-toxicity combinations of PI3K inhibitors with endocrine therapy,

analogous to conventional chemotherapy, rather than prolonged exposure, may be sufficient to increase the cure rate for ER^+ breast cancer.

Disclosure of Potential Conflicts of Interest

M.J. Ellis: commercial research grants, Novartis, Astra Zenica, and Taiho; honoraria, Novartis and Astra Zenica; consultant, Novartis, Astra Zenica, and Pfizer. The other authors disclosed no potential conflicts of interest.

Acknowledgments

Received 11/21/08; revised 2/23/09; accepted 3/2/09; published OnlineFirst 4/14/09.

Grant support: NIH grants R01 CA095614 and U01 CA114722, St. Louis Affiliate of the Susan G. Komen Breast Cancer Foundation, Breast Cancer Research Foundation, and Barnes-Jewish Hospital Research Foundation (M.J. Ellis) and award 3P50 CA68438-07S2 (J.A. Olson). The Siteman Comprehensive Cancer Center is supported in part by National Cancer Institute Cancer Center support grant P30 CA91842.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

References

1. EBCTCG. Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomised trials. Lancet 2005;365:1687–717.
2. Ellis MJ, Tao Y, Luo J, et al. Outcome prediction for estrogen receptor-positive breast cancer based on postneoadjuvant endocrine therapy tumor characteristics. J Natl Cancer Inst 2008;100:1380–8.
3. Dowsett M, Smith IE, Ebbs SR, et al. Proliferation and apoptosis as markers of benefit in neoadjuvant endocrine therapy of breast cancer. Clin Cancer Res 2006;12:1024–30s.
4. Hennessy BT, Smith DL, Ram PT, Lu Y, Mills GB. Exploiting the PI3K/AKT pathway for cancer drug discovery. Nat Rev Drug Discov 2005;4:988–1004.
5. Li J, Yen C, Liaw D, et al. PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. Science 1997;275:1943–7.
6. Barlund M, Monni O, Kononen J, et al. Multiple genes at 17q23 undergo amplification and overexpression in breast cancer. Cancer Res 2000;60:5340–4.
7. Samuels Y, Wang Z, Bardelli A, et al. High frequency of mutations of the PIK3CA gene in human cancers. Science 2004;304:554.
8. Carpten JD, Faber AL, Horn C, et al. A transforming mutation in the pleckstrin homology domain of AKT1 in cancer. Nature 2007;448:439–44.
9. Shoman N, Klassen S, McFadden A, Bickis MG, Torlakovic E, Chibbar R. Reduced PTEN expression predicts relapse in patients with breast carcinoma treated by tamoxifen. Mod Pathol 2005;18:250–9.
10. van der Hage JA, van den Broek LJ, Legrand C, et al. Overexpression of p70 S6 kinase protein is associated with increased risk of locoregional recurrence in node-negative premenopausal early breast cancer patients. Br J Cancer 2004;90:1543–50.
11. Saal LH, Holm K, Maurer M, et al. PIK3CA mutations correlate with hormone receptors, node metastasis, and ERBB2, and are mutually exclusive with PTEN loss in human breast carcinoma. Cancer Res 2005;65:2554–9.
12. Li SY, Rong M, Grieu F, Iacopetta B. PIK3CA mutations in breast cancer are associated with poor outcome. Breast Cancer Res Treat 2006;96:91–5.
13. Maruyama N, Miyoshi Y, Taguchi T, Tamaki Y, Monden M, Noguchi S. Clinicopathologic analysis of breast cancers with PIK3CA mutations in Japanese women. Clin Cancer Res 2007;13:408–14.
14. Perez-Tenorio G, Alkhori L, Olsson B, et al. PIK3CA mutations and PTEN loss correlate with similar prognostic factors and are not mutually exclusive in breast cancer. Clin Cancer Res 2007;13:3577–84.
15. Barbareschi M, Buttitta F, Felicioni L, et al. Different prognostic roles of mutations in the helical and kinase domains of the PIK3CA gene in breast carcinomas. Clin Cancer Res 2007;13:6064–9.
16. Ciraolo E, Iezzi M, Marone R, et al. Phosphoinositide 3-kinase p110β activity: key role in metabolism and mammary gland cancer but not development. Sci Signal 2008;1:ra3.
17. Jia S, Liu Z, Zhang S, et al. Essential roles of PI(3)K-p110β in cell growth, metabolism and tumorigenesis. Nature 2008;454:776–9.
18. Marone R, Cmiljanovic V, Giese B, Wymann MP. Targeting phosphoinositide 3-kinase: moving towards therapy. Biochim Biophys Acta 2008;1784:159–85.
19. Sawyer C, Sturge J, Bennett DC, et al. Regulation of breast cancer cell chemotaxis by the phosphoinositide 3-kinase p110δ. Cancer Res 2003;63:1667–75.
20. Olson JA, Budd GTLA, Carey LA, et al. Improved surgical outcomes for breast cancer patients receiving neoadjuvant aromatase inhibitor therapy: results from a multicenter phase II trial. J Am Coll Surg. In press 2009.
21. Brown LA, Hoog J, Chin SF, et al. ESR1 gene amplification in breast cancer: a common phenomenon? Nat Genet 2008;40:806–7; author reply 10–2.
22. Gazdar AF, Kurvari V, Virmani A, et al. Characterization of paired tumor and non-tumor cell lines established from patients with breast cancer. Int J Cancer 1998;78:766–74.
23. Parker JS, Mullins M, Cheang MC, et al. Supervised risk predictor of breast cancer based on intrinsic subtypes. J Clin Oncol 2009;27:1160–7.
24. Reagan-Shaw S, Ahmad N. RNA interference-mediated depletion of phosphoinositide 3-kinase activates forkhead box class O transcription factors and induces cell cycle arrest and apoptosis in breast carcinoma cells. Cancer Res 2006;66:1062–9.
25. Maira SM, Stauffer F, Brueggen J, et al. Identification and characterization of NVP-BEZ235, a new orally available dual phosphatidylinositol 3-kinase/mammalian target of rapamycin inhibitor with potent *in vivo* antitumor activity. Mol Cancer Ther 2008;7:1851–63.
26. Kyprianou N, English HF, Davidson NE, Isaacs JT. Programmed cell death during regression of the MCF-7 human breast cancer following estrogen ablation. Cancer Res 1991;51:162–6.
27. Warri AM, Huovinen RL, Laine AM, Martikainen PM, Harkonen PL. Apoptosis in toremifene-induced growth inhibition of human breast cancer cells *in vivo* and *in vitro*. J Natl Cancer Inst 1993;85:1412–8.
28. Detre S, Salter J, Barnes DM, et al. Time-related effects of estrogen withdrawal on proliferation- and cell death-related events in MCF-7 xenografts. Int J Cancer 1999;81:309–13.
29. Dowsett M, Smith IE, Ebbs SR, et al. Short-term changes in Ki-67 during neoadjuvant treatment of primary breast cancer with anastrozole or tamoxifen alone or combined correlate with recurrence-free survival. Clin Cancer Res 2005;11:951–8s.
30. Migliaccio A, Di Domenico M, Castoria G, et al. Tyrosine kinase/p21ras/MAP-kinase pathway activation by estradiol-receptor complex in MCF-7 cells. EMBO J 1996;15:1292–300.
31. Simoncini T, Hafezi-Moghadam A, Brazil DP, Ley K, Chin WW, Liao JK. Interaction of oestrogen receptor with the regulatory subunit of phosphatidylinositol-3-OH kinase. Nature 2000;407:538–41.
32. Zhao JJ, Liu Z, Wang L, Shin E, Loda MF, Roberts TM. The oncogenic properties of mutant p110α and p110β phosphatidylinositol 3-kinases in human mammary epithelial cells. Proc Natl Acad Sci U S A 2005;102:18443–8.
33. Isakoff SJ, Engelman JA, Irie HY, et al. Breast cancer-associated PIK3CA mutations are oncogenic in mammary epithelial cells. Cancer Res 2005;65:10992–1000.
34. Zhao JJ, Cheng H, Jia S, et al. The p110α isoform of PI3K is essential for proper growth factor signaling and oncogenic transformation. Proc Natl Acad Sci U S A 2006;103:16296–300.
35. Czauderna F, Fechtner M, Aygun H, et al. Functional studies of the PI(3)-kinase signalling pathway employing synthetic and expressed siRNA. Nucleic Acids Res 2003;31:670–82.
36. Pu P, Kang C, Zhang Z, Liu X, Jiang H. Down-regulation of PIK3CB by siRNA suppresses malignant glioma cell growth *in vitro* and *in vivo*. Technol Cancer Res Treat 2006;5:271–80.
37. An HJ, Cho NH, Yang HS, et al. Targeted RNA interference of phosphatidylinositol 3-kinase p110β induces apoptosis and proliferation arrest in endometrial carcinoma cells. J Pathol 2007;212:161–9.
38. Kim MS, Jeong EG, Yoo NJ, Lee SH. Mutational analysis of oncogenic AKT E17K mutation in common solid cancers and acute leukaemias. Br J Cancer 2008;98:1533–5.
39. Knight ZA, Gonzalez B, Feldman ME, et al. A pharmacological map of the PI3-K family defines a role for p110α in insulin signaling. Cell 2006;125:733–47.
40. Foukas LC, Claret M, Pearce W, et al. Critical role for the p110α phosphoinositide-3-OH kinase in growth and metabolic regulation. Nature 2006;441:366–70.