Targeting the *AIB1* Oncogene through Mammalian Target of Rapamycin Inhibition in the Mammary Gland

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Abstract

Amplified in breast cancer 1 (AIB1), an estrogen receptor (ER) coactivator, is frequently amplified or overexpressed in human breast cancer. We previously developed a transgenic mouse model in which AIB1 can act as an oncogene, giving rise to a premalignant hyperplastic mammary phenotype as well as to a high incidence of mammary tumors that are primarily ER$^+$. In this model, the AIB1 transgene is responsible for continued activation of the insulin-like growth factor-I receptor, suggesting a role for the activation of the phosphatidylinositol 3-kinase/Akt/mammalian target of rapamycin (mTOR) pathway in the premalignant phenotype and tumor development. Here we show that treatment of AIB1 transgenic mice with the mTOR inhibitor RAD001 reverts the premalignant phenotype. Furthermore, treatment of cell lines derived from AIB1-dependent mammary tumors with RAD001 in culture leads to a G_1 cell cycle arrest. Lastly, tumor growth after injection of ER$^+$ AIB1 tumor cell lines into wild-type animals is inhibited by RAD001 treatment. In this ER$^+$ model, inhibition of tumor growth by RAD001 was significantly better than inhibition by the antiestrogen 4-hydroxytamoxifen alone, whereas a combination of both RAD001 and 4-hydroxytamoxifen was most effective. Based on these results, we propose that the combination of mTOR inhibition and ER-targeted endocrine therapy may improve the outcome of the subset of ER$^+$ breast cancers overexpressing AIB1. These studies provide preclinical support for the clinical development of RAD001 and suggest that AIB1 may be a predictive factor of RAD001 response. (Cancer Res 2006; 66(23): 11381-8)

Introduction

Amplified in Breast Cancer 1 (AIB1) is a member of the p160 family of estrogen receptor (ER) transcriptional coactivators and is amplified and overexpressed in a subset of human breast and ovarian cancers. Previously, we showed that overexpression of AIB1 in transgenic mice under the control of the mouse mammary tumor virus (MMTV) promoter leads to mammary hypertrophy, hyperplasia, and abnormal involution postweaning. Furthermore, AIB1 transgenic mice showed a high incidence of multiple malignant tumors of both epithelial and mesenchymal origin. Tumors occurred most frequently in the mammary gland but also in other organs, most notably the pituitary and uterus. Thus, AIB1 is able to act as an oncogene. In addition, we found that AIB1 overexpression activates the insulin-like growth factor (IGF)/phosphatidylinositol 3-kinase (PI3K)/mammalian target of rapamycin (mTOR) pathway in primary mammary epithelial cells and mammary tumor cells derived from AIB1 transgenic mice (1).

The PI3K/Akt pathway regulates many biological processes, including proliferation, apoptosis, and growth. PI3K is frequently found activated or deregulated in oncogenesis and PI3K activity has been linked to viral oncogenes such as v-*src*, v-*abl*, v-*ros*, and v-*ras* (2–6). In addition, the PI3K pathway is found to be activated in many common human malignancies, including breast and prostate cancer, malignant gliomas, endometrial carcinoma, melanoma, and renal cell carcinoma (7, 8). In these malignancies, multiple mechanisms exist for the activation of PI3K (9, 10). PI3K participates in mTOR activation by growth factors such as hormones and insulin (11). mTOR, itself a kinase, is a key regulator of protein synthesis after mitogen or hormone stimulation. Its substrates include p70S6 kinase, a serine threonine kinase that is activated in response to a broad range of mitogenic stimuli, and eukaryotic initiation factor 4E binding protein, which is inactivated after phosphorylation by mTOR. More generally, mTOR has been found to act as sensor of energy, nutrient, and mitogen levels and as a gatekeeper for cell cycle progression (2, 12, 13). The unraveling of mTOR functions was greatly facilitated by the drug rapamycin, the first defined inhibitor of mTOR. Rapamycin is a macrolide antibiotic with antifungal and immunosuppressive properties. It is currently approved as an immunosuppressant drug for organ transplantation (14). Rapamycin binds to a highly conserved cytoplasmic receptor, FK506-binding protein-12. This FK506-binding protein-12-rapamycin complex binds to mTOR and prevents phosphorylation of downstream targets (15). Studies of rapamycin-treated yeast and mammalian cells have shown that loss of TOR function leads to arrest in early G_1 phase (16). Rapamycin has shown remarkable antitumor activity against a wide range of cancers in both *in vitro* and *in vivo* models. Apparently, mTOR inhibitors are more effective as antitumor agents in highly proliferative cancer cells of neuroectodermic origin such as neuroblastoma, medulloblastoma, and in those with high expression of IGF such as alveolar rhabdomyosarcoma (8). Furthermore, derivatives with better pharmacologic properties, including everolimus (RAD001), have been developed and are currently under evaluation in phase I and II clinical trials (8, 17). Given its importance in many types of cancer, we wanted to determine whether activation of mTOR was required for the observed premalignant and malignant phenotypes of the AIB1 transgenic mammary tumor model.

Targeting of ER$^+$ breast cancer with antiestrogens such as tamoxifen or depriving ER of estrogen through the use of

aromatase inhibitors has led to significant improvements in breast cancer prevention and therapy. However, in the advanced disease setting, these therapies are rarely curative and a significant number of women continue to die from ER$^+$ breast cancer. We therefore sought to determine whether mTOR inhibition with RAD001, either alone or in combination with tamoxifen, could inhibit the growth of ER$^+$ AIB1-dependent tumors.

Materials and Methods

Reagents. Everolimus (RAD001) was a gift from Dr. H. Lane (Novartis, Basel, Switzerland). 4-Hydroxytamoxifen (OHT) was purchased from Sigma.

Establishment of tumor cell lines. Tumor cells were isolated as described in ref. 18. Briefly, tumors were excised under sterile conditions and placed in sterile PBS to clean the blood and nontumor tissue. Tumors were placed on clean dishes, finely chopped, and incubated with collagenase for 2 to 3 hours in a 15-mL tube. Digested tumors were centrifuged at 900 rpm for 5 minutes. After resuspension of the pellet in fresh medium, tubes were left for 5 to 10 minutes to allow larger pieces to settle and the supernatant was transferred to a plate containing Ham-12 medium as described below. Several tumor cell lines were obtained in this way. Their ER status was determined by Western blot and immunohisto-chemistry and correlated with the ER status of the respective tumor a particular cell line was derived from. Cell lines were also evaluated for ER function by determining their proliferative response to estrogen. For the retroviral transduction, retroviral vector (MSCV-Puro-Luciferase) was stably infected into 293T cells using the pCL-Eco helper plasmid. Retroviral supernatants isolated 36 and 60 hours after transduction were used to infect AIB1 tumor cells. At 24 hours postinfection, the cells were selected for 2 days in growth medium containing 2 µg/mL puromycin. Cells were passaged no more than twice before injection into the mammary fat pad of FVB mice.

Cell culture. AIB1 tumor cell lines were cultured in Ham-12 media (Life Technologies, Inc.), supplemented with 5% fetal bovine serum (FBS), 100 units/mL penicillin, 100 µg/mL streptomycin, 5 µg/mL insulin, and 0.1 µg/L hydrocortisone. In all experiments, cells were maintained in 100-mm culture dishes at 37°C in a humidified 5% CO_2 atmosphere.

Dose response. Dose-response curves were determined according to the National Cancer Institute NIH guidelines.[4] In brief, cell suspensions were plated into 96-well microtiter plates at a density of 3,000 per well (100 µL) in Ham-12 media. Inoculates were allowed a preincubation period of 24 hours at 37°C for stabilization. Cells were treated in six replicates for 48 hours with RAD001, OHT, or a combination of RAD001 and OHT. Compounds were evaluated at 10-fold serial dilutions, ranging between 10^{-10} and 10^{-5} mol/L. Cell proliferation was measured by a colorimetric WST-1 assay (Roche). A plate reader was used to read the optical densities at 450 nm. After subtraction of the baseline (absorbance of the cells before treatment), the absorbance was plotted against the dose of RAD001.

Growth assay. Cells were plated in 24-well plates at a density of 5,000 per well in either HAM-12 medium without phenol red and 2% charcoal/dextran–treated serum added or Ham-12 complete medium supplemented with 5% FBS. After 24 hours, cells were treated either with RAD001 (25 nmol/L), OHT (10 µmol/L), or a combination of both. Cells were collected at 0, 24, 48, and 72 hours and counted with a hemocytometer.

Flow cytometry. Cells were fixed in 70% ethanol for 20 minutes on ice, washed with PBS, and stained with 100 µL of 500 ng/mL propidium iodide (Sigma). One-tenth volume of 1 mg/mL RNase was added to the cell suspension and samples were incubated for 1 hour at 37°C. DNA content was determined in a flow cytometer and the percent of cells in the respective cell cycle phases (G_1, S, or G_2) determined with the Modfit LT software. Averages of percent cells were determined from four independent experiments and represented as bar graph.

Immunoblot analysis. Monoclonal anti-AIB1 antibody (BD Biosciences) was used for Western blots and immunostaining. All antibodies for the PI3K pathway were purchased from Cell Signaling Technology [all antibodies were polyclonal with exception of S6 ribosomal protein (RPS6) rabbit monoclonal antibody 5G10]. Monoclonal ERα antibody was purchased from NeoMarkers. Polyclonal anti-calnexin antibody (Stressgen), anti-tubulin (Abcam), or anti-β-actin (Sigma) was used as control for protein loading. Whole mammary glands, tumors, or cells were lysed in ice-cold radioimmunoprecipitation assay buffer containing protease inhibitor cocktail (Roche). Lysates were centrifuged at 14,000 rpm for 15 minutes. Forty micrograms of protein were separated on 4% to 15% SDS-PAGE gradient gels (Bio-Rad) and transferred to nitrocellulose membranes. Blots were probed with the antibodies indicated and developed with enhanced chemiluminescence Western blotting detection reagent (Amersham Biosciences). Signal intensities were determined by densitometry and normalized using anti-calnexin, anti-tubulin, or anti-β-actin antibodies.

Mouse strains. Animal experiments were compliant with the guidelines of Dana-Farber Cancer Institute. The AIB1 transgenic mouse line (FVB-MMTV-AIB1) has previously been described (1). FVB mice were purchased from Charles River Laboratories.

RAD001 treatment of AIB1 transgenic mice *in vivo*. RAD001, 10 mg/kg/d, was administered as a microemulsion diluted in distilled, deionized water by oral gavage.

5-Bromodeoxyuridine incorporation after RAD001 treatment. Sixteen hours before sacrifice, wild-type and AIB1 transgenic mice were injected i.p. with a solution of 3 mg/mL 5-bromodeoxyuridine (BrdUrd; Sigma) in PBS. Ten microliters of BrdUrd solution were used per gram of body weight. Cross sections were stained with an anti-BrdUrd antibody (BD Biosciences) and counterstained with hematoxylin. The number of BrdUrd-positive cells was counted in 10 fields per slide under ×20 magnification. Final results represent the average of five mice per group of treatments.

Tumor cell injection. Ten-week-old female FVB mice were anesthetized by an i.p. injection of ketamine/xylazine (300 mg ketamine combined with 20 mg of xylazine in a 4-mL volume; 0.02 mL of the solution was used per 20 g body weight). Tumor cells in culture were harvested, resuspended in PBS, and 10^3 cells were injected into the fat pad of the 4th inguinal gland of each mouse. Seven days after injection, mice were divided into four groups and treated as follows: (*i*) placebo, (*ii*) 20 mg/kg/d of RAD001 (administered orally as described above), (*iii*) 100 µg of OHT (administered daily i.p), and (*iv*) 20 mg/kg/d of RAD001 plus 100 µg/d of OHT. Tumor growth was monitored twice a week by bioluminescence imaging using a cryogenically cooled charge-coupled device camera system (IVIS 100, Xenogen, Alameda, CA). Mice were anesthetized with ketamine (150 mg/kg i.p.) and xylazine (12 mg/kg i.p.), and injected with D-luciferin (50 mg/kg i.p.; Promega, Madison, WI). Surface images were acquired under dim polychromatic illumination, followed by serial acquisition of bioluminescent images without external illumination. Images were analyzed using the Living Images software package (Xenogen). Tumor volume was also measured *in vivo* every 4 days with calipers and calculated by the following formula: tumor volume = (length × width^2) / 2. After 14 days of treatment, mice were sacrificed and dissected for further analysis.

Statistical analysis. Statistical analyses were done using unpaired two-tailed Student's *t* tests and were carried out using Prism (version 4; GraphPad) or Microsoft Excel software. Statistical significance was considered to be present at levels >95% ($P < 0.05$). Each experiment was done at least thrice independently.

Results

AIB1-dependent premalignant phenotype regresses with mTOR inhibition. Mice overexpressing a human AIB1 transgene in the mammary gland show a uniform premalignant mammary gland phenotype consisting of hyperplasia, hypertrophy, and disorganized architecture. In these mice, the mammary epithelium shows increased proliferation and increased activation of the PI3K/Akt/mTOR pathway (1). We sought to determine whether the

activation of this pathway contributes to the observed hyperplasia by blocking the pathway with the mTOR inhibitor RAD001, an orally active rapamycin derivative.

Twelve-month-old transgenic mice expressing human AIB1 and syngeneic wild-type mice (FVB) were treated with placebo or RAD001. At 10 mg/kg/d, RAD001 was well tolerated with no change in body weight. The rate of cellular proliferation in the mammary gland was determined by injection of BrdUrd into live mice, followed by mammary gland dissection and staining with an anti-BrdUrd antibody. The percentage of BrdUrd-positive cells was determined as the average count over five high-power fields (Fig. 1*A-D*). Before RAD001 treatment, the percentage of BrdUrd-positive cells was ~30% in the AIB1 transgenic mammary glands, which was 10-fold greater than control wild-type mice. Following 7 days of RAD001 treatment, the number of BrdUrd-positive cells decreased to ~6% in AIB1 mice and was essentially unchanged in control mice. After 14 days of RAD001 treatment, the number of BrdUrd-positive cells in AIB1 mice returned to the basal level found in wild-type mice. Furthermore, the overall architecture of

the mammary returned to normal following RAD001 treatment (Fig. 1*C*). These data suggest that the AIB1-dependent premalignant hyperplastic phenotype requires the continuous activation of mTOR and that mTOR inhibition can revert it.

To validate the downstream signaling consequences of mTOR inhibition by RAD001 at the molecular level, protein extracts from whole mammary glands were probed with antisera recognizing phosphorylated RPS6 (pRPS6; $Ser^{235/236}$) and phosphorylated eIF4G (peIF4G; Ser^{1108}), both known substrates of mTOR (11). Phosphorylation of RPS6 was reduced ~5-fold and peIF4G was completely abolished after 14 days of RAD001 treatment (Fig. 1*E*). In contrast, RAD001 had no effect on the levels of phosphorylated GSK3 (pGSK3; $Ser^{9/21}$), a control substrate that is not a target of mTOR (Fig. 1*E*), confirming that RAD001 acted selectively to inhibit mTOR activity.

RAD001 inhibits proliferation of AIB1-dependent tumor cell lines. To facilitate *in vitro* studies, cell lines were derived from several mammary tumors arising from the AIB1 transgenic mouse model. The ERα status of these tumors and derived cell lines was

Figure 1. RAD001 treatment decreases cell proliferation in AIB1 transgenic mice. Twelve-month-old AIB1 transgenic mice were treated orally with placebo (*A*) or RAD001 (10 mg/kg) for 7 days (*B*) or 14 days (*C*). Subsequently, mice were injected with BrdUrd and sacrificed after 16 hours. Cross sections of mammary glands were prepared and stained with H&E (*left*) or with an anti-BrdUrd antibody (*right*). *D,* both wild-type and transgenic AIB1 mice were treated as above and the numbers of total cells and BrdUrd-positive cells were counted in five fields per slide. Results represent the average of five different mice per treatment. *E,* Western blot analysis of mammary gland extracts from AIB1 transgenic mice after 7 and 14 days of placebo or RAD001 treatment using antibodies for the indicated proteins. Anti-calnexin antibody was used as loading control.
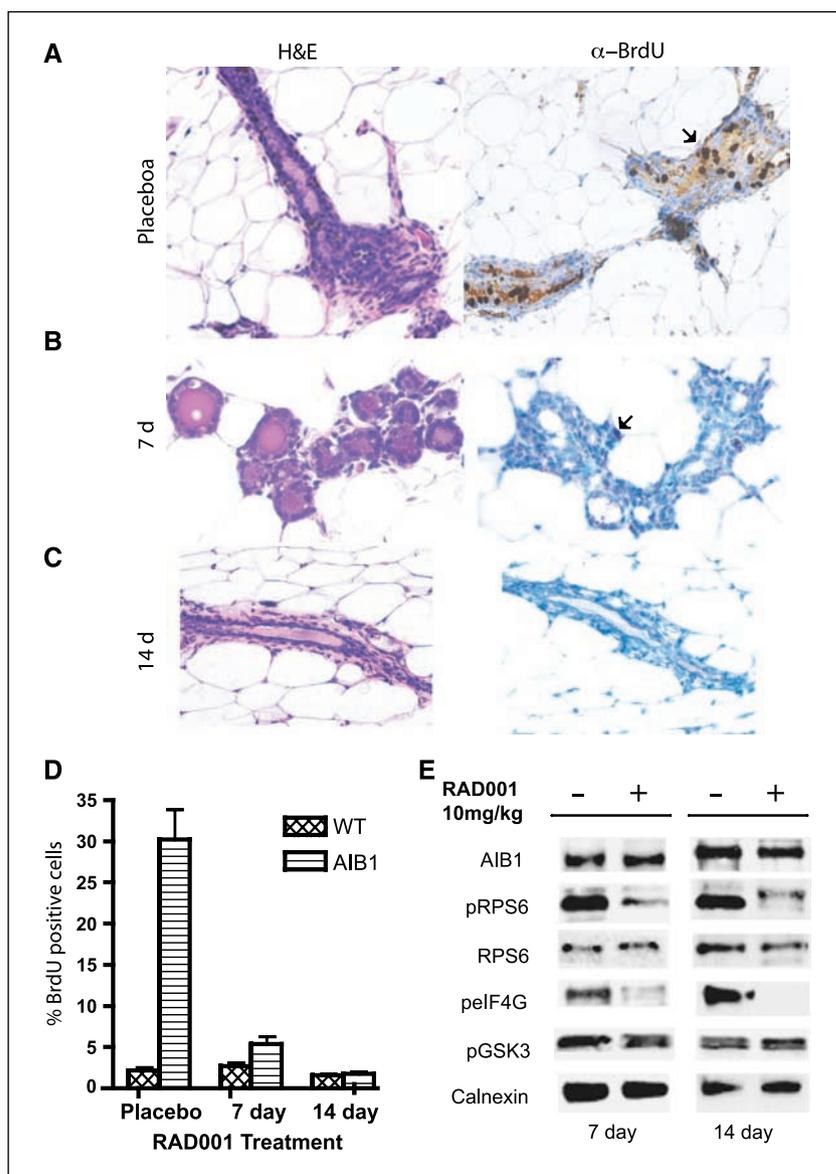
determined by ERα immunostaining and Western blot of whole cell extracts. Examples of ER⁺ and ER⁻ cell lines obtained are shown in Fig. 2A. The primary tumors as well as the cell lines both displayed evidence of activation of the PI3K/Akt/mTOR pathway, as compared with normal mammary tissue and primary epithelial cell lines derived from it (ref. 1 and data not shown). We therefore wanted to test whether mTOR activation was required for the proliferation of these tumor cell lines.

First, we established a dose-response curve for RAD001 and determined the IC_{50} to be between 50 and 100 nmol/L, indicating that RAD001 indeed did inhibit proliferation of both ER⁻ and ER⁺ cell lines (Fig. 2C). Next, we followed the growth of the tumor cell lines grown either in the presence of FBS or charcoal/dextran–treated serum and treated with 25 nmol/L RAD001 or vehicle alone. Examples of these results are shown in Fig. 2D. Of note, the ER⁻ cell line in this example required higher levels of FBS or charcoal/dextran–treated serum for proliferation and survival than the ER⁺ line. All cell lines tested required untreated FBS for effective growth and proliferation, which was significantly inhibited by RAD001. Charcoal/dextran–treated serum treatment was even more effective in suppressing proliferation than RAD001 and cells grown in charcoal/dextran–treated serum and treated with RAD001 only showed a small additional inhibitory effect in these samples, presumably because charcoal/dextran–treated serum treatment already blocked the mTOR pathway targeted by RAD001 as well as other pathways maintaining proliferation.

To determine how rapidly RAD001 was able to inhibit its molecular targets, we analyzed protein extracts obtained after 1, 4, 16, or 24 hours of treatment with RAD001 (25 nmol/L) or vehicle. Reduction of phosphorylation of RPS6 and eIF4G was observed as early as 1 hour in the ER⁻ cell line and as early as 4 hours in the ER⁺ cell line. By 24 hours, phosphorylation of these targets was essentially abolished in both cell lines (Fig. 2E).

AIB1-dependent tumor cell lines arrest in G_1 following mTOR inhibition. To determine how proliferation of AIB1-dependent tumor cell lines is inhibited by RAD001 and whether RAD001 leads to apoptosis, we carried out cell cycle analysis at the different time points following RAD001 treatment. ER⁺ AIB1-dependent tumor cells were fixed, stained with propidium iodide, and the DNA content was analyzed by flow cytometry. RAD001 led to a significant decrease of the G_2 and S peaks and an increase in the G_1 peak, indicating a G_1 arrest (Fig. 2F). We did not observe any evidence of significant apoptosis as no sub-G_1 peak was observed at any time point (data not shown). A very similar G_1 arrest was also observed in several other ER⁺ as well as ER⁻ AIB1-dependent mammary tumor cell lines tested (data not shown).

RAD001 inhibits the growth of AIB1-dependent mammary tumors. To analyze the effect of RAD001 on tumor growth *in vivo*, we modified the AIB1 tumor model to bypass the long latency of tumor development. We first determined that injection of AIB1 tumor cell lines into syngeneic wild-type mice resulted in mammary tumor formation within 1 week. We then stably infected AIB1 tumor cell lines with a retroviral expression vector (MSCV-Puro-Luciferase) to allow bioluminescent *in vivo* imaging. One of these cell lines that was ER⁺ was injected into the mammary fat pad of syngeneic mice. Mice were imaged 4 and 7 days after implantation of tumor cells. Animals with established orthotopic tumors (i.e., bioluminescence increasing between days 4 and 7) were divided into four groups (i.e., Fig. 3A, treatment day 0) and treated with vehicle or RAD001 at 20 mg/kg daily. Treatments were

continued for 14 days, and tumor burden was assessed by bioluminescence imaging (Fig. 3A) and confirmed by terminal quantification of volume and mass of resected tumors. The average tumor mass of RAD001-treated mice was 12.7-fold smaller ($P = 0.0023$) as compared with the tumor mass of the placebo-treated mice (Fig. 3B and C). Very similar results were found using tumor volume (data not shown).

After 14 days of RAD001 treatment, tumors were dissected and prepared for routine histology. H&E staining of tumor sections showed that tumors of RAD001-treated mice displayed few cells and islands or nodules of cells with no structure, representing tumor necrosis. In addition, areas with dark apoptotic debris were observed (Fig. 3D, *top*). In contrast, tumors from placebo-treated mice were more cellular and revealed cohesive sheets of tumor cells and well-illustrated cytologic features of malignancy, such as increased nuclear/cytoplasmic ratio, nuclear irregularity, hyperchromatism, pleomorphism, and high number of mitotic figures (Fig. 3D, *bottom*).

Increased growth inhibition after treatment of AIB1-dependent mammary tumors with RAD001 in combination with OHT. Drugs targeting ER, such as OHT, play an important role in the treatment of women with ER⁺ breast cancers. Therefore, we wanted to test the effects of combined targeting of ER and mTOR in ER⁺, AIB1-dependent tumors. We treated mice with RAD001 alone (described above), with OHT (100 μg/d) alone, or with a combination of RAD001 plus OHT. The OHT-treated tumors were 2.6-fold smaller ($P = 0.0239$) than tumors in placebo-treated mice whereas tumors treated with RAD001 plus OHT were 21.1-fold smaller ($P = 0.0021$) than placebo-treated mice and also significantly smaller than with either treatment alone. In addition, RAD001-treated tumors were 4.9-fold smaller ($P = 0.0117$) than tumors treated with OHT, indicating that RAD001 treatment is more effective than OHT treatment and that both treatments together provide additive benefits in this model (Fig. 3A-C).

As with the *in vitro* treatments described above, the specificity of RAD001 for its molecular targets was confirmed by Western blot. Protein extracts from tumors were probed with antisera recognizing pRPS6 and peIF4G. RAD001 treatment reduced phosphorylation of pRPS6 and peIF4G whereas it had no effect on the levels of pGSK3 (Fig. 3E). Interestingly, in our model we did not see any evidence for increased phosphorylation of Akt at Ser[473] (pAkt; Fig. 3E), an effect previously attributed to mTOR inhibition resulting from the inhibition of a negative feedback loop (17).

RAD001 counteracts the endometrial hyperplasia caused by tamoxifen in the uterus. An unwanted side effect of the treatment of women with breast cancer with tamoxifen has been its estrogenic activity in the uterus leading to endometrial hyperplasia and, rarely, endometrial cancer (19). Therefore, we examined the effects of OHT and RAD001 treatment on the uterus. As predicted from previous studies, uterine size and mass was increased by OHT treatment (Fig. 4A). In contrast, RAD001 treatment led to a significant decrease in uterine size. Interestingly, in mice treated with the combination of both RAD001 and OHT, uterine size and mass was significantly reduced compared with OHT alone, suggesting that RAD001 is able to counteract the estrogenic effects of OHT in the uterus (Fig. 4A). This was confirmed by H&E staining, which showed that the histology of the uteri of OHT plus RAD001–treated animals was indistinguishable from that of placebo-treated animals, whereas OHT resulted in uterine hyperplasia (Fig. 4B).
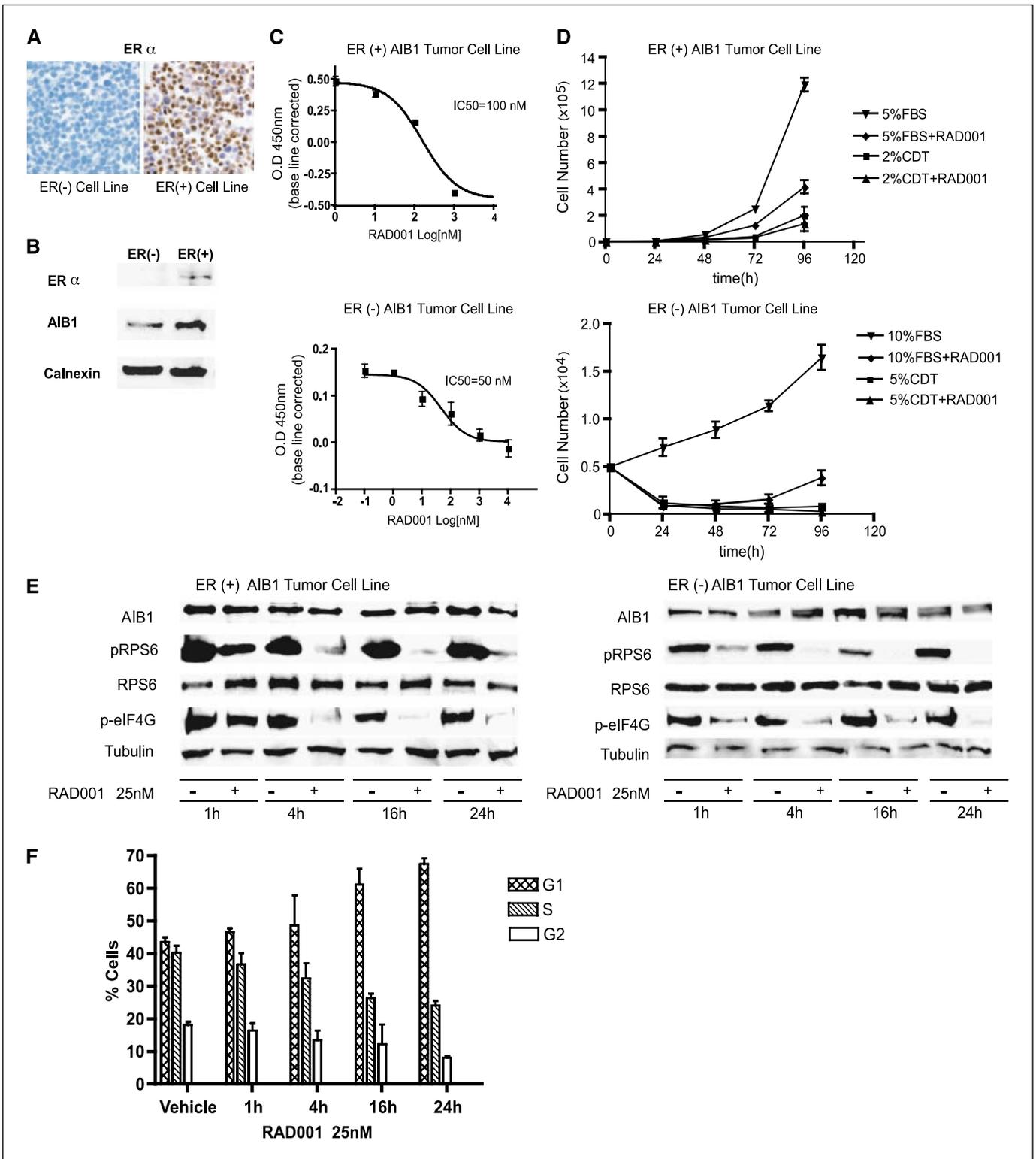
A

ER α

ER(-) Cell Line   ER(+) Cell Line

B

ER(-)   ER(+)

ER α

AIB1

Calnexin

C

ER (+) AIB1 Tumor Cell Line

IC50=100 nM

O.D 450nm (base line corrected)

RAD001 Log[nM]

ER (-) AIB1 Tumor Cell Line

IC50=50 nM

O.D 450nm (base line corrected)

RAD001 Log[nM]

D

ER (+) AIB1 Tumor Cell Line

Cell Number (x10⁵)

- ▼ 5%FBS
- ◆ 5%FBS+RAD001
- ■ 2%CDT
- ▲ 2%CDT+RAD001

time(h)

ER (-) AIB1 Tumor Cell Line

Cell Number (x10⁴)

- ▼ 10%FBS
- ◆ 10%FBS+RAD001
- ■ 5%CDT
- ▲ 5%CDT+RAD001

time(h)

E

ER (+) AIB1 Tumor Cell Line

AIB1
pRPS6
RPS6
p-eIF4G
Tubulin

RAD001 25nM   - +   - +   - +   - +
1h   4h   16h   24h

ER (-) AIB1 Tumor Cell Line

AIB1
pRPS6
RPS6
p-eIF4G
Tubulin

RAD001 25nM   - +   - +   - +   - +
1h   4h   16h   24h

F

% Cells

- G1
- S
- G2

Vehicle   1h   4h   16h   24h
RAD001 25nM

Figure 2. Response of AIB1 tumor cell lines to RAD001 treatment. ERα status in AIB1 tumor cell lines was determined by ERα immunostaining (*A*) and Western blot (*B*) of whole-cell extracts of ER⁺ and ER⁻ cell lines. *C*, dose-response curve of RAD001 treatment. An ER⁺ (*top*) and an ER⁻ (*bottom*) AIB1 tumor cell line were treated with the indicated doses of RAD001 for 48 hours. Cell numbers were analyzed by WST assay, and the absorbance at 450 nm was plotted against the RAD001 dose after subtraction of the absorbance at time 0 (before treatment). *D*, time course of cell growth after RAD001 treatment. Tumor cell lines were grown either in phenol red–free Ham-12 medium supplemented with 2% charcoal/dextran–treated serum or regular medium supplemented with 5% FBS. RAD001 at 25 nmol/L or vehicle was added. At the times indicated, cells were harvested and counted manually. Growth curves were plotted. Both ER⁺ (*top*) and ER⁻ (*bottom*) tumor cell lines were analyzed. *E*, Western blot analysis. Whole-cell extracts from ER⁺ (*left*) and ER⁻ (*right*) AIB1 tumor cell lines were prepared after 1, 4, 16, or 24 hours of RAD001 treatment. Forty micrograms of protein extracts were separated on a 4% to 15% SDS-PAGE gradient gel, transferred to a nitrocellulose membrane, and probed with antibodies for the indicated proteins. *F*, cell cycle analysis. An ER⁺ AIB1 tumor cell line was treated with RAD001 for the times indicated and cells were stained with propidium iodide and analyzed by flow cytometry. Cell numbers in the respective cell cycle phases were determined from the histogram using Modfit software.
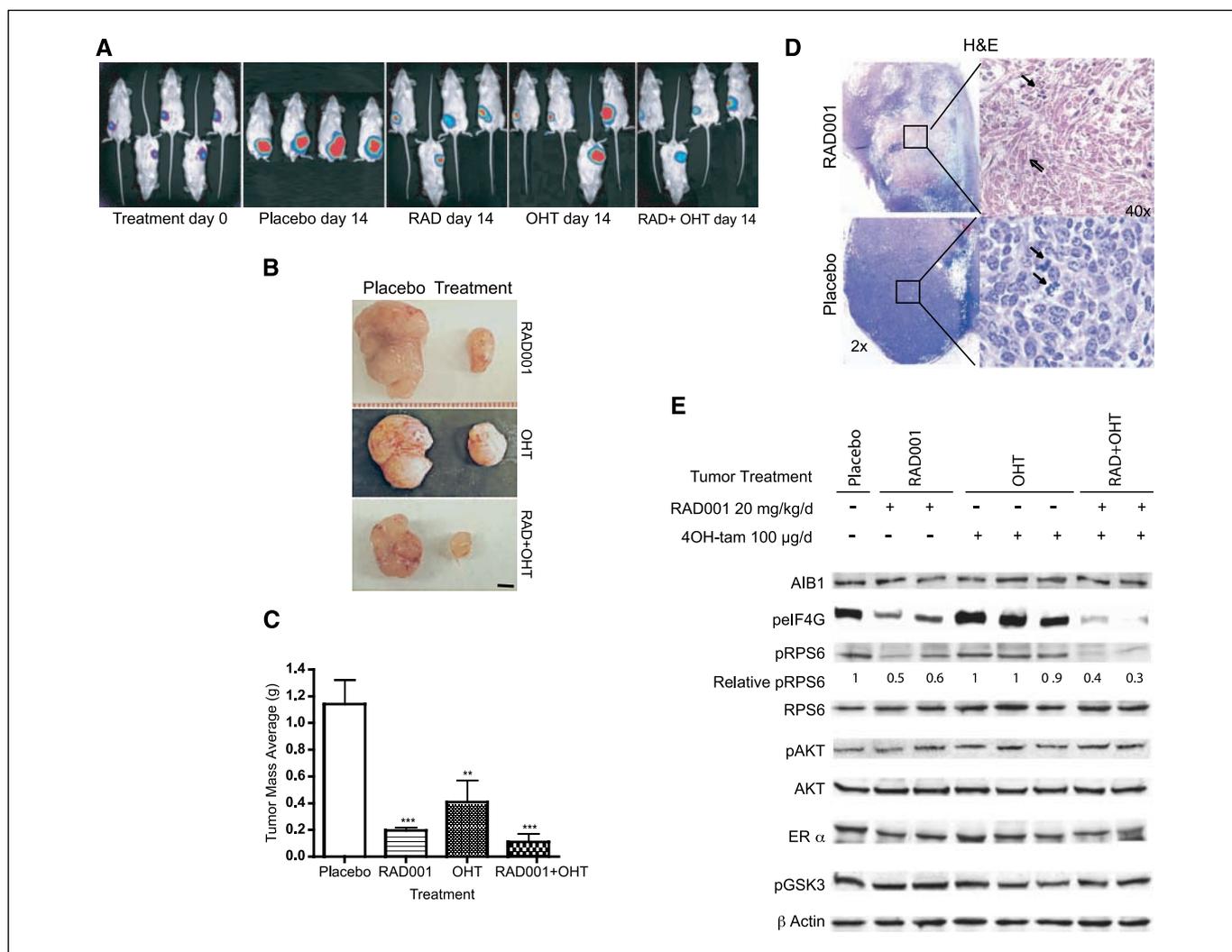
Figure 3. *A,* bioluminescence *in vivo* imaging of tumors after treatment. Four mice for each group were injected with an ER⁺ AIB1 tumor cell line expressing an exogenous *luciferase* gene. Seven days after tumor cell injection, treatment started with placebo, RAD001, OHT, or a combination of RAD001 and OHT. *Far left,* tumors after 7 days of injection before treatments started. Surface images were acquired under dim polychromatic illumination, followed by serial acquisition of bioluminescent images without external illumination. *B,* photographs of tumor specimens obtained after dissection of mice treated as indicated. Bar, 4 mm. *C,* the mass and volume of each tumor specimen was determined. The graph summarizes the tumor mass average of four mice per treatment. *D,* histologic analysis of H&E-stained tumor sections from mice treated with RAD001 (*top*) or placebo (*bottom*). The RAD001-treated tumors show nodules of necrotic areas (*open arrow*) and apoptotic debris (*solid arrow*). In contrast, placebo-treated tumors contain a high proportion of cells displaying mitotic figures (*solid arrow*) and hyperchromatic and pleomorphic nuclei. *E,* Western blot analysis of tumor samples after different treatments. Forty micrograms of protein extracts from tumors were separated on a 4% to 15% SDS-PAGE gradient gel, transferred to a nitrocellulose membrane, and probed with the antibodies indicated.

Discussion

Current efforts in anticancer drug development are based on inhibiting the activity of key targets required for the maintenance of the transformed state. These targets are either the activated oncogenes themselves or, in the cases where the oncogenes are not easily drugable, more approachable targets downstream of the activated oncogene. We have explored this second approach in an attempt to develop strategies for treating breast cancers dependent on the *AIB1* oncogene.

We have previously shown that aberrant AIB1 expression in the mammary gland of transgenic mice leads to mammary hyperplasia and mammary tumors (1). Both the hyperplastic premalignant mammary epithelium and mammary tumors in this model show activation of the IGF-I/PI3K/Akt/mTOR pathway, suggesting that inhibition of any of these factors might influence development of AIB1-dependent hyperplasia and might block the growth of AIB1-

dependent tumors. Whereas PI3K and Akt have multiple targets to regulate growth, proliferation, and survival, in this study, we focused on the mTOR branch of the PI3K/Akt pathway that is believed to regulate cell growth through control of protein synthesis (20, 21). We did this by using the mTOR inhibitor RAD001 (everolimus), a rapamycin derivative currently in clinical development (14).

We found that mTOR inhibition by RAD001 was able to reverse the premalignant hyperplastic phenotype of the mammary gland in AIB1 transgenic mice, also referred to as mammary intraepithelial neoplasia (22), supporting the conclusion that the persistence of the premalignant phenotype requires continuous mTOR activity. We also found that AIB1-dependent mammary tumor cell lines grown in culture were sensitive to RAD001. In these cell lines, RAD001 inhibited proliferation by arresting cells in the G_1 phase of the cell cycle, in agreement with the effects of RAD001 or

rapamycin in other tumor models (23, 24). Thus, our results indicate that proliferation of cell lines derived from AIB1-dependent primary tumors requires mTOR activity. Furthermore, our data indicate that the ER$^+$ mammary tumor cell lines derived from AIB1 transgenic animals are dependent on ER signaling for survival because treatment with the estrogen modulator OHT led to apoptosis, as has been seen in some human breast cancer cell lines (25, 26).

To evaluate the effectiveness of RAD001 in the treatment of tumors *in vivo*, we modified the AIB1-dependent tumor model by injecting tumor cell lines into normal mice. This allowed for rapid screening of potential drug candidates and for monitoring their effectiveness through *in vivo* imaging. Using this model, we were able to show that RAD001 treatment *in vivo* shows a significant antitumor effect.

These results have several potential implications for the treatment of women with breast cancer. First, a considerable proportion of human breast cancers show AIB1 amplification and/or overexpression. AIB1 mRNA levels were found to be increased in up to 60% of human breast tumors (27). Our study suggests that such tumors may benefit from the use of inhibitors of the PI3K/Akt/mTOR pathway and specifically from mTOR inhibition with RAD001. The results from our model suggest that signaling pathways activated by overexpressed AIB1 are required for tumor maintenance. Furthermore, our model raises the possibility that the PI3K pathway may be activated in human primary tumors overexpressing AIB1. In this regard, it would be interesting to determine the relationship between AIB1 over-expression and PI3K-activating mutations.

Second, a subset of ER$^+$ human breast cancers is resistant to tamoxifen. A recent report indicates that resistance in some cases is linked to breast cancers showing increased levels of AIB1 and HER2 (28). It is conceivable that in these cases, AIB1 plays a similar role to that seen in our model and would suggest that RAD001 might provide a possible treatment for such tumors. Furthermore, our data indicate that RAD001 treatment might be administered in conjunction with OHT because, in our model, RAD001 was superior to treatment with OHT alone and augmented its antitumor effects in combination. In addition to its increased antitumor efficacy, a combination treatment of RAD001 and OHT might also prevent the formation of OHT resistance by targeting the two pathways simultaneously. Lastly, such a combination treatment might have beneficial effects with regards to endometrial proliferation, a side effect of OHT treatment that has been a concern (29) because RAD001 was able to counteract OHT-induced endometrial hyperplasia in our model.

A recent study examined possible reasons for the apparent resistance of some human tumors to mTOR inhibition despite the
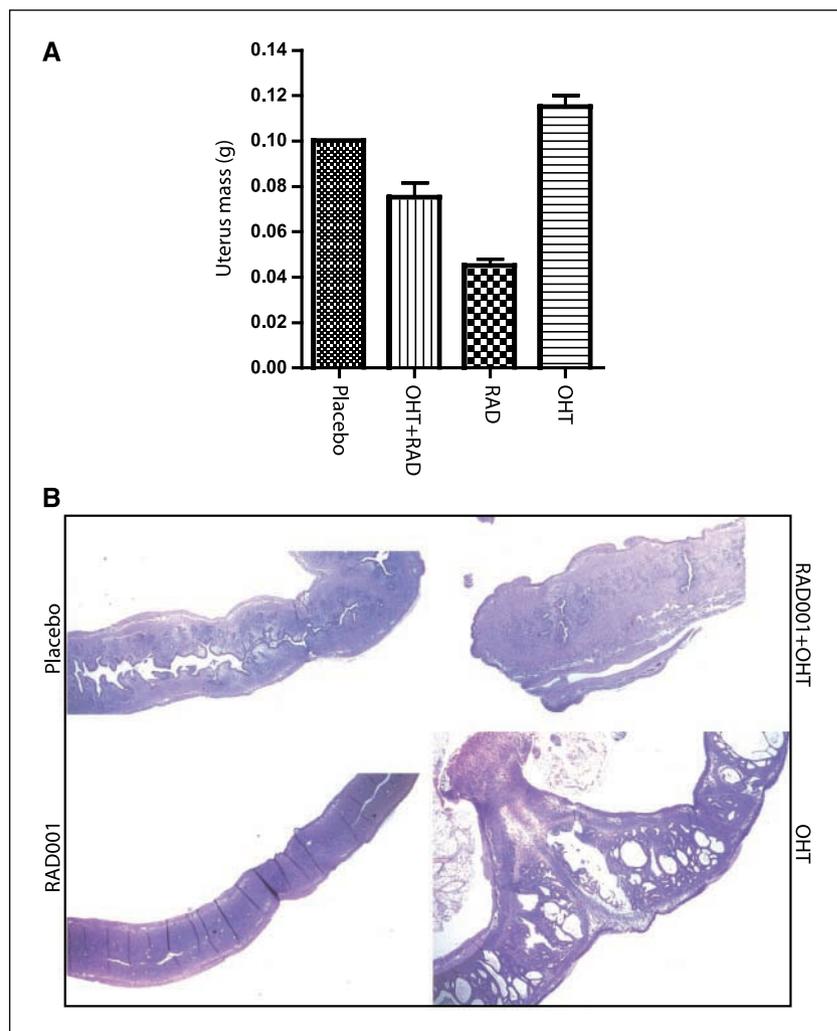

Figure 4. Effect of implanted tumor treatment on uterus size. *A,* after 14 days of the indicated treatment, mice were sacrificed, uterus specimens isolated, and their mass determined. The graph represents the average uterus mass of four specimens per group. *B,* histologic analysis of uterus sections stained with H&E.

successful inhibition of human tumor cell lines *in vitro* (17). These authors report that mTOR inhibition leads to feedback activation of the PI3K pathway including Akt by reversing a feedback inhibition apparently mediated through mTOR. Cell lines and samples of primary human tumors after treatment with mTOR inhibitors were found to have increased levels of pAkt. Whereas mTOR, a downstream target of the PI3K pathway, was still inhibited, other branches of the PI3K pathway downstream of Akt apparently were able to signal proliferation. In our system, we did not observe increased Akt activation after mTOR inhibition. Levels of pAkt as well as of pGSK3, a target of Akt but not mTOR signaling, were unchanged after RAD001 treatment of cell lines *in vitro* or of tumors *in vivo* (Fig. 3*E*). The absence of feedback activation in our system gives rise to the possibility that AIB1-overexpressing breast cancers may also be sensitive to mTOR inhibition with drugs such as RAD001. This awaits clinical trials in which the status of the PI3K pathway is explored as a determinant of response to mTOR inhibitors. Our results suggest that AIB1 may be an important predictive factor in these clinical settings.

Acknowledgments

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