BMS-536924 Reverses IGF-IR-Induced Transformation of Mammary Epithelial Cells and Causes Growth Inhibition and Polarization of MCF7 Cells

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Abstract

Purpose: This study aimed to test the ability of a new insulin-like growth factor receptor (IGF-IR) tyrosine kinase inhibitor, BMS-536924, to reverse the ability of constitutively active IGF-IR (CD8-IGF-IR) to transform MCF10A cells, and to examine the effect of the inhibitor on a range of human breast cancer cell lines.

Experimental Design: CD8-IGF-IR-MCF10A cells were grown in monolayer culture, three-dimensional (3D) culture, and as xenografts, and treated with BMS-536924. Proliferation, cell cycle, polarity, and apoptosis were measured. Twenty-three human breast cancer cell lines were treated in monolayer culture with BMS-536924, and cell viability was measured. MCF7, MDA-MB-231, and MDA-MB-435 were treated with BMS-536924 in monolayer and 3D culture, and proliferation, migration, polarity, and apoptosis were measured.

Results: Treatment of CD8-IGF-IR-MCF10A cells grown in 3D culture with BMS-536924 caused a blockade of proliferation, restoration of apical-basal polarity, and enhanced apoptosis, resulting in a partial phenotypic reversion to normal acini. In monolayer culture, BMS-536924 induced a dose-dependent inhibition of proliferation, with an accumulation of cells in G0/G1, and completely blocked CD8-IGF-IR – induced migration, invasion, and anchorage-independent growth. CD8-IGF-IR-MCF10A xenografts treated with BMS-536924 (100 mg/kg/day) showed a 76% reduction in xenograft volume. In a series of 23 human breast cancer cell lines, BMS-536924 inhibited monolayer proliferation of 16 cell lines. Most strikingly, treatment of MCF7 cells grown in 3D culture with BMS-536924 caused blockade of proliferation, and resulted in the formation of hollow polarized lumen.

Conclusions: These results show that the new small molecule BMS-536924 is an effective inhibitor of IGF-IR, causing a reversion of an IGF-IR – mediated transformed phenotype.

The insulin-like growth factor (IGF) family consists of two ligands (IGF-I and IGF-II), two receptors (IGF-IR and IGF-IIR), six high-affinity binding proteins (IGFBP 1 to 6), and several IGFBP-related proteins (IGFBP 1-6). IGF-I mediates cell signaling through the IGF-IR, which upon binding IGF-I undergoes conformational changes resulting in the activation of the intrinsic tyrosine kinase domain and subsequent downstream signaling pathways such as Erk1/2 and Akt that lead to cell proliferation and survival.

Expression of IGF family members is often altered in numerous neoplasms, including breast cancer. IGF-IR is present in human breast cancer cell lines and is frequently overexpressed and auto-phosphorylated in breast cancer (6, 7). IGF-IR is a potent oncogene, with overexpression in NIH-3T3 fibroblasts causing transformation and tumor growth in vivo (8). Indeed, IGF-IR is actually required for transformation, with fibroblasts derived from IGF-IR null mice (R-cells) being resistant to transformation by a variety of viral and cellular oncogenes (9–11).

Recent studies have shown that IGF-IR is a critical regulator of mammary epithelial transformation, and that overexpression...
Translational Relevance

The insulin-like growth factor receptor (IGF-IR) is critical for cell transformation, and within the last few years several drugs targeting IGF-IR have entered clinical trials and are showing promising early results. In this report we show that a new IGF-IR tyrosine kinase inhibitor, BMS-536924, is a potent inhibitor of IGF-IR – induced transformation of human mammary epithelial cells, being able to completely reverse all measures of transformation in vitro and reducing xenograft tumor burden in vivo. Supporting these proof-of-concept studies, BMS-536924 also affects cell viability of a range of human breast cancer cell lines. This work suggests that targeting IGF-IR may be an effective strategy for the treatment of human breast cancer.

in the mouse mammary gland is sufficient to induce mammary epithelial hyperplasia and tumor formation (12, 13). Consistent with this, two studies found that overexpression of IGF-IR in human mammary epithelial cells (MCF10A) disrupted acini formation due to hyperproliferation and increased survival (14, 15). We have recently shown that overexpression of a constitutively active IGF-IR (CD8-IGF-IR) in MCF10A cells likewise disrupted mammary acini formation, but also caused an epithelial to mesenchymal transition and full transformation, with growth as xenografts in immunocompromised mice, an uncommon feature following transformation of MCF10A cells with a single oncogene (16).

Due to IGF-IR’s critical role in proliferation and survival, the receptor has become a major focus for anticancer therapy (17, 18). Various strategies, including antisense technology (19), dominant-negative IGF-IR (20), inhibitory antibodies (21–25), and small molecule inhibitors (26–29) have been used to inhibit breast cancer cell growth in vitro or in vivo by disrupting IGF-IR function. Indeed, clinical trials of antibodies and small molecule inhibitors are currently ongoing as an approach for the therapeutic blockade of the IGF-IR in human cancer (18, 30).

Here we report the ability of a new small molecule inhibitor targeting IGF-IR, BMS-536924, to reverse IGF-IR – mediated transformation of mammary epithelial cells and affect breast cancer cell growth and migration. BMS-536924 completely reversed all measures of CD8-IGF-IR – induced transformation in vitro, and caused a partial phenotypic reversion to more normal acini formation in Matrigel culture. Furthermore, the inhibitor blocked proliferation of a wide range of breast cancer cells. Most strikingly, treatment of MCF7 cells grown in three-dimensional cultures with BMS-536924 caused blockade of proliferation and restoration of apical-basal polarity, resulting in a partial phenotypic reversion to normal acini similar to MCF10A cells. These results show that the new small molecule BMS-536924 is an effective inhibitor of IGF-IR, and may be effective in the treatment of IGF-IR – stimulated human breast cancer.

Materials and Methods

Cell culture. MCF10A cells were cultured in complete growth medium consisting of DMEM/F12 supplemented with 5% horse serum (Sigma), 20 ng/mL epidermal growth factor (Sigma), 10 μg/mL insulin (Sigma), 0.5 μg/mL hydrocortisone (Sigma), 100 ng/mL cholera toxin (Sigma), and 100 units/mL penicillin-streptomycin (Invitrogen). CD8-IGF-IR-MCF10A and pBabe-MCF10A were generated by infection of retrovirus containing the CD8-IGF-IR-pBabe-puro or pBabe-puro as previously described (16). All breast cancer cell lines were obtained from the American Type Culture Collection, except H3396, which was obtained from the Pacific Northwest Institute. Breast tumor cell lines were maintained in RPMI 1640 media, 10% heat-inactivated fetal bovine serum (FBS), 100 μg/mL penicillin, and 100 μg/mL streptomycin (Invitrogen). The MCF7/Her2 cell line was made stable transfection of the human epidermal growth factor receptor 2 (HER2) gene into MCF7 cells. MDA-MB-435 cells were originally classified as a breast cancer cell line, but have since been shown to be derived from the M14 melanoma cancer cell line and thus must be considered to be of melanoma origin (31).

IGF-1 and BMS-536924 treatment and preparation of total cell lysates. For testing the inhibition of the IGF-IR pathway in pBabe-MCF10A control cells, 1 × 10⁶ pBabe-MCF10A cells were seeded onto 60-mm dishes. After 24 h, the medium was changed to serum-free medium and incubated overnight at 37°C for 24 h. Cells were then preincubated with or without 1 μmol/L BMS-536924 for 1 h in serum-free medium followed by stimulation with IGF-1 (50 ng/mL) for 10 min. Cell monolayers were washed twice with PBS and harvested for immunoblot analysis. For dose-dependent inhibitor treatment, 1 × 10⁷ CD8-IGF-IR-MCF10A cells were plated onto 60-mm cell culture dishes and grown in complete medium for 24 h. The medium was replaced with complete growth medium plus BMS-536924 diluted in DMSO at the following concentrations: 0, 0.01, 0.1, and 1 μmol/L. After 24 h the cells were harvested for immunoblot analysis. For time-dependent inhibitor treatment, 1 × 10⁷ CD8-IGF-IR-MCF10A cells were plated onto 60-mm cell culture dishes and grown in complete medium for 24 h. Medium was changed to complete growth media plus 1 μmol/L BMS-536924. Cells were harvested after 10 min and 1, 8, 24, and 48 h. For studies assessing the effect of the inhibitor on epidermal growth factor receptor (EGFR), 1 × 10⁶ pBabe-MCF10A cells were seeded onto 60-mm dishes. After 24 h, the medium was changed to serum-free medium and incubated overnight at 37°C for 24 h. Cells were then preincubated with increasing concentrations of BMS-536924 for 1 h in serum-free medium followed by stimulation with epidermal growth factor (EGF; 20 ng/mL) for 10 min. Cell monolayers were washed twice with PBS and harvested for immunoblot analysis.

3D Matrigel culture. Three-dimensional (3D) cultures were grown in 8-well chamber slides (Falcon) as described previously (16) using growth factor – reduced Matrigel (BD Biosciences). Cells were treated with assay medium containing 2% Matrigel and 1 μmol/L BMS-536924. Assay medium containing 2% Matrigel was replaced every 4 d. For BMS-536924 treatment, day 0 was designated the next day after plating cells. Acini structures were harvested and immunostained with antibodies to Ki-67 (Dako), laminin-V (BD Biosource), activated cleaved caspase-3 (Cell Signaling), and GM130 (BD Biosource) as described previously (16).

Immunoblot analysis. To evaluate phosphorylation levels of the selected IGF-IR signaling molecules, monolayer cultures were lysed with 200 μL 5% SDS. Total protein extract (50-75 μg) was resuspended in denaturing sample loading buffer, separated by 8% SDS-PAGE, and electrophoretically transferred to a nitrocellulose membrane overnight at 4°C. The membrane was blocked with PBS plus 0.05% Tween-20 (PBST) containing 5% nonfat milk for 1 h. The following antibodies were utilized: anti-pY1162/1163-IGF-IR (#44-804G, Biosource; 1:500), anti-IGF-IRβ (#sc-713, Santa Cruz Biotechnology; 1:500), antiphospho-Erk1/2 (#9101, Cell Signaling Technology; 1:1,000), anti-Erk1/2 (#9102, Cell Signaling Technology; 1:1,000), anti-pS473-AKT (#4068, Cell Signaling, 1:1,000), anti-pS21/9-GSK3α/β (#9271, Cell Signaling; 1:1,000), and anti-GSK3α/β (#9313, Cell Signaling; 1:1,000), and anti-β-actin (#A1978, Sigma; 1:4,000). Antibodies were incubated in blocking solution overnight at 4°C. Subsequently, the membrane was washed
three times for 5 min each with PBST and then incubated with a horseradish peroxidase–linked secondary antibody (Amersham Pharmacia Biotech) at a dilution of 1:4,000 in blocking solution. After the membrane was washed three times for 5 min each with PBST, bands were visualized by enhanced chemiluminescence according to the manufacturer's protocol (Pierce Biotechnology) and captured using an Alpha Innotech 7000 (Alpha Innotech).

**Cell cycle analysis.** For cell cycle analysis, 5\times10^5 CD8-IGF-IR-MCF10A cells and pBabe-MCF10A control cells were seeded in duplicate onto 60-mm dishes. Day 0 was designated as the day after plating cells. Cells were then incubated overnight with serum-free medium. At day 1, medium was replaced either by complete medium or serum-free medium containing 0, 0.1, 0.5, and 1 \mu mol/L BMS-536924 and incubated for 24 h. Cells were washed with PBS, trypsinized, and resuspended in 2 mL 0.9% NaCl. Ninety percent ice cold ethanol was added dropwise to fix cells for 30 min at room temperature. Cells were centrifuged at 1,000 rpm, stained with 50 \muL of 0.9% NaCl. Ninety percent ice cold ethanol was added dropwise to fix cells for 30 min at room temperature. Cells were centrifuged at 1,000 rpm, stained with 50 \muL of propidium iodide, and 1 mg/mL RNase A (Sigma) was added. Cells were incubated for 30 min at 37 °C. Single-color fluorescent flow cytometry was done with a FACScalibur flow cytometer (Becton Dickinson). The histograms were analyzed with ModFit LT software (Verify Software House, Topshire, ME).

**Monolayer growth, soft agar, wound healing, and invasion assays.** All assays were done as described previously (16). Cells were cultured with the increasing concentrations of BMS-536924 in complete growth medium or serum-free medium, and cell number was counted at day 4 using a Beckman Coulter Z Series (Beckman Coulter). For breast cancer cell line studies, cells were seeded at 1,000 to 12,000 cells per well depending on the cell line in 96-well microtiter plates and incubated overnight. Compound was serially diluted and added. After 72 h exposure, a cytotoxicity evaluation was done using an MTS tetrazolium dye conversion assay. The IC_{50} values were calculated and expressed graphically for each cell line exposed to the compound. Anchorage-independent colony formation was assessed as described previously. Every 4 d growth medium (200 \muL) containing 0, 0.1, 0.5, and 1 \mu mol/L BMS-536924, respectively, was added. Colonies were stained with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; 5 mg/mL in PBS) by incubation for 4 h at 37 °C, photographed using an Alpha Imager 7000 (Alpha Innotech), and then counted. The wound healing assay was done as described previously, with proliferation inhibited with 5 \mu mol/L mitomycin C. The invasion assay was done as described previously. The lower compartment was filled with 800 \muL complete medium or complete medium containing 1 \mu mol/L BMS-536924.

**Fig. 1.** BMS-536924 inhibits IGF-IR signaling in pBabe-MCF10A cells and inhibits phosphorylation of CD8-IGF-IR. A, pBabe-MCF10A cells in serum-free medium were pretreated with BMS-536924, and then incubated with or without BMS-536924 in the presence or absence of IGF-I (50 ng/mL). Cells were lysed and analyzed by immunoblot as described in Materials and Methods and probed using IGF-IR p-specific antibodies as well as total/phospho-specific antibodies for AKT, ERK1/2, and GSK3β. β-actin was used as a loading control. B, CD8-IGF-IR-MCF10A cells were incubated in serum-free medium and then exposed to increasing concentrations of BMS-536924 for 24 h and harvested for immunoblot analysis. C, CD8-IGF-IR-MCF10A cells were cultured in serum-free medium and then incubated with 1 \mu mol/L BMS-536924 and harvested after 10 min, 1 h, 8 h, 24 h, or 48 h for immunoblot analysis. D, pBabe-MCF10A cells were incubated in serum-free medium and pretreated for 1 h with increasing concentrations of BMS-536924 (0.01, 0.1, 0.25, 0.5, 0.75, 1 \mu mol/L), followed by stimulation with EGF (20 ng/mL) for 15 min. Cells were lysed and analyzed by immunoblot.
BMS-536924 blocks acinar proliferation, partially restores polarization, and induces apoptosis in CD8-IGF-IR-MCF10A acini. A, pBabe-MCF10A and CD8-IGF-IR-MCF10A acini were plated in Matrigel and treated every 4 d with or without 1 μmol/L BMS-536924. Acini were cultured for 12 d and stained with antibodies against Ki67 (green) or laminin V (red). Nuclei were counterstained with To-Pro3. Confocal microscopy visualized cross-sections through the middle of the acini with 40× magnification. Pictures show representative acini. B, quantification of the number of cells per acinus was done by image analysis. Data represent means ± SE from 10 different acini. The percentage of Ki67-positive cells within acini was scored after the indicated number of days in culture. Values represent the means ± SE of 10 acini. C, pBabe-MCF10A and CD8-IGF-IR-MCF10A were cultured for 12 d and then treated with 1 μmol/L BMS-536924. After 4 d treatment, acini were stained as in A. D, the percentage of CC3-positive cells within the acini was scored after the indicated number of days in culture. Values represent the means ± SE of 10 acini.
Xenograft studies. All procedures were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals, and were approved by the Institutional Animal Care and Use Committee of Baylor College of Medicine. Mice were maintained on a 12-h light, 12-h dark schedule with ad libitum access to laboratory chow (Pico Lab Rodent Diet 20, Lab Diet 5053, PMI Nutrition International Inc.) and water. 5 × 10^5 pBabe-MCF10A or CD8-IGF-IR-MCF10A cells were injected orthotopically into the thoracic mammary gland of athymic nude mice (with or without Matrigel). The pBabe-MCF10A cells did not grow. When CD8-IGF-IR-MCF10A cells were between 100 and 200 mm^3 they were randomized to receive either vehicle or BMS-536924 (100 mg/kg) daily by gavage. Tumor size was measured by digital caliper, and tumor volumes were estimated according to the formula for an ellipse: (short dimension)^2 × (long dimension)/2. Mice did not lose weight during treatment, and there were no outward signs of toxicity.

Immunohistochemistry. Tumor sections were cut at intervals of 100 μm, stained by H&E, and then examined microscopically. Serial sections (5-μm thick) were placed on Superfrost Plus slides (Fisher Scientific), deparaffinized, and gradually hydrated. Immunohistochemistry was done for mouse cleaved caspase-3 (CC3; 1:100 Covance) using a Vectastain ABC peroxidase immunodetection kit (rat IgG as a negative control) and a mouse on mouse (M.O.M.) immunodetection kit (both purchased from Vector Laboratories).

Results

BMS-536924 inhibits IGF-1–stimulated IGF-IR signaling in MCF10A cells and blocks constitutive IGF-IR activity in CD8-IGF-IR-MCF10A. BMS-536924 was identified in a research program aimed at the development of small molecule ATP-competitive inhibitors of IGF-IR. BMS-536924 inhibits IGF-IR kinase activity with an IC50 of 80 nmol/L, but also inhibits a small number of other kinases including insulin

![Graph A](image1.png)  
**A.** BMS-536924 induces a dose-dependent inhibition of proliferation with a decrease in S-phase cells, and inhibits IGF-IR–induced migration and invasion. A, monolayer growth assay. pBabe-MCF10A and CD8-IGF-IR-MCF10A were exposed to increasing concentrations of BMS-536924 in serum-free medium for 4 d. The panel displays percentage of growth inhibition with an IC50 of 0.4 μmol/L BMS-536924 for CD8-IGF-IR-MCF10A. B, cell cycle analysis of CD8-IGF-IR-MCF10A and pBabe-MCF10A in serum-free medium with increasing concentrations of BMS-536924. C, single-parameter histogram of DNA shows the discrimination of cell populations existing in G0/G1, S, and G2/M phases of the cell cycle. D, confluent monolayers of CD8-IGF-IR-MCF10A cells were incubated overnight in either full medium or serum-free medium. One hour before treatment proliferation was inhibited by 1 μmol/L mitomycin C. Monolayers were lightly scratched and cultured in serum-free medium or complete medium containing 1 μmol/L mitomycin C or 1 μmol/L mitomycin C and 1 μmol/L BMS-536924. Photographs were taken immediately (0 h) and 24 h postscratch at 10× magnification and the percentage of wound closure was quantified. E, matrigel invasion assay showed that BMS-536924 blocks CD8-IGF-IR-MCF10A cells from penetrating the membrane. Cells were counted in a minimum of 4 high-powered fields (HPF). Data are presented as number of cells per chamber (number of cells/HPF), and represent means ± SE of duplicate experiments.
Effect of New IGF-IR Inhibitor on Breast Cancer Cells

Mammary gland. Oncexenografts reached a volume of 100 to 200 mm³; they were treated and untreated CD8-IGF-IR-MCF10A xenografts were harvested after 3 wk of treatment and embedded in paraffin. Five-micrometer tumor sections were stained by H&E or processed for immunohistochemistry using an anti-CC3 antibody. Pictures show representative tumor cross-sections at 40× magnification.

Fig. 4. BMS-536924 inhibits transformation in vitro and in vivo. A, for anchorage-independent growth assay, 1 x 10⁴ cells were suspended in their growth medium containing 0.38% agarose and increasing concentrations of BMS-536924 and seeded in 6-well plates coated with a basal layer of complete medium containing 0.7% agarose. Colony assay was set up in triplicate. 3 wk later, colonies were stained with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), counted, and plotted against increasing concentrations of BMS-536924. B, 6-week-old mice were injected with CD8-IGF-IR-MCF10A cells in the #3 mammary gland. Once xenografts reached a volume of 100 to 200 mm³ they were randomized and treated once daily with 100 mg/kg BMS-536924. Data represent the mean ± SE of xenograft volumes. C, treated and untreated CD8-IGF-IR-MCF10A xenografts were harvested after 3 wk of treatment and embedded in paraffin. Five-micrometer tumor sections were stained by H&E or processed for immunohistochemistry using an anti-CC3 antibody. Pictures show representative tumor cross-sections at 40× magnification.

CD8-IGF-IR disrupts normal mammary acini formation in 3D Matrigel, which is partially reversed by BMS-536924. Previous studies have shown that abnormal expression of oncogenes can induce hyperproliferation, disrupt apico-basal polarity, and suppress apoptosis to alter normal mammary acinar morphogenesis of MCF10A cells in 3D Matrigel (34). Similarly, receptor (32). To investigate the ability of BMS-536924 to inhibit IGF-IR activity, tyrosine phosphorylation of IGF-IR was examined in the presence or absence of the inhibitor and IGF-I (Fig. 1). MCF10A cells showed no detectable phosphorylation of IGF-IR when cells were cultured in serum-free medium without addition of growth factors. In the presence of IGF-I, however, key tyrosine residues in the kinase domain of the β-subunit of IGF-IR became phosphorylated (Fig. 1A). Preincubation of cells with 1 μmol/L BMS-536924 completely blocked the ability of IGF-I to stimulate IGF-IR phosphorylation. There was no change in the amount of total IGF-IR between those different treatment groups as determined by immunoblotting for total receptor levels. We next determined the effect of BMS-536924 on key signaling molecules downstream of IGF-IR. MCF10A cells in serum-free medium showed weak phosphorylation of ERK1/2 and GSK3β, but p-AKT was not detectable. IGF-I stimulation resulted in increased phosphorylation of ERK1/2, GSK3β, and AKT. BMS-536924 inhibited this ligand-induced phosphorylation, consistent with the blockade of IGF-IR phosphorylation and activation. Basal levels of GSK3β and ERK1/2 phosphorylation were reduced by BMS-536924 potentially due either to low-level autocrine (IGF-I or IGF-II) activation of IGF-IR or due to BMS-536924 inhibition of other signaling pathways. We did not observe any appreciable change in the level of total ERK1/2, AKT, or GSK3β among the various treatment groups.

CD8-IGF-IR is a chimeric receptor consisting of the extracellular domain of human CD8α fused to the β-subunit of IGF-IR. Disulfide bonding between CD8α subunits is believed to cause the IGF-IR kinase domains to have constitutive activity (12). To study the role of IGF-IR in mammary epithelial cell transformation, and the ability of BMS-536924 to reverse this, MCF10A human immortalized mammary epithelial cells were previously infected with a retrovirus encoding CD8-IGF-IR and stable clones isolated (thereafter called CD8-IGF-IR-MCF10A; ref. 16). Treatment of the CD8-IGF-IR-MCF10A cells with BMS-536924 resulted in a dose-dependent inhibition of phosphorylation with partial inhibition at 1 μmol/L and 0.1 μmol/L, but complete receptor inhibition at a concentration of 1 μmol/L (Fig. 1B). Maximal inhibition of phosphorylated IGF-IR was observed as early as 10 minutes following incubation (Fig. 1C). BMS-536924 retained its ability to inhibit IGF-IR phosphorylation for up to 48 hours. Furthermore, addition of BMS-536924 time-dependently inhibited AKT phosphorylation starting at 1 hour. By 48 hours, AKT activation was completely blocked.

MCF10A cells are dependent upon EGF for proliferation, and typically require EGF in the culture medium for growth (33). To assure that any further work with the inhibitor was not due to nonspecific blockade of EGFR, we examined EGF activation of EGFR in the presence of increasing concentrations of BMS-536924. BMS-536924 up to a concentration of 1 μmol/L (which was maximal for blocking IGF-IR; see Fig. 1A and B) had no effect upon EGF-mediated activation of EGFR (Fig. 1D). EGFR-mediated activation of AKT and ERK1/2 was also not affected by BMS-536924.

CD8-IGF-IR disrupts normal mammary acini formation in 3D Matrigel, which is partially reversed by BMS-536924. Previous studies have shown that abnormal expression of oncogenes can induce hyperproliferation, disrupt apico-basal polarity, and suppress apoptosis to alter normal mammary acinar morphogenesis of MCF10A cells in 3D Matrigel (34). Similarly,
transformed breast epithelial cells grown in 3D Matrigel show phenotypes similar to those of transformed MCF10A cells, with uncontrolled proliferation and lack of polarity. Targeting β1 integrin can lead to a reversion of the malignant phenotype and formation of normal acini (35, 36). In our previous study we reported that CD8-IGF-IR-MCF10A cells grown in Matrigel formed large, multilobulated structures with enhanced proliferation and luminal filling (16). Herein, we further analyzed CD8-IGF-IR-mediated disruption of MCF10A acini morphogenesis and found that pBabe-MCF10A cells underwent growth arrest similar to that described by others (14, 15, 34), whereas in stark contrast, CD8-IGF-IR-MCF10A cells were highly proliferative with the high rate of proliferation over an extended period of time (Supplementary Fig. S1). This high degree of proliferation likely contributed to expansion and overgrowth of the acini. Consistent with IGF-IR stimulating survival and luminal filling, CD8-IGF-IR showed little or no apoptosis in the luminal space as assessed by CC3 staining (data not shown and Fig. 2C). Additionally, pBabe-MCF10A cells showed a polarized layer of cells as described by others (37), whereas CD8-IGF-IR-MCF10A cells lost polarity as indicated by aberrant deposition of lamininV in the luminal space (Supplementary Fig. S1).

These studies showed that overexpressed IGF-IR caused dramatic alterations in mammary acinar morphogenesis involving enhanced proliferation, suppressed apoptosis, and a loss of polarization. To investigate the effect of BMS-536924 on proliferation and apicobasal polarization, pBabe-MCF10A and CD8-IGF-IR-MCF10A cells were cultured in Matrigel and treated with 1 μmol/L BMS-536924 every 4 days. BMS-536924 inhibited growth with both pBabe-MCF10A and CD8-IGF-IR-MCF10A acini being smaller in size and a reduction of more than 80% of Ki67-positive cells (Fig. 2A and quantified in B). The size reduction was more dramatic in CD8-IGF-IR–overexpressing cells but most strikingly, BMS-536924 treatment of CD8-IGF-IR-MCF10A cells resulted in the formation of acini that resembled the MCF10A acini, and we didn’t observe any large multilobular structures (Fig. 2A). BMS-536924 treatment of CD8-IGF-IR-MCF10A cells resulted in acini with distinct polarization and lamininV deposition to the basal surface of the acini and a disappearance of lamininV from the lumen (Fig. 2A). Therefore, BMS-536924 is able to partially reverse IGF-IR–mediated disruption of proliferation and polarization and results in the formation of acini that resemble normal MCF10A cells. These results are similar to those results originally observed with targeting β1-integrin that led to a reversion of the malignant phenotype and formation of normal acini (35, 36).

**BMS-536924 induces apoptosis in CD8-IGF-IR-MCF10A acini.**

BMS-536924 induces filling of the luminal space, in part, by blocking proliferation (14, 15, 34). We therefore tested whether BMS-536924 had the ability to enhance apoptosis in the center of the acini. CD8-IGF-IR-MCF10A and pBabe-MCF10A were cultured in matrigel for 12 days, and then treated for 4 days with 1 μmol/L BMS-536924. At day 16 acini were harvested, fixed, and stained for CC3 as a marker for apoptosis, and Ki67 as a marker of proliferation. At day 16, pBabe-MCF10A acini showed little or no proliferation either in the presence or absence of BMS-536924 (Fig. 2C). In contrast, CD8-IGF-IR-MCF10A acini were large and hyperproliferative, and treatment with BMS-536924 caused a dramatic reduction in Ki67 staining (Fig. 2C). CC3 was not detectable in untreated pBabe-MCF10A acini whereas treatment with BMS-536924 resulted in the detection of cells positive for CC3, indicating a role for IGF-IR in the survival of MCF10A cells in culture. More importantly, treatment of CD8-IGF-IR-MCF10A cells with BMS-536924 resulted in a dramatic induction of apoptosis, with up to 40% of CC3-positive cells (Fig. 2C and quantified in D). Interestingly, the majority of the staining was in the center of the largely misshapen acini, but we also noted apoptotic cells in the outer layer. Despite this large increase in apoptosis, we didn’t observe a complete elimination of cells in the center of the lumens. Even longer incubation with BMS-536924 for 6 days after 12 days of culturing CD8-IGF-IR-MCF10A cells in matrigel didn’t allow luminal clearance (data not shown), suggesting either that the increased apoptosis is not sufficient alone to clear the luminal space or that a longer incubation is needed to see this effect.

**BMS-536924 inhibits growth of CD8-IGF-IR-MCF10A cells and causes a G0/G1 block.**

MCF10A cells require serum and EGF for proliferation (38). In serum-free medium MCF10A cells undergo growth arrest. To assess the antiproliferative ability of BMS-536924, we did a monolayer growth assay on pBabe-MCF10A and CD8-IGF-IR-MCF10A cells. In serum-free medium, pBabe-MCF10A cells were mostly refractory to BMS-536924, whereas CD8-IGF-IR-MCF10A cell growth was inhibited in a dose-dependent manner with a complete blockade of proliferation at an IC50 of 0.48 μmol/L BMS-536924 (Fig. 3A). The lack of response of pBabe-MCF10A cells to BMS-536924 was due to the fact that in serum-free medium very few cells (~1.5-2.0%) were in S-phase (Fig. 3B); most were found in G0/G1 (data not shown). In contrast, a large fraction of CD8-IGF-IR-MCF10A cells were found in S-phase (~24%), and this fraction was reduced in a dose-dependent manner with BMS-536924, with 1 μmol/L causing an 89% reduction in S-phase fraction (Fig. 3B). At the same time there was a dramatic increase (from 66% to 91.53%) of cells in G0/G1 after BMS-536924 treatment (Fig. 3C). Interestingly, when the same assays were done in cells in complete growth medium, BMS-536924 had little effect on proliferation or S-phase fraction (data not shown), suggesting only a 9% decrease in S-phase cells. This indicates that IGF-1 or CD8-IGF-IR is not
sufficient to induce proliferation in this setting and that in complete growth medium, other growth factors are sufficient for cell growth, e.g. EGF. This data are also consistent with data from Fig. 1D showing that BMS-536924 (1 μmol/L) is selective for IGF-IR and doesn’t block EGFR. We noted that during the fluorescence-activated cell sorting experiments, cell death was negligible as nearly 100% of cells were gated for analysis, and we did not detect a sub-G₁ population indicative of apoptosis (Fig. 3C). This is in contrast to the ability of BMS-536924 to induce apoptosis in 3D culture and suggests that CD8-IGF-IR may be required for survival signals in 3D but not 2D culture. This has previously been reported by Dr. Baserga’s group (39).

**BMS-536924 blocks CD8-IGF-IR-induced migration and invasion.** To measure the effect of BMS-536924 on cellular migration in CD8-IGF-IR-MCF10A cells, a scratch-in assay was done. In the absence of BMS-536924, CD8-IGF-IR-MCF10A cells were able to migrate into a scratch in either serum-free medium (68% of scratch filled and Fig. 3D) or complete growth medium (58% of scratch filled, data not shown). Treatment with 1 μmol/L BMS-536924 completely blocked migration in both serum-free medium (Fig. 3D) and complete growth medium (data not shown). In contrast to the proliferation assays (Fig. 3A), in which BMS-536924 had little effect on growth of CD8-IGF-IR-MCF10A cells grown in complete medium, the complete blockade of migration in cells grown in complete medium suggests that CD8-IGF-IR alone is both sufficient and required for migration even in the presence of serum or other growth factors. We also tested the effect of BMS-536924 on CD8-IGF-IR-MCF10A cell invasion using a modified Boyden chamber assay with Matrigel-coated wells (Fig. 3E). pBabe-MCF10A cells showed a poor ability to invade through the Matrigel-coated chamber whereas CD8-IGF-IR-MCF10A cells were highly invasive. BMS-536924 completely blocked invasion of CD8-IGF-IR-MCF10A through Matrigel-coated wells.

**BMS-536924 blocks CD8-IGF-IR-MCF10A soft-agar growth and causes regression of xenografts in vivo.** CD8-IGF-IR-MCF10A cells exhibit several hallmarks of transformation. Consistent with this, CD8-IGF-IR-MCF10A cells were able to grow in soft agar assays, whereas pBabe-MCF10A cells failed to grow in soft agar consistent with their nontransformed phenotype (Fig. 4A). Increasing doses of BMS-536924 caused a dose dependent reduction in CD8-IGF-IR-MCF10A colony formation, with a low concentration of BMS-536924 (0.1 μmol/L BMS) causing significantly smaller colonies (Fig. 4A), and 0.5 μmol/L BMS-536924 completely inhibiting colony formation. We next examined the effects of BMS-536924 in *vivo*. Injection of CD8-IGF-IR-MCF10A resulted in the appearance of palpable tumors that rapidly grew to ~100 to 200 mm³ in size. Interestingly, despite the *in vitro* transformed phenotype of CD8-IGF-IR-MCF10A cells, xenografts stopped growing once they reached 100 to 200 mm³. Histologic analysis of the xenografts showed them to be poorly differentiated carcinomas that were relatively benign and noninvasive. Similar results following transformation of human immortalized mammary epithelial cells have been previously reported (40). Despite this lack of growth past 100 to 200 mm³, we tested the ability of BMS-536924 to cause regression of these xenografts. Therefore, when xenografts had reached 100 to 200 mm³, mice were treated daily with either vehicle or BMS-536924 (100 mg/kg) and tumor volume measured. Within 1 week of treatment xenograft volume was reduced and after 2 weeks an average reduction of 76% tumor volume was noted (Fig. 4B). Treated xenografts showed large areas of necrosis with apoptotic cells as assessed by CC3 staining (Fig. 4C).

**BMS-536924 inhibits growth of a wide range of breast cancer cell lines.** To assess the ability of BMS-536924 to inhibit growth of human breast cancer cell lines, the minimal and maximal IC₅₀ were determined on 23 different cell lines (Fig. 5A). MCF7 cells were the most sensitive breast cancer cell line with an IC₅₀ of 1.2 μmol/L BMS-536924. To confirm the ability of BMS-536924 to inhibit IGF-IR activity of MCF7 cells, tyrosine phosphorylation of the IGF-IR was examined in the presence or absence of the inhibitor and IGF-I (Fig. 5B). MCF7 cells showed no detectable phosphorylation of the IGF-IR when cultured in serum-free medium. However, in the presence of IGF-I the IGF-IR receptor was phosphorylated (Fig. 5B). Preincubation of cells with 1 μmol/L BMS-536924 completely blocked the ability of IGF-I to stimulate IGF-IR phosphorylation. Furthermore, addition of BMS-536924 inhibited downstream activation of AKT (Fig. 5B). In stark contrast to the sensitivity of MCF7 cells to BMS-536824, MCF7 cells that overexpressed HER2/ErbB2 were completely resistant (IC₅₀ >9.9 μmol/L; Fig. 5A). Among the different tumor cell lines sensitivity varied widely; of the 23 cell lines, 17 responded with an average IC₅₀ of 5.24 μmol/L, whereas 7 were resistant to BMS-536924 with IC₅₀ of >9.9 μmol/L. Many of these resistant cell lines overexpressed ErbB2/HER2 (such as MCF7/HER2), consistent with other reports that Her2 can cause resistance to IGF-I inhibition and favoring the notion of cotargeting of these pathways in breast cancer.

To determine the correlation between the sensitivity of cell lines to BMS-536924 and level of IGF-I receptor protein expression, we examined publicly available data from Neve et al. (41). IGF-IR protein levels (derived from densitometric quantitation of immunoblots) were available on 13 cell lines for which we had the IC₅₀ for BMS-536924. Dichotomization of the cell lines based upon the mean IC₅₀ (5.89 μmol/L) showed that significant higher (P < 0.05) IGF-IR levels were correlated sensitivity to the drug (Fig. 5C).

**BMS-536924 inhibits migration of MDA-MB-231 cells and blocks MCF7 soft agar growth.** To assess whether BMS-536924 may affect other biological properties of breast cancer cells, we examined the influence of the inhibitor upon migration and anchorage-independent growth. MDA-MB-231 cell migration was measured using a scratch-in assay (Fig. 5D). After 24 hours, 42% of cells migrated into the gap. BMS-536924 (2 μmol/L) completely inhibited migration of MDA-MB-231 cells in the scratch-in assay (Fig. 5D). Of note, that maximal concentration for inhibition of migration was still lower than the IC₅₀ required for inhibition of monolayer proliferation (IC₅₀ > 4.82 μmol/L; Fig. 5A). In soft agar assays, MCF7 cells showed a dose-dependent decrease in colony number and colony size (Fig. 5E), which is consistent with the results seen in CD8-IGF-IR-MCF10A. Effects on colony formation were observed at concentrations as low as 0.1 μmol/L BMS-536924, with a complete blockade at 1 μmol/L BMS-536924. Similar to results in MDA-MB-231 cells, the IC₅₀ for growth inhibition of MCF7 cells in soft agar growth was lower (approximately 10-fold) than for monolayer growth.

**BMS-536924 decreases proliferation and cell number in MCF7 and MDA-MB-435 cells grown in 3D culture, and in MCF7 cells causes lumen formation.** In 3D culture, breast cancer cells show
uncontrolled proliferation, suppression of apoptosis, and a lack of polarity that result in the cells forming clumps that have little or no resemblance to MCF10A acini. To investigate the effect of BMS-536924 on proliferation and apicobasal polarization, we analyzed 3D cultures of MCF7 cells and MDA-MB-435 cells cultured in Matrigel and treated with 1 μmol/L BMS-536924 every 4 days. BMS-536924 inhibited growth with more than a 27% reduction compared with vehicle control. (Fig. 6A and B). To determine the effect of treatment on cell number as a whole, we counted the total number of cells per acinus and compared untreated and treated acini. We found that IGF-IR inhibition resulted in a significant decrease in total

![Fig. 6. BMS-536924 decreases proliferation and cell number in MCF7 and MDA-MB-435 acini and reverses MCF7 acini formation. A. MCF7 cells were placed in morphogenesis assays, treated every 4 d with BMS-536924, and then fixed and immunostained at day 12 with antibodies to Ki67, active caspase-3, or GM130. Nuclei were counterstained with TOPRO-3. B. The number of cells per acinus was quantified. Values represent the mean ± SE of 10 acini. C, MDA-MB-435 acini were treated every 4 d with BMS-536924 and immunostained with antibodies to Ki67, activated caspase-3, or GM130. Nuclei were counterstained with TOPRO-3 (blue). D, the number of cells per acinus was quantified. Values represent the mean ± SE of 10 acini.](image-url)
cell number (Fig. 6B). Most strikingly, however, BMS-536924 treatment of MCF7 cells resulted in the formation of acini that resembled the MCF10A acini with hollow lumen (Fig. 6A). Staining with an antibody for GM130, a matrix protein in the cis-Golgi compartment (42), showed distinct acinar polarization. Similar to the results observed with targeting CD8-IGF-IR in MCF10A cells, BMS-536924 was able to reverse disruption of polarization, allowing formation of acini that resemble normal MCF10A cells. We next examined the effects of BMS-536924 on MDA-MB-435 acini formation. BMS-536924 was able to suppress proliferation indicated by a 41% reduction compared with vehicle (Fig. 6C and D). However, treatment did not cause polarization in MDA-MB-435 acini, as GM130 was dispersed. In both cell lines we observed no change in apoptosis as indicated by CC3 staining (Fig. 6A and C), perhaps because apoptosis occurred at an earlier stage of acinar morphogenesis.

Discussion

In the present study we showed several significant findings. First, BMS-536924 fulfills the key characteristics expected from an IGF-IR inhibitor, completely blocking IGF-IR tyrosine kinase activity. Second, treatment of CD8-IGF-IR-MCF10A acini with BMS-536924 caused a partial phenotypic reversion to normal acini with blockade of proliferation, restoration of apico-basal polarization, and enhanced luminal apoptosis. Third, BMS-536924 completely blocked and/or reversed all aspects of IGF-IR-induced transformation including IGF-IR-mediated migration, invasion, anchorage-independent growth, and xenograft growth. Finally, BMS-536924 was effective at inhibiting growth of a wide range of breast cancer cell lines, and caused MCF7 cells in 3D culture to form polarized hollow lumen.

Previously, Bissell’s group showed that phenotypic reversion of malignant T4-2 cells was possible by inhibition with antibodies directed against EGFR or β1-integrin (43). Different antibodies reversed invasive and metastatic breast cancer cells to a near normal phenotype of growth-arrested acini structures, or caused cell death. Phenotypic reversion of T4-2 cells was also achieved by treatment with a PI3 kinase inhibitor (44). Similar to the studies by Bissell, we found that BMS-536924 caused a phenotypic reversion of CD8-IGF-IR acini structures to near-normal polarized MCF10A acini. Most strikingly, multi-acini structures didn’t develop after treatment with BMS-536924. Consistent with this, BMS-536924 was able to inhibit proliferation and partially restore polarization. In contrast to the partial reversion in 3D culture, BMS-536924 completely reversed all measures of transformation in vitro.

BMS-536924 showed a lower IC_{50} against CD8-IGF-IR-MCF10A and MCF7 cells grown in anchorage-independent versus monolayer cultures. This is consistent with studies showing that IGF-IR is not an essential requirement for monolayer growth (45, 46) but that IGF-IR is a strict requirement for anchorage-independent growth (39). Presumably there are IGF-IR-dependent survival pathways that are selectively active in anchorage-independent conditions and are inhibited by BMS-536924. In addition, BMS-536924 was effective at blocking migration of MDA-MB-231 cells at a concentration below the IC_{50} for monolayer proliferation. Our results thus reaffirm the need to test inhibitors in multiple settings and not only in monolayer proliferation assays.

Overexpression of a dominant-acting oncogene can cause cells to become “addicted” to the oncogene (47). This is in part revealed by overexpression of the CD8-IGF-IR in MCF10A cells. Thus, CD8-IGF-IR allows MCF10A cells to grow in serum-free medium, but they are now completely reliant upon IGF-IR for proliferation, because blockade causes a complete loss of cells in S-phase. Interestingly, BMS-536924 did not block proliferation or S-phase fraction of CD8-IGF-IR-MCF10A cells grown in complete medium, indicating that in this setting other factors confer proliferation. In contrast to this, BMS-536924 completely inhibited migration in both serum-free medium and complete medium, indicating that active IGF-IR is both sufficient and required for migration in both conditions.

The xenograft results are particularly striking when contrasted to the partial reversion in 3D culture, BMS-536924 completely blocked and/or reversed all aspects of transformation of malignant T4-2 cells grown in vivo. In addition, BMS-536924 causes a phenotypic reversion of CD8-IGF-IR-induced transformed acini structures, accompanied by suppression of proliferation, induction of apoptosis, and a partial restoration of polarization. Finally BMS-536924 can restore polarization in MCF7 cells, and affects growth of a range of breast cancer cell lines, highlighting a possible role in breast cancer therapy.

Disclosure of Potential Conflicts of Interest

J.M. Carboni, R.M. Attar, M.M. Gottardis, and C.R. Fairchild are employed by Bristol-Myers Squibb. A.V. Lee is a member of the speakers bureau of Bristol-Myers Squibb.

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BMS-536924 Reverses IGF-IR-Induced Transformation of Mammary Epithelial Cells and Causes Growth Inhibition and Polarization of MCF7 Cells
