Dual IGF-1R/InsR inhibitor BMS-754807 synergizes with hormonal agents in treatment of estrogen-dependent breast cancer

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ABSTRACT

Insulin like growth factor (IGF) signaling has been implicated in the resistance to hormonal therapy in breast cancer. Using a model of post-menopausal, estrogen-dependent breast cancer, we investigated the anti-tumor effects of the dual IGF-1R/InsR tyrosine kinase inhibitor BMS-754807 alone and in combination with letrozole or tamoxifen. BMS-754807 exhibited anti-proliferative effects in vitro that synergized strongly in combination with letrozole or 4-hydroxytamoxifen and fulvestrant. Similarly, combined treatment of BMS-754807 with either tamoxifen or letrozole in vivo elicited tumor regressions not achieved by single agent therapy. Notably, hormonal therapy enhanced the inhibition of IGF-1R/InsR without major side effects in animals. Microarray expression analysis revealed downregulation of cell cycle control and survival pathways and upregulation of erbB in response to BMS-754807 plus hormonal therapy, particularly tamoxifen. Overall, these results offer a preclinical proof of concept for BMS-754807 as an anti-tumor agent in combination with hormonal therapies in hormone-sensitive breast cancer. Cooperative cell cycle arrest, decreased proliferation, and enhanced promotion of apoptosis may contribute to anti-tumor effects to be gauged in future clinical investigations justified by our findings.
INTRODUCTION

Hormonal therapies are front line systemic therapies for patients with estrogen responsive breast cancer (ERBC). The selective estrogen receptor modulator (SERM), tamoxifen, for instance has demonstrated improved survival in breast cancer patients for over 25 years(1). However, resistance to therapies targeting the estrogen receptor signaling pathway represents a major clinical hurdle(2).

Mounting data suggests that the insulin-like growth factor (IGF) system is a major determinant in the development of resistance to therapies targeting estrogen signaling(3). As an estrogen dependent gene, IGF-1 receptor (IGF-1R) expression is modulated by estrogen signaling(4). In addition, IGF-1, by a number of mechanisms, regulates estrogen receptor dependent transcription(5). The combination of IGF-1 and estradiol synergistically stimulate growth of ERBC and crosstalk pathways between these systems have implicated the IGF-1 system as a mechanism of resistance to endocrine therapy in breast cancer(6-9). Furthermore, the proliferative effects of IGF-1 can be attenuated by tamoxifen and cells that have been selected to become resistant to tamoxifen have increased responsiveness to the proliferative effects of IGF-1(10). Recently, data has suggested direct interactions between estrogen and IGF-1R may be important for mitogenic estrogen receptor signaling(11).

Thus, targeting both the IGF signaling pathway and the estrogen receptor pathway is an attractive strategy for enhancing the clinical activity of endocrine therapy, as well as preventing or delaying the development of resistance. Currently, it is unclear whether estrogen deprivation or estrogen receptor inhibition would have a greater anti-tumor effect in combination with IGF-1 blockade. This distinction becomes important as the two classes of approved endocrine therapies (aromatase inhibitors and selective estrogen receptor modulators, respectively) function by these differing mechanisms. Preclinical data with a monoclonal antibody directed at the IGF-1R has demonstrated enhancement of tamoxifen activity in vivo (12). However, in post-menopausal breast cancer patients, aromatase inhibitors are often used as first line hormonal therapy due to superior activity over tamoxifen(13, 14). Thus, to optimize the selection of the most appropriate agent to investigate in combination with IGF-1 blockage, preclinical assessment of activity in an in vivo model is necessary.
In regards to blocking IGF signaling, the majority of current strategies aimed at blocking the IGF system focus on the IGF-1 receptor (IGF-1R). The IGF-1R is a transmembrane tyrosine kinase that is the major signaling receptor for the IGF-1 pathway. The functional receptor consists of two subunits (α and β) in a heterodimeric structure. Upon activation by the mitogenic ligands IGF-1 and IGF-2, the IGF-1R becomes autophosphorylated, stimulating the activation of downstream intracellular pathways (namely, the PI3K/AKT and Ras/MEK/ERK pathways) that lead to tumor proliferation, survival and metastasis. Additionally, the IGF-1R half-receptor can dimerize with the insulin receptor (InsR) tyrosine kinase, which shares a high degree of homology to the IGF-1R. Dimerization of these “hybrid-receptors’ have different biological activity and ligand specificity. In particular, the fetal or A isoform of the InsR appears to have a more mitogenic role in cancer cell proliferation than its purely metabolic isoform B. The varying biological activities of the InsR isoforms are likely related to their differing affinities for IGF-1 system ligands. For instance, while the metabolic InsR isoform B only binds insulin at physiologic concentrations, the InsR isoform A is able to bind and be activated by IGF-2. Thus, InsR isoform A through dimerization with IGF-1R or homo-dimerization may provide mitogenic stimuli to cancer cells through activation by IGF-2. Accumulated data has implicated the InsR isoform A, or the InsR total content, as being important in breast cancer progression and survival. More recent data suggest it may also be a mechanism of resistance to therapies that specifically target the IGF-1 receptor, such as monoclonal antibody therapies. Patients with node negative breast cancers whose tumors express high InsR content have worse disease free survival than patients with even moderate InsR content. Early studies have also shown that approximately 80% of breast cancers have an InsR content higher than the median content found in the normal breast and approximately 20% of cancers show InsR over 10-fold higher than the median value of the normal breast tissue. Early studies targeting the IGF-1 receptor in patients with refractory tumors have demonstrated that monoclonal antibody therapies may induce upregulation of insulin secretion, suggesting a compensatory mechanism which could possibly activate InsR signaling as a mechanism of resistance. Thus, if InsR isoform A expression is an important mechanism of proliferation of breast cancer cells and an important mechanism of resistance to IGF-1 targeted antibody therapy, dual kinase inhibitors of InsR and the IGF-1R may have a therapeutic advantage.

Based on these data, we conducted a pre-clinical study investigating the efficacy of a small molecule inhibitor of the IGF-1R and InsR, BMS-754807, in the estrogen-dependent, aromatase-expressing breast cancer model, MCF-7/AC-1 both with and without hormonal therapy. Our hypothesis was that complete IGF blockade would increase the
antitumor activity of hormonal therapy in ERBC. We have also used this model system to evaluate the tolerability of these treatments in vivo and perform correlative studies to identify potential biomarkers of anti-tumor activity in response to IGF blockade.

MATERIALS AND METHODS

Reagents

Phenol red–free modified IMEM, DMEM, penicillin/streptomycin solution, 0.05% trypsin-EDTA solution, Dulbecco's PBS, and geneticin (G418) were obtained from Life Technologies (Carlsbad, CA). Fetal bovine serum (FBS) and charcoal/dextran–treated FBS were obtained from Hyclone/Thermo Scientific (Rockford, IL). Matrigel was purchased from BD Biosciences (Sparks, MD). Androstenedione, 4-hydroxytamoxifen (for in vitro use), tamoxifen (for in vivo use) and hydroxypropyl cellulose was purchased from Sigma Co. (St. Louis, MO). Enhanced chemiluminescence (ECL) kits and Hybond-ECL nitrocellulose membranes were purchased from Amersham Biosciences/GE Healthcare (Piscataway, NJ). IGF-1 LR3 was purchased from GroPep (Thebarton, Australia). Antibodies against p-AKT, AKT, p-IGF-IRβ/InRβ, IGF-IRβ, p-MAPK, MAPK were purchased from Cell Signaling Technology (Danvers, MA). An antibody against β-actin was purchased from Sigma-Aldrich (St. Louis, MO). Antibodies against insulin Rβ, ERα were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). An antibody against Ki-67 was purchased from Abcam (Cambridge, MA). Horseradish peroxidase–conjugated anti-mouse and anti-rabbit antibodies were purchased from Invitrogen (Carlsbad, CA). MCF-7 human breast cancer cells stably transfected with the human aromatase gene were provided by Dr. Angela Brodie and Shiuan Chen (Beckman Research Institute of City of Hope, Duarte, California) as previously reported (26). Cells authentication was performed using 16 loci short-tandem repeat profiling at the completion of included studies. Letrozole (Femara, CGS 20267) was kindly provided by Dr. D. Evans (Novartis Pharma, Basel, Switzerland).

Cell culture

MCF-7 human breast cancer cells stably transfected with the human aromatase gene (MCF-7/AC-1 cells) as previously described (25) were routinely maintained in DMEM with 10% fetal bovine serum, 1% penicillin/streptomycin solution, and 750 μg/mL G418. The culture medium was changed twice weekly.
MTS proliferation assay

The effect of the various drugs and hormones on MCF-7/AC-1 cellular growth was examined using the MTS proliferation assay (CellTiter 96 Aqueous, Promega, Madison, WI), as previously described(27). Briefly, cells growing in regular media were transferred to IMEM containing 5% charcoal-stripped serum and 1% penicillin/streptomycin (charcoal-stripped serum in medium, CSSM) for 72 hours. This medium was used for all cell growth assays. The cells were detached from their flask using trypsin and seeded in 96-well plates at 1.0 x 10^3/mL (100 μL) on day 0. Twenty-four hours later, several concentrations of the hormones or drugs were added at doses indicated in the text. The cells were incubated for 6 days in the absence and presence of the tested drugs and hormones. At the end of the treatment, the MTS dye reduction was assessed as per the product information label. Proliferation was calculated as a percentage of the non-drug-treated controls. Experiments were done in at least triplicate. To evaluate the effect of combination treatment with BMS-754807 and tamoxifen or letrozole or fulvestrant, the method of Chou and Talalay was used to determine synergy as described previously(28). Median effect analysis was done using CalcuSyn software (Biosoft, Cambridge, UK). Mean values of the combination index at the affected fractions of 50% (Fa50) and 75% (Fa75) are shown. A CI value significantly less than 1 indicates synergism, a CI not significantly different from 1 indicates addition, and a CI significantly higher than 1 indicates antagonism.

Postmenopausal intratumoral aromatase mice xenograft model

Female ovariectomized BALB/c athymic nude mice 4–6 weeks of age were obtained from Harlan Laboratories (Indianapolis, IN) The animals were housed in a pathogen-free environment under controlled conditions of light and humidity and received food and water. All animal studies were carried out according to the guidelines approved by the Animal Care Committee of the Mayo Clinic, Rochester MN. Animals were allowed to acclimatize for 48 h after shipment before tumor inoculation was performed. For inoculation, subconfluent MCF-7/AC-1 cells were suspended in Matrigel (10 mg/mL) at 2.5×10^7 cells/mL. Each mouse was injected s.c. with 100 μL of cell suspension on each flank. Tumors were measured weekly with calipers, and volumes were calculated with the formula 4/3π x r_1^2 x r_2 (r_1 < r_2), where r_1 is the smaller radius. Treatments began when the tumors reached a measurable size (250-300 mm3). Mice randomized to treatment groups using JMP (SAS, Cary, NC).

Mice received subcutaneous injection with vehicle, non-steroidal aromatase inhibitor letrozole (10 μg/day), and antiestrogen tamoxifen (500 μg/day), which were all prepared as suspensions in 0.3% hydroxypropyl cellulose; IGF-
1R/InsR inhibitor BMS-754807 (50mg/kg/day) was prepared in PEG400:water (80:20) and was administered by daily
gavage; the combinations of letrozole, and/or tamoxifen with BMS-754807 were given at the full doses. All treatments are
given once daily for 28 days continuously. All animal groups were supplemented with androstenedione daily for the
duration of the experiment.

After 28 days of treatment, one of the two flank tumors was surgically excised from each tumor-bearing animal. Animals
were then maintained observed until the study endpoint (300% tumor growth from treatment baseline) at which point the
remaining tumor was resected at the time of animal sacrifice. After each resection, tumors were carefully excised from
mouse tissue with portions frozen in OCT medium (Tissue-Tek, Redding, CA) or placed in formalin for further analyses.
Differences between the groups were analyzed by ANOVA with multiple comparison post-tests using Prism (Graph Pad,
LaJolla, CA).

Mouse weight and blood glucose measurements

Blood glucose levels in all mice were measured from lateral tail vein pricks in the morning using an Ascensia Elite XL
glucose meter (Bayer) and Glucometer Elite test strips (Fisher Scientific). One glucose measurement required
approximately 3 uL of blood. Depending on the volume of blood that flowed out by a tail vein prick, 1 or 2 glucose
measurements were taken. In the case of 2 measurements, the average was used in the calculations. Mouse weight was
measured every other day.

Immunohistochemical (IHC) staining

At autopsy, the tumors were resected and processed for routine gross and microscopic examination. The tumor tissues
were fixed in 10% neutral-buffered formalin and embedded in paraffin. Serial sections (5 μm thick) were cut on a
microtome and mounted on glass slides. For histopathological examination, every fourth section was de-waxed in
Histoclear (National Diagnostic, Atlanta, GA, USA) and hydrated in graded alcohol solutions and distilled water for H&E
staining and examined under a light microscope.

For IHC staining, prior to use, the slides were washed three times (5 min each) in xylene to remove paraffin, three times
(5 min each) with 100, 90, and 70% ethanol (in that order), and hydrated in distilled water before tissue treatment.
Antigens were retrieved by treatment for 45 min in 0.001 M sodium citrate buffer (pH 6) in a water bath (95–99 °C). The
endogenous peroxides activity was inhibited with 3% hydrogen peroxide for 5 min. Non-specific binding was blocked by incubation for 30 min with 2% BSA in PBS; specimens were washed five times in PBS and incubated -overnight at 4 degrees with different antibodies, including anti-human Ki-67 (1:500). After five further washes in PBS, specimens were incubated with second antibodies (Invitrogen, Carlsbad, CA). Staining reaction was carried out using horseradish peroxidase-mediated AEC (aminoethyl carbazole) or DAB immunostaining. (Zymed Laboratories, San Francisco, CA). Red or brown deposits indcated the sites of positive immunostaining and were counterstained with Mayer’s hematoxylin solution by standard procedures.

**Western blot analysis**

Inhibition of IGF-1R and insulin receptor phosphorylation and ERK/Akt pathways by BMS-754807 alone or combination with 4-OH Tamoxifen(10 μmol/L); Letrozole(10 μmol/L) or Fulvestrant(100nmol/L) were determined by Western blot in Briefly, cells were cultured in IMEM steroid-reduced medium without phenol red for 24 hours, then subconfluent MCF-7/AC-1 cells were treated with either DMSO, BMS-754807 (10 μmol/L), or BMS-754807 (10 μmol/L) plus hormones for 24 h in serum-free conditions. For the final 15 min drug treatment, 10 nmol/L LongR3 IGF-I was added to the medium. Lysates were then prepared and analyzed by Western blotting.

Western blotting was performed as previously described (27). Briefly, proteins were extracted from the tumor tissues by homogenization in buffer containing 50 mM Tris (pH 7.4), 1 mM EDTA, 150 mM NaCl and protease/phosphatase inhibitors (1 μg/ml phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin and 1μg/ml leupeptin). The homogenates were centrifuged at 2000g for 15 min at 4°C. After centrifugation at 10,000 x g for 5 min, the supernatants were separated and their protein concentrations were measured. The protein lysates were separated by 10% SDS-PAGE, transferred onto Immuno-Blot polyvinylidene difluoride (PVDF) membrane (catalog no. 162-0177, Bio-Rad), and Western blot analysis was performed as described previously(27). The membranes were blocked with 5% milk in TBS (10 mM Tris-HCl (pH 8.0) and 150 mM NaCl) plus 0.05% Tween-20 overnight at 4°C and then incubated in 5% milk containing primary antibodies (1:1000/1500 for both antibodies or 1:2500 dilution for Actin) overnight at 4°C. After incubation, membranes were washed three times (15 min each) with 5% milk, incubated with goat anti-rabbit IgG conjugated with horseradish peroxidase (1:5000) in 5% milk for 1 h at room temperature, and washed three times (15 min each) in TBS. The bands were detected using an ECL kit (Amersham, Arlington Heights, IL). Densitometry was performed using Genetools software (Syngene, Frederick, MD).
RNA isolation and Gene Expression Profiling

Total RNA was isolated using Trizol reagents (Cat#15596-026, Invitrogen, Carlsbad, CA). 1 μg of total RNA isolated from tumors surgically excised from each tumor-bearing animal after 28 days of treatment were used to generate gene expression data using Affymetrix HT-HG-U133A GeneChip® (Affymetrix, Santa Clara, CA) according to the manufacturer’s instructions. The microarray data were analyzed by ANOVA using Partek® software to identify genes differentially expressed between different treatment groups. Gene expression data was analyzed by Ingenuity Pathway Analysis to identify pathways that were up or down regulated in response to in vivo treatments (Ingenuity Systems, Redwood City, CA). DNA microarray data is available through the Gene Expression Ominibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo/) using the GEO accession # GSE33366.

Statistical analysis

Error bars represent standard error of the mean. Differences in the mean of two samples were analyzed using Student's unpaired t-test. For comparisons between multiple samples, ANOVA with Tukey’s multiple comparisons post-test was used. For p-values, differences <0.001 was considered extremely significant (***) ; 0.001 to 0.01 very significant (**); 0.01 to 0.05 significant (*). P-values >0.05 were considered not significant (ns).
RESULTS

MCF-7/AC-1 cells are estrogen-driven breast cancers cells *in vitro* and *in vivo*. The MCF-7/AC-1 cells were employed in a series of experiments due to their responsiveness to estrogen and sensitivity to clinically estrogen-targeting agents, including the SERM tamoxifen, the aromatase inhibitor, letrozole, and the pure antiestrogen, fulvestrant. To confirm that the MCF-7/AC-1 cells were estrogen-dependent *in vitro*, MCF-7/AC-1 cells were cultured in IMEM containing 5% charcoal-stripped serum with or without the aromatase substrate androstenedione (1nmol/L). After 6 days incubation, androstenedione stimulated a 4-fold growth in MCF-7/AC-1 cells (Fig. 1A). Furthermore, this androstenedione-induced, estrogen-dependent cell growth can be blocked by 4-hydroxytamoxifen in a dose dependent manner (Fig. 1B). In *vivo*, AC-1 cells reliably formed xenografts that were palpable in 3-4 weeks upon androstenedione supplementation (Fig 1C). Without androstenedione, xenografts did not reliably form. The uteri of AC-1 tumor-bearing mouse were substantially larger in the group receiving androstenedione compared to controls (Fig. 2A), supporting the evidence that androstenedione was being converted to estrogens *in vivo* and contributing to the proliferative effects on mouse endometrium (Fig. 2B). Thus, in our model, the tumors being generated are estrogen dependent and reliably heterotransplanted only in animals receiving androstenedione, supporting functional aromatase expression and estradiol production.

**Synergistic effect of BMS-754807 in combination with tamoxifen or letrozole on growth of MCF-7/AC-1 cells *in vitro***. Based on the findings above, MCF-7/AC-1 cell proliferation was assessed by treatment with various concentrations of BMS-754807, 4-hydroxytamoxifen, letrozole and fulvestrant either as single agent or 4 hydroxytamoxifen, letrozole or fulvestrant in combination with BMS-754807 at a fixed ratio. At doses of the single agents that had modest anti-proliferative effects, the combination treatment appeared to have a significant anti-proliferative effect (P < 0.001; Fig. 3A-3C). In comparison to the single agent anti-proliferative effects, the combination of BMS-754807 with letrozole, 4-hydroxytamoxifen or fulvestrant was strongly synergistic at the 50% and 75% fraction affected (P < 0.001; Fig. 3D).

**Enhanced effects of combined IGF blockade and hormonal therapy on AKT and ERK pathway signaling**. To investigate possible mechanisms for the synergy between BMS-754807 and hormonal therapies, we investigated the effects of BMS-754807 with and without hormonal therapies on the major proliferation and survival pathways of IGF signaling- AKT and ERK1/2 (Fig. 4, Supplemental Figure 2). BMS-754807 alone very effectively inhibited pIGF-1R and
pAKT and had modest effects on pERK1/2 in MCF-7/AC-1 cells \textit{in vitro}. All hormonal therapies investigated enhanced the ability of BMS-754807 to decreased phosphorylation of IGF-1R and AKT in the low nanomolar range. 4-Hydroxytamoxifen appeared to be the most effective at enhancing the inhibition of AKT phosphorylation in combination with BMS-754807. Fulvestrant was the most effective hormonal therapy at enhancing the inhibition of IGF-1R phosphorylation by BMS-754807. Only letrozole appears to substantially reduce the phosphorylation of ERK1/2 in combination with BMS-754807. Total levels of IGF-1R, AKT, ERK1/2 and InsR were unchanged by BMS-754807 alone or in combination with 4-hydroxytamoxifen, letrozole, or fulvestrant. However, the estrogen receptor levels were decreased in the presence of fulvestrant and letrozole.

**Effect of combination of BMS-754807 and tamoxifen or letrozole on growth of MCF-7/AC-1 cells xenografts \textit{in vivo}.** To determine if the \textit{in vitro} synergy translated into enhanced tumor regression and improvement in time to tumor progression, the effects of BMS-754807 in combination with hormonal therapy was investigated compared to single agents \textit{in vivo}. As both tamoxifen and letrozole represent front-line breast cancer therapies with differing mechanisms (SERM vs. aromatase inhibitor, respectively), these agents were selected for \textit{in vivo} investigations. MCF-7/AC-1 cells were injected s.c. into both flanks of athymic nude mice supplemented with androstenedione daily (100 μg/mouse/d s.c.). Once the tumors reached a measurable size of 250-300 mm$^3$ (after 4-5 weeks), the mice were randomly assigned to six treatment groups so that mean tumor volumes were not significantly different at the start of treatment: control (vehicle alone), letrozole (10μg/day s.c.), tamoxifen (500μg/day s.c.), and BMS-754807 (50mg/kg/day), BMS-754807 plus letrozole or plus tamoxifen. All treatments are given once daily for 28 days continuously. All groups' animals were supplemented with androstenedione daily during the whole experiments.

At the end of 28 days treatment, the tumors in the control group (203%) doubled in size after 4 weeks whereas the tumors in the letrozole (p < 0.0005 vs. control), tamoxifen (p < 0.05 vs control) or BMS-754807 (p < 0.005) groups demonstrated significant growth inhibition compared to controls. There was no significant difference in the growth of the tumors in the single agent treated groups. The tumors in mice receiving BMS-754807 with tamoxifen had significantly greater antitumor activity compared either agent alone (Fig. 5A, p < 0.005 for BMS-754807/tamoxifen vs. BMS-754807; p<0.0005 for BMS-754807/tamoxifen vs. tamoxifen). Similarly, tumors in mice receiving BMS-754807 with letrozole had greater antitumor activity than either agent alone (Fig. 5B, p < 0.005 for BMS-754807/letrozole vs. BMS-754807; p<0.05 for
BMS-754807/letrozole vs. letrozole). After 28 days treatment, there is not significant difference between BMS/Tamoxifen and BMS/Letrozole (p=0.0514). After 28 days of treatment, the control cohort animals were sacrificed; in the experimental groups, one of the two flank tumors, which were the biggest was surgically excised from each tumor-bearing animal. The mice were then followed without any treatment except daily androstenedione until the correlative endpoint (300% tumor growth from treatment baseline) was met. The tumor volumes were followed by once a week measurements. Times to the endpoint after 28 days treatment with BMS-754807/tamoxifen was significantly improved over tamoxifen alone (Fig. 5C; p= 0.0159) and BMS-754807 alone (p=0.0031). There was no significant improvement in time to endpoint for the treatment of BMS-754807/letrozole over letrozole alone or BMS-754807 alone (Fig. 5D; p >0.05).

Effects of BMS-754807 on mice blood glucose and weight change. As BMS-754807 is a dual inhibitor with activity against the IGF-1R and InsR kinase, we explored the tolerability of daily BMS-754807 as a single agent and in combination with hormonal therapy by assessing blood glucose and animal weight during the treatment. Comparisons between all treatment groups demonstrated no significant difference between any of the treatment groups during the course of treatment (Fig. 6A; p= 0.5622). In contrast, treatment with BMS-754807/tamoxifen resulted in significant weight loss compared to all other treatment groups (Fig. 6B; p< 0.0001). However, the weight at treatment end was not different between the treatment groups (p = 0.2657).

Gene expression profiling reveal modulation of cell cycle and pro-survival pathway genes
To define the mechanisms of enhanced activity in combination of targeting both the estrogen receptor (ER) and IGF-1R/InsR, We conducted a microarray study on tumors that were surgically excised from tumor-bearing animal after 28 days of treatment. Statistical analyses were performed to identify 868 probe sets representing 698 unique genes that were significantly modulated by any treatment with false discovery rate (FDR)=5% and two-fold changes when compared to the untreated control (Supplemental Table 1). Both hormonal agents had similar change in gene expression with some in different directions, and the combination of BMS-754807 with tamoxifen further enhanced the changes in gene expression patterns (Fig. 7A). Both letrozole and tamoxifen decreased IGF-1R expression and the latter had more significant effects; while ER expression was only down regulated by tamoxifen not by letrozole (Fig. 7B). BMS-754807 did not alter expression of either IGF-1R or ER expression, nor did it substantially effect the changes in expression due to hormonal therapy. Significant changes were demonstrated after study treatments in genes involved in survival
proliferation (Fig. 7 C, D). Among the cell cycle and survival genes, BIRC5 (gene for survivin), BCL2, CCNA2 (gene for Cyclin A2), and MKI67 (gene for Ki-67) were down regulated by study treatments, though these effects were most profound in the BMS-754807 + tamoxifen treatment group, particularly for MKI67. Receptor tyrosine kinases EGFR, ERBB2, ERBB4, MET, EPHA4 and TGFBR2 were upregulated in response to BMS-754807 + tamoxifen and, to a lesser extent, other study treatments. For example, both hormonal agents increased ERBB4 expression, which was enhanced by BMS-754807; EPHA4, MET and TGFBR2 expression were only affected by tamoxifen not by letrozole, BMS-754807 in combination with tamoxifen extended the up-regulation of these genes; whereas EGFR expression only modestly changed in response to single drug treatment but increased substantially by tamoxifen/ BMS-754807 in combination.

Ingenuity Pathway Analysis was performed to compare the involvement of biological functions and canonical signaling pathways of genes modulated by each treatment condition (Supplemental Figure 1). The top significant differences in biological functions (e.g. cell cycle, growth and proliferation, cell death) and canonical signaling pathways (e.g. ATM signaling and p53 signaling) were shown in Supplementary Figure 1A and 1B, respectively. Genes that were further modulated by tamoxifen and BMS754807 were involved in cell cycle regulation (e.g., CCNA2, CCNB1, CCNB2, CCNE2, CDC6, CDC7 CDK2, CHEK1 and CHEK2), anti-apoptosis (e.g., BAG3, BCL2, and BIRC5), and cell proliferation (e.g. Ki67 and PCNA).
DISCUSSION

We have demonstrated that targeting IGF signaling has antitumor activity in combination with hormonal therapies. This is clinically significant, as resistance to hormonal therapy is a common clinical problem that limits survival in patients with breast cancer(2). As agents targeting IGF-1R/InsR, such as BMS-754807 are currently undergoing clinical investigation, these data would support the combination of IGF targeted therapy with hormonal therapy. Interestingly, BMS-754807 appears to synergize with hormonal therapies agents that target ER signaling by different mechanisms with similar efficiency. This would suggest that IGF signaling has a role in estrogen signaling downstream of the ER. Indeed, despite having similar in vitro and in vivo efficacy during treatment, letrozole in combination with BMS-754807 decreased ER expression, while tamoxifen in combination with BMS-754807 led to increased ER expression by western blotting (Fig 4). This contrasted the RNA expression of ER in both cases, suggesting compensatory mechanisms of regulation. In regards to the IGF targeting component, initial data suggests that the method of IGF targeting does indeed matter. Preliminary data with the same MCF-7/AC-1 model suggests that using a monoclonal antibody against the IGF-1R does not enhance the efficacy of hormonal therapy in vivo(29, 30). Though unclear as to why this may be different, monoclonal antibody therapy against the IGF-1R induced upregulation of InsR A expression, which is an IGF signaling receptor(31). This upregulation of InsR-A expression is not substantially modulated in response to BMS-754807 either as a single agent or in combination with hormonal therapy. This may also explain the recent negative clinical trial of the anti-IGF-1R monoclonal antibody, AMG-479, in combination with hormonal therapy in patients with breast cancer(32). These data in summation would suggests that targeting the InsR is a critical component of enhancing the antitumor effects of hormonal therapy targeting in breast cancer.

In regards to targeting the InsR, there are hypothetical concerns regarding the safety and tolerability of this approach(33). This largely contributed to the initial suggestion in the field of selectively targeting IGF signaling with an anti-IGF-1R monoclonal antibody to avoid metabolic dysregulation and represented the most viable strategy at the time. However, ironically, among the most common side effects from anti-IGF-1R targeted monoclonal antibodies is hyperglycemia(22, 34). Importantly, early investigations with IGF-1R/InsR targeting small molecule inhibitors have demonstrated reasonable tolerability with mild adverse event profiles that include manageable hyperglycemia (35-37). Clinical investigations will be needed to determine if this is tolerable and effective method of IGF signaling blockade in combination with hormonal therapy.
The mechanism of synergy between anti-estrogen hormonal therapy and BMS-754807 appears multifactoral. We suggest several mechanisms based on the expression analyses that demonstrate differences between genes that are modulated in response to BMS-754807 with tamoxifen and compare this to the effect of BMS-754807 with letrozole, which had no improvement in time to study endpoint (Fig. 5 C, D). This is an intriguing comparison as the improvements in the primary endpoints were similar for BMS-754807 with either tamoxifen or letrozole (Fig. 5 A, B). One can speculate that this results from the described ‘withdrawal’ response to tamoxifen. Tumor response after withdrawal of tamoxifen was first described by Legault-Poisson et al.(38) and then by other investigators (39-43), Prospective data has also demonstrated that up to 40% of patients progressing on tamoxifen had a response or prolonged stability after tamoxifen withdrawal (44). Withdrawal responses from aromatase inhibitors, however, are not as well described.(45, 46). These clinical findings may explain our results following the completion of therapy.

Overall, the DNA microarray data from BMS-754807/tamoxifen-treated tumors suggested a stronger repression of proliferative, cell cycle, DNA damage and survival markers than other treatments investigated, including BMS-754807/letrozole. BMS-754807 in combination with tamoxifen had a substantial decrement in Ki-67 gene expression at the end of treatment in comparison all other treatments, including BMS-754807 in combination with letrozole (Fig. 7 C). It is possible that the enhance effects of the Ki67 downregulation after 28 days of treatment with BMS-754807 and tamoxifen led to the extended benefits of the combination beyond the end of treatment that were not seen with BMS-754807 in combination with letrozole. Future studies will be needed to determine if this effect contributed to the long term benefits of the BMS-754807 plus tamoxifen combination. In addition, canonical pathway analyses for hereditary breast cancer, ATM, DNA damage and cell cycle check point signaling were strikingly upregulated in response to BMS-754807 + tamoxifen. Western blotting also suggested that the combination of BMS-754807 with 4-hydroxytamoxifen maximally blocked survival signaling through AKT (Fig. 4). Thus, future biomarker evaluations in studies investigating BMS-754807 in combination with hormonal therapy should include evaluation of cell cycle, DNA damage, survival and proliferation markers, including Ki-67.
Expression of the erbB family of receptors, as well as other receptor tyrosine kinases identified in Fig. 7D, may also be important biomarkers of resistance and represent adaptive mechanisms of escape in response to IGF and estrogen targeted therapy. Modulation of erbB receptors has been previously described as a adaptive pathway to IGF blockade and may be sufficient for tumor cells to overcome IGF targeting strategies, such as BMS-754807(27, 47). As erbB-targeted therapies are available, this may support the rationale for combinations of BMS-754807 with either EGFR- or HER2-targeted therapy in tumors expressing these proteins.

In summary, IGF-1R/InsR inhibition with BMS-754807 enhances the antitumor effects of hormonal therapy in vitro and in vivo. These preclinical data led us propose a phase II non-comparative clinical trial investigating the combination of BMS-754807 and letrozole or single agent BMS-754807 in patients with locally advanced/metastatic, estrogen receptor-positive, non-steroidal aromatase inhibitor resistant breast cancer (ClinicalTrials.gov number NCT01225172) which is ongoing. These preclinical data also suggest that BMS-754807 with other hormonal agents including tamoxifen or fulvestrant warrant further investigation.
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(none)
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REFERENCES


TABLES

(none)
FIGURE LEGENDS

Figure 1. MCF-7/AC-1 cells are estrogen-driven in vitro and estrogen-dependent in vivo. Cells were cultured in IMEM steroid-reduced medium without phenol red for 2 days before plating. Cell proliferation was measured using an MTS assay, as described in methods.

A) Effect of presence of androstenedione (Ad) on MCF-7/AC1 cell growth. Cell growth is expressed as the percentage of the cells compared with the control wells (untreated cells). Columns, mean; bars, SE.

B) Antiproliferative effect of increasing concentrations of 4-hydroxytamoxifen in the presence of 1 nmol/L androstenedione on MCF-7/AC1 cells. Cell proliferation is expressed as the percentage of the cells compared with the control wells (1 nM androstenedione treated cells). CSSM, untreated cells cultured in steroid-reduced medium. Columns, mean; bars, SE.

C) MCF-7/AC-1 xenografts, each mouse received s.c. inoculations in two sites per flank with 100 µL of MCF-7/AC-1 cell suspension containing 2.5 × 10⁶ cells. The mice were injected daily with supplemental androstenedione (100 µg/day) or vehicle from day 0. Tumors were measured with calipers weekly throughout experiment. Points, mean; bars, SE.

Figure 2. Uterotropic effects of mice bearing MCF-7/AC-1 tumors, with and without androstenedione. Each mouse received s.c. inoculations in two sites per flank with 100 µL of MCF-7/AC-1 cell suspension containing 2.5 × 10⁶ cells. The mice were injected daily with supplemental androstenedione (100 µg/day) or vehicle from day 0. At day 28, the mice were sacrificed and uteri were removed from the mice, weighed, formalin-fixed and embedded in paraffin.

A) Morphological appearances of two representative uteri from ovariectomized MCF-7/AC-1 xenografts bearing mice were shown with or without androstenedione for 28 days.

B) Analysis of cell proliferation. Uterine cross-sections were examined by Ki-67 immunostaining. Reddish nuclear deposits indicate the sites of positive immunostaining (20X).

Figure 3. Antiproliferative effects of BMS-754807 and/or hormonal therapy in vitro. MCF-7/AC1 cells were cultured and after 6 days treatment cell proliferation measured as above. Proliferation was assessed in the presence of BMS-754807, hormonal therapies and a fixed ratio of increasing amount of combinations, as indicated. Cell proliferation is expressed as the percentage of the cells compared with the control wells (1 nM androstenedione treated cells).
A) 4-OH Tamoxifen (** P < 0.001); B) Letrozole (** P < 0.001); C) Fulvestrant (**** P < 0.0001). Points, mean; bars, SE.

D) Combination index values at the 50% (Fa50) and 75% (Fa75) fraction affected were plotted for the treatment combinations. All CI values plotted were significantly synergistic (P < 0.001). Columns, mean; bars, SE.

**Figure 4.** Inhibition of IGF-1R/InsR, ERK/Akt phospho signaling pathways by BMS-754807 alone or combination with 4-OH Tam (10 µmol/L); Letrozole (10 µmol/L) or Fulvestrant (100nmol/L). Briefly, cells were cultured in IMEM steroid-reduced medium without phenol red with 1 nM androstenedione for 24 hours, then subconfluent MCF-7/AC-1 cells were treated with either DMSO, BMS-754807 at the indicated concentrations, or BMS-754807 plus hormones for 24 h in serum-free conditions. For the final 15 min of drug treatment, 10 nmol/L LongR3 IGF-I was added to the medium. Lysates were then prepared and analyzed by Western blotting, as described in the Methods.

**Figure 5.** In vivo antitumor activity of single agent BMS-754807, letrozole, tamoxifen or in combinations.
Ovariectomized female nu/nu mice between the ages of 7-8 weeks old were inoculated with MCF7/AC1 tumor cells in each flank with supplemental androstenedione (100 µg/day). Once the bilateral flank tumors grew such that the average of their left and right tumor volumes are between 250-300 mm3, mice were randomized to each treatment group and given various treatments for a total of 28 days; treatment groups consisted of 8-10 mice/group, and the experiments were repeated three times. The tumor volumes were measured twice weekly. At day 28, the average of the left and right tumor volumes of each mouse and percent change from its pre-treatment average volume was determined, then one of the two flank tumors was surgically excised from each tumor-bearing animal. Animals were then observed until study endpoint. Tumor volume was measured at the intervals indicated and expressed as percentage change in tumor volume relative to start of treatment (Baseline, day 0).

A, B) At the end of study treatment, both the combination of letrozole + BMS-754807 and tamoxifen + BMS-754807 had significantly improved anti-tumor activity compared to the respective single agents. (Bilateral tumor measurements). Points, mean; bars, SE.

C, D) Time to endpoint following treatment with BMS-754807 and/or hormonal therapy. (Single remnant tumor measurements) Points, mean; bars, SE.
Figure 6. Glucose homeostasis and animal weight during in vivo therapy. During administration of 28 days of vehicle, BMS-754807, letrozole, tamoxifen or their combinations, both A) glucose B) weight of all animals on study were measured and recorded. Investigators were blinded to treatment groups by using implanted identification chips. Points, mean; bars, SE

Figure 7. Genes significantly modulated by either single hormonal agent or in combination with BMS-754807. RNA isolated from resected tumors at treatment end (Day 28) was used for DNA microarray experiments, as described in Methods.

A) Heat Map demonstrating the expression pattern changes and clustering of 698 genes by protocol treatment

B) Changes in IGF-1R and ER expression by protocol treatment. Columns, mean; bars; SD

C) Changes in cell cycle, proliferation, and pro-survival genes that represent the most significant changes in response to protocol treatment.

D) Changes in receptor tyrosine kinase genes that represent the most significant changes in response to protocol treatment.
Figure 2

A

+AC1

+AC1/Ad

H&E

Ki-67

B

+AC1

+AC1/Ad
Figure 4

- BMS-807
- p-IGF-1R
- Total IGF-1R
- p-AKT
- Total AKT
- p-ERK1/2
- Total ERK1/2
- Total ER
- Total InsR
- Actin
Figure 5: Graphs comparing tumor volume (% of baseline) over weeks of treatment for different drug regimens. The graphs show the effects of Control, Letrozole, BMS-754807, and BMS-754807/Tamoxifen treatments on tumor volume reduction.
Figure 7, A

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Dual IGF-1R/InsR inhibitor BMS-754807 synergizes with hormonal agents in treatment of estrogen-dependent breast cancer

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