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Experimental Treatment of Estrogen Receptor (ER) Positive Breast Cancer with Tamoxifen and Brivanib Alaninate, a VEGFR-2/FGFR-1 Kinase Inhibitor: a potential clinical application of angiogenesis inhibitors

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Abstract

Purpose—Tamoxifen, a selective estrogen receptor modulator (SERM), and brivanib alaninate, a VEGFR-2 inhibitor, are two target specific agents that result in a substantial decrease in tumor growth when given alone. Tamoxifen activates SERM stimulated breast and endometrial tumor growth. Tamoxifen and brivanib alaninate have side effects that can affect therapeutic outcomes. The primary goal of the current study was to evaluate the therapeutic effects of lower doses of both agents when given in combination to mice with SERM sensitive, estrogen stimulated tumor xenografts (MCF-7 E2 tumors). Experiments were conducted to evaluate the response of SERM stimulated breast (MCF-7 Tam, MCF-7 Ral) and endometrial tumors (EnCa 101) to demonstrate the activity of brivanib alaninate in SERM resistant models.

Experimental Design—In the current study, tumor xenografts were minced and bi-transplanted into the mammary fat pads of athymic, ovariectomized mice. Preliminary experiments were conducted to determine an effective oral dose of tamoxifen and brivanib alaninate that had minimal effect on tumor growth. Doses of 125 µg of tamoxifen and 0.05 mg/g of brivanib alaninate were evaluated. An experiment was designed to evaluate the effect of the two agents together when started at the time of tumor implantation. An additional experiment was done in which tumors were already established and then treated, to obtain enough tumor tissue for molecular analysis.

Results—Brivanib alaninate was effective at inhibiting tumor growth in SERM sensitive (MCF-7 E2) and SERM stimulated (EnCa 101, MCF-7 Ral, MCF-7 Tam) models. The effect of the low dose drug combination as an antitumor strategy for SERM sensitive (MCF-7 E2) in early

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treatment was as effective as higher doses of either drug used alone. In established tumors, the combination is successful at decreasing tumor growth, while neither agent alone is effective. Molecular analysis revealed a decreased phosphorylation of VEGFR-2 in tumors that were treated with brivanib alaninate and an increase in VEGFA transcription to compensate for the blockade of VEGFR-2 by increasing the transcription of VEGFA. Tamoxifen increases the phosphorylation of VEGFR-2 and this effect is abrogated by brivanib alaninate. There was also increased necrosis in tumors treated with brivanib alaninate.

Conclusion—Historically, tamoxifen has a role in blocking angiogenesis as well as the blockade of the ER. Tamoxifen and a low dose of an angiogenesis inhibitor, brivanib alaninate, can potentially be combined not only to maximize therapeutic efficacy but also to retard SERM resistant tumor growth.

Introduction

Angiogenesis is a major requirement for tumors to grow successfully and spread. Early work (1–3) characterized many of the factors involved in the regulation of angiogenesis and how these factors can become dysregulated in tumor pathogenesis (4–6). One of the most important factors in the positive modulation of angiogenesis is the vascular endothelial growth factor (VEGF) family of growth factors and their corresponding receptors. Angiogenesis in tumors is different from physiologic angiogenesis seen with normal development and wound healing. In wound healing, angiogenesis is a carefully orchestrated process and occurs in a short time. In contrast, the blood vessels that form in the tumor bed are thin, disorganized, and leaky. The growth of such vessels persists over years as long as viable tumor tissue is present (3).

In estrogen receptor (ER) positive breast cancer, it is clear that adjuvant anti-estrogenic therapy must be extended to 5 years and beyond to prevent recurrence and improve survival (7–9). However, toxicities, development of resistance to anti-hormonal therapy, and side effects from therapy such as clots and endometrial cancer with tamoxifen (10,11) and fractures and joint pain with aromatase inhibitors (11,12) often limit long-term treatment. Clearly new treatment strategies need to be developed to enhance the activity of anti-hormonal therapy by improving efficacy. Estrogen enhances the angiogenic cascade critical for tumor growth, primarily through the release of VEGF (13–16). Tamoxifen has a historical role in the prevention of tumor angiogenesis as it was one of the three drugs in the “Navy Regimen” developed by Folkman (17). Tamoxifen is also reported (18) to reduce angiogenesis for ER negative tumors. An anti-estrogen for the treatment of ER positive breast cancer can potentially regulate VEGF production. However, with the development of acquired resistance (10) in breast and endometrial tumors it is axiomatic that SERM (tamoxifen and raloxifene) stimulated tumors must induce angiogenesis to grow. We hypothesize that limiting angiogenesis with angiogenic drugs during anti-hormonal therapy could potentially improve adjuvant therapeutic regimens. However, there are significant toxicities with current anti-angiogenic drugs that limit their usefulness for long term therapy.

Several anti-angiogenic agents are either used in clinical practice or are in clinical trials. Most notably, bevacizumab, a monoclonal antibody that binds to VEGFA and as a result, prevents phosphorylation and activation of its target receptors, vascular endothelial growth factor receptor 1 and 2 (VEGFR-1 and VEGFR-2), has shown promise in combination with chemotherapy for breast cancer (19–21). In a phase 3 trial of 722 patients, the disease free survival time in patients with metastatic breast cancer has been shown to double (5.9 vs. 11.8 months) when treated with paclitaxel in conjunction with bevacizumab (21). Unfortunately, the overall survival does not change when bevacizumab is included as a part of therapy. Toxicities such as infection (9.3 vs. 2.9%), proteinuria (3.6% vs. 0.0%),

hypertension (14.8% vs. 0.0%), and cerebrovascular ischemia (1.9% vs. 0.0%) also limit long-term therapy (21). Part of the problem with the therapeutic use of monoclonal antibodies is that VEGFA is not the only ligand that can bind to these receptors. Other members of the VEGF family such as VEGF C and D can bind to VEGFR-2, while VEGF B has been shown to bind and activate VEGFR-1 (19,22). With this in mind, other agents, which target the tyrosine kinase domain of the receptor, have been developed and several pre-clinical and clinical trials are investigating the use of such agents (2,23). Many of the newer agents that are being developed also target other growth factor receptor tyrosine kinases such as PDGF, FGFR, and c-Kit with the idea that blocking several receptors will prevent resistance to therapy that results from activation of alternate pathways by co-regulatory proteins (2).

One dual-targeting drug is brivanib alaninate (BMS 582664, Bristol Myers-Squibb, Princeton, NJ), a VEGFR-2/FGFR-1 inhibitor. Pre-clinical studies in-vivo, have shown that brivanib alaninate is effective in reducing the growth of a lung tumor xenograft, L2987, a panel of human derived hepatocellular carcinomas (24), and an ER negative breast tumor H3396 (25). Pharmacological studies in a phase I clinical trials have shown that doses of brivanib alaninate below 800mg/day are tolerable, but have associated toxicities such as hypertension (>150/100), elevated transaminases, fatigue, and dizziness as the dosage increases from a baseline of 180 mg/day. Several phase 1 clinical trials are underway in patients with a variety of solid tumors (26,27).

We have addressed the hypothesis that combining tamoxifen, a selective estrogen receptor modulator (SERM) with a sub-therapeutic dose of brivanib alaninate would be a beneficial strategy for long-term therapy in the treatment of breast cancer. We report the first studies testing the efficacy of brivanib alaninate to control tumor growth of ER regulated SERM sensitive (MCF-7 E2) and SERM stimulated (MCF-7 Ral, MCF-7 Tam), and endometrial (EnCa 101) tumors. We find that the combination of tamoxifen and brivanib alaninate in a laboratory model provided a therapeutic advantage for the control of breast tumor growth over tamoxifen or brivanib alaninate alone.

Materials and Methods

Tumor Xenografts

SERM sensitive tumors were previously developed by injecting the mammary fat pads of ovariectomized, BALB/c athymic mice (Harlan Sprague Dawley, Madison, WI) with 1×10^7 WS8 human breast cancer cells (28). Tumor growth was sustained with 0.3 cm silastic capsules containing estradiol (Sigma, St. Louis, Mo) delivering 83.8 ± 34.6 pg/mL estrogen over an 8-week period (29). Over time, the tumors were serially passaged by bi-transplanting the established tumors into the mammary fat pads of estradiol treated mice. The development and characterization of the SERM stimulated EnCa 101 endometrial cancer model (30), MCF-7 Ral model (31), and MCF-7 Tam model (32) have been reported previously.

For the experiments in the current study, athymic ovariectomized CrTac: NCr-Foxn1nu mice were obtained from Taconic (Hudson, NY). Mice were placed under anesthesia, using a mixture of isoflurane and 100% oxygen delivered via inhalation. Healthy tumor tissue was sectioned into 1 mm^3 pieces and implanted bilaterally into the mammary fat pads. Estradiol capsules (0.3 cm silastic capsule) were placed subcutaneously on the dorsal surface of the mice to maintain tumor growth.

Tumors were measured with calipers once a week. Cross sectional areas (CSA) were calculated by measuring the length and width of the tumors and then using an Excel

(Microsoft) spreadsheet to calculate the CSA (length (cm) $\times$ width (cm) $\times \pi/4$). Growth curves were derived from the determining the average CSA per treatment group per week. In the case of EnCa 101 endometrial tumors, growth characteristics were atypical with a prolonged latent period of tumor spreading subcutaneously with an eventual rapid hemorrhagic growth phase reminiscent of the “angiogenic trigger”. Tumor volumes were measured for EnCa 101 using the formula $4/3 \pi r^3$.

Six sets of experiments were completed. The first experiment was specifically conducted to evaluate where VEGFR-2 and VEGFA are expressed and how expression changes in response to hormonal and anti-hormonal manipulation. Experiments 2–5 were conducted to determine dosing of brivanib alaninate to prevent the growth in MCF-7 E2, a SERM sensitive tumor, and MCF-7 Ral, MCF-7 Tam, and EnCa 101 SERM stimulated tumors. The fourth experiment determined the dosing of tamoxifen to block estradiol stimulated tumor growth in MCF-7 E2 tumors. The fifth and sixth experiments determined the effects of combined therapy when started 24 hours after initial tumor implantation versus giving the drug to animals with established tumors for a 2-week time period.

Drug Preparation

Bristol Myers Squibb (Princeton, NJ) provided brivanib alaninate in powder form. The drug was suspended in a citric acid buffer solution and the pH was gradually titrated to a pH of 3.5 after the drug dissolved. The final concentration was 10 mg/mL

Tamoxifen (Sigma Chemical Co, St. Louis, MO) was weighed and suspended in 10 % Tween 80/Polyethylene glycol (PEG) 400 (99.5% PEG 400/0.5% Tween 80) and 90% carboxymethylcellulose (CMC, 1% CMC dissolved in double distilled water). The final concentration of the tamoxifen solution was 2.5 mg/mL and administered by gavage at the doses indicated. Administration of tamoxifen to animals bearing EnCa 101 tumors was at a dose of 500 μ g/mouse by gavage.

Raloxifene (Evista, Eli Lilly, IN) was prepared by placing 5 raloxifene tablets in a conical tube and dissolving them via centrifugation in 27 mL double distilled water. Once the tablets were dissolved, 3 mL of 90% CMC and 10% PEG 400/Tween 80 was added to the raloxifene solution. The final concentration was 10 mg/mL. Raloxifene was administered at a daily dose of 1.5 mg/mouse by gavage.

Estradiol capsules were prepared by plugging one end 0.3 cm length of medical grade silastic tubing and filling it with 17β -estradiol (Sigma Chemical Company, St. Louis, MO) mixed 1:3 with elastomer. Capsules sealed by placing elastomer at the open ends and then sterilized with radiation (20,000 rads) (33).

Fulvestrant (Faslodex, AstraZeneca, Wilmington, DE) was purchased from the pharmacy at Fox Chase Cancer Center as a solution of fulvestrant suspended in EtOH and castor oil (50 mg/ml).

Drug Administration

Brivanib alaninate was dosed orally 7 days a week, according to the weight of each mouse. Mice were weighed once weekly. For the high dose, a 20 g mouse was given 200 μ L (2 mg) and for the low dose a 20 g mouse was given 100 μ L (1 mg). Tamoxifen was also administered 7 days a week. Dosing of tamoxifen was as follows: 125 μ g (50 μ L), for 250 μ g (100 μ L), or 500 μ g (200 μ L). Fulvestrant was administered as 2mg (40 μ L) injections 5 days per week.

Experiment 1: The effect of hormonal manipulation on VEGFA and VEGFR-2 expression

Tumors were grown in the presence of estradiol (0.3 cm silastic capsule) until the tumors reached 0.4 cm². The mice were then treated with different drug regimens for 2 weeks. The treatments after tumors reached 0.4 cm² were as follows:

1. continue estradiol (0.3 cm silastic capsule)
2. withdraw estradiol
3. estradiol + 125 µg tamoxifen daily
4. withdraw estradiol (0.3 cm silastic capsule) and 2 mg/40 µL fulvestrant injections given subcutaneously daily

Experiment 2: Effects of different doses of brivanib alaninate on SERM sensitive MCF-7 E2 tumors

We evaluated the effects of a high dose and low dose of brivanib alaninate. The brivanib alaninate treatment was started 24 hours after tumor implantation. Treatment groups (5 animals) were as follows:

1. Estradiol (0.3 cm silastic capsule) + placebo given orally (citric acid buffer: pH=3.5)
2. Estradiol (0.3 cm silastic capsule) + low dose brivanib alaninate given orally (.05mg/g)
3. Estradiol (0.3 cm silastic capsule) + high dose brivanib alaninate given orally (0.1mg/g)

Experiment 3: The effects of brivanib alaninate on SERM stimulated tumors

Experiment 3A: The effects of different doses of brivanib alaninate on SERM resistant MCF-7 Ral tumors—We evaluated the effects of a high dose and low dose of brivanib alaninate. The brivanib alaninate treatment was started 24 hours after tumor implantation. Treatment groups (5 animals) were as follows:

- Placebo (citric acid buffer)
- 1.5 mg raloxifene
- 2 mg Fulvestrant- pure anti-estrogen (subcutaneous)
- 1.5 mg Raloxifene + high dose VEGFR antagonist (0.1 mg/g)

Experiment 3B: The effect of brivanib alaninate on established SERM resistant MCF-7 Ral tumor models—Tumors were grown up to an average 0.5cm² CSA. The mice were randomized to receive 2 weeks of therapy with the high dose brivanib alaninate.

Groups: 10 mice each after randomization:

- 1.5 mg Raloxifene
- 1.5 mg Raloxifene + high dose brivanib alaninate (0.1mg/g)

Experiment 3C: The effect of brivanib alaninate on SERM resistant MCF-7 Tam tumors—We examined the effects of the high dose brivanib alaninate on another SERM resistant model. There were two components to this experiment. The first was to determine

whether brivanib alaninate inhibited tumor growth and the second was to determine whether brivanib alaninate was effective in established tumors.

1. 1.5 mg tamoxifen first 48 days of the experiment (8 mice, 16 tumors)--this group was used for the second part of the experiment
2. Placebo: citric acid buffer (0.15mL) (5 mice, 10 tumors)
3. 1.5 mg tamoxifen + 0.1mg/g brivanib alaninate (4 mice (6 tumors))

Once the tumors in group one reached a CSA of 0.5 cm², 48 days after tumor implantation, group 1 was subdivided:

1. continue 1.5 mg tamoxifen for 2 more weeks (4 mice, 8 tumors)
2. 1.5 mg tamoxifen + high dose brivanib alaninate (0.1mg/g) for 2 weeks (4 mice, 8 tumors)

Experiment 3D: The effect of brivanib alaninate on EnCa Tam endometrial tumors

This experiment determined whether brivanib alaninate inhibited the growth of endometrial tumors that normally grow with 500 µg of tamoxifen daily. Tumors initially are not evident until after one month after which they grow rapidly. Twenty mice were treated with tamoxifen for 40 days and then randomized into two groups. After randomization, treatments were given for 3 weeks.

Control Group: 500 µg tamoxifen daily(10 mice)

Experimental Group: 500 µg tamoxifen daily + 0.1 mg/g brivanib alaninate (started on day 40) (10 mice)

Experiment 4: Determination of Tamoxifen Dosing in SERM sensitive MCF-7 E2 tumors

We determined a dose response curve of various oral doses of tamoxifen to determine the lowest dose that was effective in decreasing the rate of tumor growth.

1. No estradiol
2. Estradiol (0.3 cm silastic capsule)
3. Estradiol (0.3 cm silastic capsule) + 500 µg tamoxifen given orally
4. Estradiol (0.3 cm silastic capsule) + 250 µg tamoxifen given orally
5. Estradiol (0.3 cm silastic capsule) + 125 µg tamoxifen given orally

Experiment 5: The combined effect of a lower dose of tamoxifen and brivanib alaninate in SERM sensitive MCF-7 E2 tumors

This experiment determined the combined effects of a sub-maximal dose of tamoxifen and a sub-maximal dose of brivanib alaninate on estrogen stimulated tumor growth. Drug dosing was commenced 24 hours after tumor implantation.

1. control with estradiol (0.3 cm silastic capsule)
2. experimental group with estradiol (0.3 cm silastic capsule) + 125 µg tamoxifen given orally
3. experimental group with estradiol (0.3 cm silastic capsule) + low dose brivanib alaninate (.05mg/g dose) given orally
4. experimental group with estradiol (0.3 cm silastic capsule) + 125 µg tamoxifen given orally+ low dose brivanib alaninate (0.05 mg/g dose) given orally.

Experiment 6: The combined effect of a lower dose of tamoxifen and brivanib alaninate in established SERM sensitive MCF-7 E2 tumors

Experiment number eight was similar to experiment number seven with one exception. The tumors were grown to an average CSA of 0.43mm^3 and drug therapy was given for two weeks.

Western Immunoblotting

Tumors were harvested and placed in foils and frozen immediately in liquid nitrogen. Tumors were kept at -80°C until they were processed. For processing, tumors were placed in liquid nitrogen and homogenized using a mortar and pestle. The extract was suspended in RIPA buffer (Sigma, St. Louis, MO) with protease (Roche, Nutley, NJ) and phosphatase (Calbiochem, San Diego, CA) inhibitors. The mixture was briefly sonicated and centrifuged for 10 minutes at $5000 \times g$. The supernatant was removed and protein concentration was determined using the Bradford assay (BCA assay, Pierce, Rockford, IL) with a Spectramax machine (Molecular Devices, Sunnyvale, CA). Equal amounts ($25 \mu\text{g}$) and concentrations of protein were loaded into 4–12% Nupage Bis-tris (Invitrogen, Carlsbad, CA) gels, and transferred to nitrocellulose membranes. Immunoblotting was carried out with the following antibodies: Total VEGFR-2 (1:1000, rabbit polyclonal, Cell Signaling Technologies, Beverly, MA), phospho-VEGFR-2 Tyr⁹⁵¹ (1:200, rabbit polyclonal, Santa Cruz, Biotechnology, Santa Cruz, CA), Total FGFR-1 and Total VEGFR-3 (1:200, rabbit polyclonal, Santa Cruz Biotechnology, Santa Cruz, CA), Total VEGFR-1 (rabbit polyclonal, 1:200, Labvision, Fremont, CA), Total ER alpha (ER α) (rabbit polyclonal, 1:200, G20, Santa Cruz, Santa Cruz, CA), Phospho-ER α (rabbit monoclonal, 1: 2000, Ser 118, clone NL 44, Upstate, Billerica, MA), β -actin (mouse monoclonal, 1:30,000, Sigma-Aldrich, St. Louis, MO)

Real time Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from frozen tumor tissues using RNA mini easy kit (Qiagen, Venlo, The Netherlands) as per the manufacturer's instructions. Two micrograms of total RNA was reverse transcribed using a cDNA high capacity reverse transcription kit (Applied Biosystem, Carlsbad, CA) in $20 \mu\text{l}$ of total volume, as per manufacturer's instruction. The resulting cDNA was diluted to a total volume of $200 \mu\text{l}$ using sterile water. The real time PCR was carried out on an ABI 7900 HT Fast Real Time PCR system using 1X SYBR green PCR master mix (Applied Biosystem, Carlsbad, CA) and 100 nM of forward and reverse primers. All the forward and reverse primers (Table 1) were designed using Primer Express 3 software (Applied Biosystem, Carlsbad, CA) except ER α (34) and mouse and human 36B4 (35,36). The fold change in expression of each gene was calculated by the $\Delta\Delta\text{Ct}$ (37) method using 36B4, a ribosomal phospho-protein as an internal control.

Immunohistochemistry (IHC)/Histology

Staining (IHC) was done to determine VEGFR-2 and VEGFA expression on tumor tissue from experiments 2 and 6. Tumors were placed in formalin for 48 hours and subsequently embedded in paraffin. Fixation was done with phosphate buffered formaldehyde 10% (F79-4, Fisher Scientific, Pittsburgh, PA). Xenografts were placed in the fixative for 48 hours and subsequently embedded in paraffin. Paraffin sections were dewaxed using xylenes and hydrated using a series of ethanol. Antigen retrieval was performed with citrate buffer pH 6 during 10min in a microwave oven (1500 W, 2 minutes at high and 8 minutes at the lowest power) Endogenous peroxidases were quenched with 0.3% hydrogen peroxide in methanol during 30 minutes. Sections were incubated overnight with the primary antibody raised against VEGFR-2 and VEGFA. Total VEGFR-2 (55B11) rabbit monoclonal antibody from Cell Signaling Technology (Beverly, MA) and anti-VEGF (A-20) purified rabbit

polyclonal antibody from Santa Cruz (Santa Cruz, CA) were diluted 1/100 (2µg/ml) in phosphate buffered saline (PBS), washed the next day with PBS, incubated with biotinylated secondary antibodies (Vector Labs), incubated with Vecta Elite ABC kit (Vector Labs), developed with a DAB kit (Vector Labs) and lightly counterstained with Gill's hematoxylin. Negative controls were stained without primary antibody or with the corresponding concentration of rabbit IgG isotype. Specimens were documented photographically using a Nikon Optiphot microscope, equipped with an Optronics CCD camera. The stained sections were scored on the basis of staining intensity. The vast majority of tissues stained diffusely and all or more than 70% of the tumor tissue was stained in the positive specimens. The score was defined as weak (1+), positive (2+) or strong (3+).

For CD31 staining, in Experiment 3C, the sections were washed in PBS and then treated with 3% H₂O₂ for ten minutes to block endogenous peroxidase activity and were blocked with normal rabbit serum. Then, the sections were incubated with rat anti-mouse CD31 (PECAM-1) monoclonal antibody (BD Pharmingen, San Diego, CA) at a 1:300 dilution overnight at 4°C. Negative controls were incubated with the rat serum IgG at the same protein concentration. All sections were washed in PBS containing 0.05% Tween-20, and were then incubated with a second antibody, mouse anti-rat IgG (Vector laboratories, Burlingame, CA) at a 1:200 dilution for 30 minutes at room temperature again followed by washing with PBS containing 0.05% Tween-20. The sections were incubated in a 1:400 dilution of Extravidin Peroxidase (Sigma, St. Louis, MO) for 30 min. After washing in PBS containing 0.05% Tween-20, the sections were incubated in peroxidase substrate (Vector laboratories, Burlingame, CA) for five min. After washing we used a Biotinyl-Tyramide enhancement kit (TSA/Biotin Tyramide Reagent Pack, Perkin Elmer, Waltham, MA) according to the manufacturer's instructions. The sections were washed in PBS containing 0.05% Tween-20 and were counterstained with Gill's hematoxylin.

For general morphological evaluations, sections from each tumor were stained with hematoxylin and eosin (H and E).

Statistical Analysis

Tumor growth data were analyzed using random effects growth curve models, where tumor cross sectional area was fit assuming a quadratic function of time. Let A_{ijt} be the cross sectional area of tumor i on mouse j , in treatment group k , measured t days after treatment (or control) initiation. The growth curve model was of the following form.

$$A_{ijkl} = \beta_{0j} + t\beta_{1ij} + t^2\beta_{2ij} + \sum_{z=1, \dots, K} \gamma_{0z} I(k=z) + \sum_{z=1, \dots, K} t\gamma_{1z} I(k=z) + \sum_{z=1, \dots, K} t^2\gamma_{2z} I(k=z) + \varepsilon_{ijkl}$$

where the β 's were assumed random terms with mean zero, the γ 's were fixed effects and K is the number of treatment groups. Random effects were included to allow deviation of individual tumors from the mean growth of the group and to account for within-animal clustering. The estimated curves were plotted and the fit examined. Linear contrasts were used to estimate mean tumor size differences (and associated standard error) at a specified time t between any two pre-specified experimental groups. Wald tests were used to test the null hypothesis of equal tumor size between two experimental groups at time t . For experiments with randomization and treatment initiated after day 0, only observations taken after randomization were analyzed. For example, in experiment 8, only observations after initiation of brivanib alaninate or tamoxifen treatment (≥ 35 days) were analyzed. Bonferroni corrections were used to adjust for multiple testing within each experiment for these analyses. The experiment-wise type I error was 5%. The RNA expression data measured by RTPCR with high/low dose of VEGFR-2 inhibitor and combination treatment were analyzed

using Wilcoxon rank-sum tests. The RTPCR analyses were confirmatory and, therefore no adjustment of the type I error for multiple testing was used. All tests were 2-sided. Statistical analyses were performed using STATA version 10.1. For the CD31 counts that were done for the MCF-7 Tam model, the statistical analysis was done using a two tailed Student's t-test and a p-value that was less than 0.05 was considered significant.

Results

Immunohistochemistry

Immunohistochemistry was performed on representative MCF-7 E2 tumors to determine whether the VEGFR-2 receptor was expressed in response to estradiol and two weeks of tamoxifen. We also determined VEGFR-2 receptor expression in response to estradiol, estradiol withdrawal and the treatment with the pure anti-estrogen, fulvestrant. This analysis demonstrated the presence of VEGFR-2 on both tumor cells and on endothelial cells (Figure 1A). In addition, VEGFR-2 and VEGFA expression was increased on tumor cells in the presence of estradiol. It is interesting to note that the combination of estradiol and two weeks of 125 µg tamoxifen did not apparently change VEGFR-2 or VEGFA expression in comparison to estradiol treatment alone. However, as noted in Figure 6A, tamoxifen was not effective at controlling established estradiol stimulated tumor growth during the two-week treatment period. With estradiol withdrawal alone, and the subsequent destruction of the ER with fulvestrant, there was very little expression of VEGFR-2 or VEGFA on the tumor cells (Figure 1A and B).

Effects of different doses of brivanib alaninate in SERM sensitive MCF-7 E2 tumors

We evaluated the effects of a low dose (0.05 mg/g) and high dose (0.1 mg/g) of brivanib alaninate on estradiol stimulated tumor growth. The high dose was based on data demonstrating the highest effective dose with minimal toxicity and the low dose that was chosen was half of the high dose and the minimally effective dose as determined by Bristol Myers Squibb (Princeton, NJ) (38). Statistical comparisons were done to determine whether there was a difference in the average CSA of tumors treated with estradiol vs. those that received the high dose (0.1mg/g) or low dose (0.05mg/g) of brivanib alaninate in the presence of estradiol. Estradiol caused tumor growth, while the high dose of brivanib alaninate produced a dramatic decrease in estradiol-stimulated growth (Figure 2A). The average difference in tumor CSA at 6 weeks in the mice that received the high dose of the brivanib alaninate and estradiol vs. estradiol was -0.37cm^2 ($p=0.001$, $\alpha=0.025$). There was no significant difference (0.13cm^2) in the average CSA of tumors treated with estradiol only and those treated with estradiol and the low dose of brivanib alaninate ($p=0.202$, $\alpha=0.025$).

The tumor tissue was further evaluated with H and E staining (Figure 2B). The purpose of this analysis was to detect differences in the amount of necrotic tissue. In tumors in which angiogenesis and thus, oxygen and nutrient delivery is blocked, there would be a decrease in tumor cell viability and hence an increase in necrosis. In tumors that received brivanib alaninate, there was an increase in tissue necrosis as exemplified by the areas that stain pink only. The necrosis was most prominent in the tumors treated with the high dose of the brivanib alaninate. There was mild necrosis in the tumors that were treated with the low dose of the brivanib alaninate.

Western immunoblotting of tumor extracts did not reveal a difference in total VEGFR-2, but there was less phosphorylation at the tyrosine 951 residue of VEGFR-2 in brivanib alaninate treated animals (Figure 2C). The presence of ER and phospho-ER demonstrated active tumor tissue and an activated ER. There was very little VEGFR-1, VEGFR-3, or FGFR-1 (data not shown) detected by immunoblotting. The use of RTPCR analysis confirmed a

significant increase in VEGFA ($p=0.02$) and a non-significant increase in human VEGFR-2 in tumors that were treated with the high dose of brivanib alaninate (Figure 2D). There was a significant decrease in mouse VEGFR-1 and mouse VEGFR-2 in tumors that were treated with the higher ($p=0.02$, $p=0.04$) dose of brivanib alaninate. Estrogen receptor mRNA decreased slightly, but significantly (Figure 2C) in those tumors that were treated with the higher dose of brivanib alaninate ($p=0.04$), but there was no increase in ER protein by western blotting analysis (Figure 2C). There was a significant decrease in transcription of VEGFC mRNA ($p=0.04$) in tumors treated with the lower dose of brivanib alaninate (Figure 2D). There was very little or no VEGFB, VEGFD, mouse VEGFR-3, or human VEGFR-1 present in the tumors as evidenced by high CT values (>35) detected by RTPCR analysis (data not shown).

Effect of brivanib alaninate on SERM stimulated tumor growth

To establish that an inhibitor of VEGFR-2 would block the growth of SERM stimulated tumors and as a consequence, would have the potential to retard the development of acquired SERM resistance in ER positive cancers, a series of models and designs was explored. The MCF-7 Ral tumor model (39) grows without raloxifene, and to a greater extent in the presence of raloxifene. Fulvestrant retards tumor growth (39). This is illustrated in Figure 3A. Statistical comparisons were done to determine whether there was a difference in the average CSA of tumors treated with raloxifene vs. those treated with placebo, fulvestrant or high dose brivanib alaninate (0.1 mg/g). Raloxifene stimulated tumor growth was significantly decreased in the presence of high dose brivanib alaninate (0.1 mg/g) administered with raloxifene and the difference in average CSA was 0.391 cm^2 after 8 weeks ($p<0.001$, $\alpha=0.016$). A similar difference in average CSA (0.366 cm^2) was also observed with tumors treated with raloxifene vs. tumors treated with fulvestrant ($p<0.001$, $\alpha=0.016$). There was no significant difference between the average CSAs of tumors (0.212 cm^2) in the presence or absence of raloxifene ($p=0.024$, $\alpha=0.016$). The addition of high dose brivanib alaninate (0.1mg/g) to a daily regimen of 1.5 mg of raloxifene (0.1mg/g) caused a rapid decrease in tumor growth (decrease in average CSA = -0.294 cm^2) ($p<0.001$, $\alpha=0.025$) in established raloxifene stimulated tumors (Figure 3B) over a two week period. At the time of randomization, the group that was treated with raloxifene (1.5 mg) and brivanib alaninate (0.1mg/g) demonstrated no difference in average CSA (-0.0146 cm^2) than those that received raloxifene (1.5mg) only ($p=0.73$, $\alpha=0.025$).

Our MCF-7 Tam SERM stimulated model showed similar effects with brivanib alaninate. Statistical comparisons were done to determine whether there was a difference in the average CSA of tumors treated with 1.5 mg tamoxifen daily vs. vehicle or 1.5 mg tamoxifen + 0.1mg/g brivanib alaninate. The difference in CSA between those tumors that received 1.5 mg tamoxifen daily vs. 1.5 mg tamoxifen and the high dose brivanib alaninate (0.1mg/g) daily ($p<0.001$, $\alpha=0.025$) was 0.395 cm^2 . A similar, difference in CSA (0.484 cm^2) was observed between tumors treated with tamoxifen alone vs. control treated with vehicle only ($p<0.001$, $\alpha=0.025$). The tamoxifen (1.5 mg/daily) treated group was then randomized to continue 1.5 mg/day tamoxifen or 1.5 mg/day tamoxifen + high dose brivanib alaninate for two weeks. At the time of randomization, the group that was treated with tamoxifen (1.5 mg) and brivanib alaninate (0.1mg/g) demonstrated no difference in average CSA than those that received tamoxifen (1.5mg/g) only ($p=0.76$, $\alpha=0.25$). The addition of brivanib alaninate (0.1 mg/g) caused rapid tumor regression (difference in average CSA = -0.261 cm^2) of established tumors after two weeks of treatment ($p<0.001$, $\alpha=0.025$) (Figure 3C). There was a significant decrease in blood vessel density (CD31 counts) in the group that received 0.1 mg/g brivanib alaninate and 1.5 mg tamoxifen for 2 weeks (average MVD/sq. mm=76) in comparison with the group that continued receiving 1.5 mg tamoxifen (average MVD sq/mm=156) ($p=0.003$).

Finally, the tamoxifen stimulated EnCa 101 endometrial tumor model (30) was also used to evaluate the efficacy of brivanib alaninate (0.1 mg/g). Animals with bi-transplanted tumors were treated with 500 µg of tamoxifen daily by oral gavage for 40 days and then randomized. One group received 500 µg of tamoxifen and 0.1mg/g brivanib alaninate daily. The other group continued to receive 500 µg of tamoxifen. At the time of randomization, the group that was treated with tamoxifen (500 µg) and brivanib alaninate (0.1mg/g) had a larger average volume (difference=40 mm³) than those that received tamoxifen (500 µg) only (p=0.002, α =0.025). Despite this initial difference, over a three week period, animals treated with tamoxifen alone subsequently had an average tumor volume (difference = 0.168 mm³) that was much greater than those animals treated with brivanib alaninate in combination with tamoxifen (p<0.001, α =0.025) (Figure 3D). All models demonstrated that a VEGFR-2 inhibitor, brivanib alaninate would prevent the growth of SERM stimulated tumors.

Determination of Tamoxifen Dosing in SERM sensitive MCF-7 E2 tumors

We determined an anti-estrogenic dose of tamoxifen that would be approximately 50% effective in blocking estradiol stimulated tumor growth. Previously, 1.5 mg/day of tamoxifen has been used to almost completely block estrogen stimulated tumor growth (40). The differences in the CSAs of tumors treated with estradiol and 125 µg tamoxifen (−0.368 cm², p=0.01, α =0.016), estradiol and 250 µg tamoxifen (−0.479 cm², p=0.001, α =0.016) or estradiol and 500 µg tamoxifen (−0.479 cm², p<0.001, α =0.016) vs. estradiol alone were significant (Figure 4). A dose of 125 µg was chosen for further testing in combination with brivanib alaninate to determine whether there would be an improvement in therapeutic efficacy.

The combined effect of a lower dose of tamoxifen and brivanib alaninate in SERM sensitive MCF-7 E2 tumors

We hypothesize that a sub-therapeutic dose of brivanib alaninate may enhance a sub-optimal effective daily dose of tamoxifen (125 µg) and thus improve tumor growth control. The strategy of limiting angiogenesis would optimize long-term anti-estrogen therapy. Statistical comparisons were done to determine whether there was a difference in the average CSA of tumors treated with 125 µg tamoxifen + 0.05 mg/g brivanib alaninate vs. 125 µg tamoxifen or 0.05 mg/g brivanib alaninate. The results illustrated in Figure 5 demonstrated that the combination of 125 µg of tamoxifen and 0.05mg/g of brivanib alaninate significantly improved the anti-tumor action tamoxifen or brivanib alaninate alone after 6 weeks. The difference in average CSAs of tumors treated with 125 µg tamoxifen and 0.05mg/g brivanib alaninate vs. those treated 125 µg tamoxifen (−0.128 cm², p=0.01, α =0.025) was significant. Similarly, there was a significant difference in the CSA of those tumors treated with the combination therapy and those treated with brivanib alaninate (−0.449 cm², p< 0.001, α =0.025).

The combined effect of a lower dose of tamoxifen and brivanib alaninate in established SERM sensitive MCF-7 E2 tumors

The goal of this experiment was to obtain sufficient tumor tissue for molecular analysis to evaluate the actions of tamoxifen and brivanib alaninate. Results are summarized in Figure 6 and Figure 7. The short-term combination of brivanib alaninate and tamoxifen decreased tumor size during the two-week period, whereas neither tamoxifen alone nor the brivanib alaninate alone prevented an increase in established tumor size (Figure 6A). Statistical comparisons were done to determine whether there was a difference in the average CSA of tumors treated with 125 µg tamoxifen + 0.05 mg/g brivanib alaninate vs. 125 µg tamoxifen or 0.05 mg/g brivanib alaninate. There was no difference in size at the time of randomization (tamoxifen vs. combination therapy (p=0.87) and brivanib vs. combination therapy

($p=0.29$). The average CSA was significantly different between tumors treated with 125 μg tamoxifen vs. those treated with 125 μg tamoxifen and .05 mg/g brivanib alaninate (-0.292 cm^2 , $p=0.01$, $\alpha=0.025$). The same observation was noted for those tumors treated with 0.05 mg/g brivanib alaninate vs. those treated with 0.05 mg/g brivanib alaninate and 125 μg tamoxifen (-0.341 cm^2 , $p=0.007$, $\alpha=0.025$).

Consistent with our findings, illustrated in Figure 2B, representative histological analysis in this experiment confirmed (Figure 6B) increased necrosis in tumors that received only brivanib alaninate or brivanib alaninate plus tamoxifen.

Western immunoblotting (Figure 6C) demonstrated a decrease in phosphorylation of the VEGFR-2, but not total VEGFR-2 in the two groups that received brivanib alaninate. Total ER expression was reduced in the group receiving tamoxifen and the brivanib alaninate compared to tamoxifen alone

RTPCR analysis (Figure 6D) demonstrated an increase in mRNA for mouse VEGFR-1 and mouse VEGFR-2 in tumors that receive brivanib alaninate with ($p=0.002$, $p=0.002$) or without ($p=0.001$, $p=0.001$) tamoxifen. VEGFA mRNA is increased with tamoxifen ($p=0.01$), brivanib alaninate ($p=0.001$) or both drugs ($p=0.002$) in combination. VEGFC increased with the tamoxifen treated group ($p=0.001$), but decreased in the groups treated with the brivanib alaninate ($p=0.004$). ER mRNA levels increased ($p=0.04$) with the tamoxifen treated group, but decreased with the group that received both the VEGFR inhibitor and tamoxifen ($p=0.04$).

We further validated our molecular studies with immunohistochemistry. There was little change in total VEGFR-2 (Figure 7A), which was consistent with the findings in Western immunoblotting. VEGFA staining intensity increased in the tumors treated with tamoxifen and brivanib alaninate, which is consistent with the increased VEGFA mRNA seen in RTPCR analysis (Figure 7B). The nuclear staining of the VEGF in the presence of brivanib (Fig 7C) could be consistent with the report by Rosenbaum-Dekel (41) with the nuclear localization of L-VEGF, but no specific antibody was available to test the hypothesis.

Discussion

We report the first study to explore the potential of combining tamoxifen with low dose brivanib alaninate to block the growth of ER positive breast cancer. Previous studies have demonstrated the efficacy of brivanib in mouse models of human hepatocellular carcinoma (24) and to inhibit growth in ER negative H3396 xenografts in athymic mice (25). Our strategy is to employ an antiestrogen (tamoxifen) to block estrogen stimulated VEGF production and to use a combination with blockers of VEGFR-2 to reduce angiogenic survival mechanisms in both the tumor and endothelial cells to enhance tumor cell death. Our results demonstrate that the strategy is feasible. We have advanced the idea with the demonstration that a VEGFR-2 inhibitor, brivanib alaninate can not only inhibit the growth of small SERM stimulated implants derived from MCF-7 cells with acquired resistance to tamoxifen and raloxifene, but also can inhibit SERM stimulated growth of established tumors in athymic mice (Figure 3A–C). Additionally, brivanib alaninate inhibits tamoxifen stimulated endometrial cancer (EnCa 101) growth (Figure 3D). Thus, the ability of a VEGFR-2 inhibitor to block the growth of tumors with acquired SERM resistance supports the idea that this strategy might improve adjuvant therapies.

Angiogenesis is important for tumor growth and metastasis. Stable transfection of MCF-7 cells with the VEGF gene results in hormone independent growth in vivo and tamoxifen resistance (42). This is supported by the recent work by Aesoy and coworkers (43) using an anti-estrogen resistant cell line (LCC2) in vitro that has constitutive VEGF secretion relative

to wildtype MCF-7 cells. MCF-7 cells respond to 4-hydroxytamoxifen with a reduction in VEGF, but the anti-estrogen resistant variant LCC2 does not. Estrogen has been shown to increase the synthesis of VEGFA (13) and anti-estrogens inhibit the process (13,43). This observation was validated in our tumor models as the expression of VEGFA and VEGFR-2 is increased in the presence of estrogen and decreased with estrogen withdrawal (Figure 1A and B). As there is strong evidence for the estrogen mediated regulation of angiogenesis, combining an anti-estrogen with an anti-angiogenic inhibitor to diminish tumor growth is a reasonable therapeutic approach.

There are fewer side effects such as malignant hypertension with angiogenesis inhibitors when used a lower doses (44). At higher doses, therapeutic efficacy may be diminished when drug dosing is reduced or abbreviated. Therefore, we advanced the concept of dual inhibition of angiogenesis further and tested a combination of sub-effective tamoxifen (125 μ g) daily and the sub-therapeutic VEGFR-2 inhibitor brivanib alaninate (0.05mg/g daily). The combination significantly decreased tumor growth compared with estradiol and either drug alone. This was true for the prevention of early tumor development following initial implantation (Figure 5) or during the short-term treatment of established tumors (Figure 6 and Figure 7). Thus, we have shown that using a combination of lower, more tolerable doses of two drugs that are as efficacious as higher, less tolerable doses of either drug used alone, is a viable alternative for adjuvant therapy.

Drug treatments were evaluated in established tumors to provide tissue to investigate molecular mechanisms. Total VEGFR-2 levels did not change in the tumors with treatment (Figure 6C and Figure 7A), but the phosphorylation patterns were different (Figure 6C). Brivanib alaninate inhibits phosphorylation of the VEGFR-2 receptor. This confirmed the reported mechanism of action (24) of brivanib alaninate as an inhibitor of the VEGFR-2 tyrosine kinase. Treatment of established tumors with tamoxifen alone increased phosphorylation of VEGFR-2 and this increase in phosphorylation was inhibited when brivanib alaninate was combined with tamoxifen. Thus, it is possible to explain why a significant decrease in tumor size resulted from the use of a two-drug combination rather than a single drug that was individually ineffective in established tumors.

Similarly, transcription of VEGFC mRNA increased during tamoxifen treatment, but this was abrogated with brivanib alaninate. This is an important finding because VEGFC also activates VEGFR-2 (22). There was a compensatory rise in VEGFA with tamoxifen, brivanib alaninate, or the combination of the two drugs. However, with the combination of tamoxifen and brivanib alaninate, the compensatory mechanisms of the tumor to overcome blockade of the ER and VEGFR-2 failed as evidenced by increased tumor necrosis. The compensatory rise in VEGFA was validated by IHC in tumors treated with the combination of tamoxifen and brivanib alaninate. Overall, our findings confirm and extend the recent findings of Aesoy and co-workers (43) who demonstrate a breast cancer cell survival of VEGF/VEGFR-2/p38 feedback loop in cells resistant to anti-estrogens.

Classically, the VEGF pathway in tumors has been thought to result from VEGF secretion from tumor cell activation of VEGF receptors on endothelial cells. However, accumulating evidence suggest that VEGFR-2 is most likely found on both cancer cells and endothelial cells (43,45–47). By using IHC to localize VEGFR-2 in the MCF7 tumor model, there is demonstrable expression of VEGFR-2 on the breast cancer cells (Figure 1A). Moreover, there is evidence of estrogen mediated regulation of VEGFR-2 expression on tumor cells as VEGFR-2 expression decreases with the withdrawal of 17- β estradiol. Ryden (48) also demonstrated that VEGFR-2 is expressed on tumor material from patients. These findings strengthen the argument to target VEGFR-2 in breast cancer.

By using RTPCR to differentiate between mouse and human VEGFR-2, we were able to evaluate the response to therapy in the endothelial (mouse) and the tumor cell (human) components. Interestingly, when the brivanib alaninate is started at the time of implantation there is a significant decrease in mouse VEGFR-1 and VEGFR-2. There was a trend towards an increase in human VEGFR-2 in mice treated with the higher dose of brivanib alaninate, with a significant decrease in mouse VEGFR-2 mRNA. When the angiogenesis inhibitor was given to mice with established tumors, there was a trend towards a decrease in human VEGFR-2 mRNA with a significant increase in mouse VEGFR-1 and VEGFR-2 mRNA. Thus, when the human VEGFR-2 is blocked, this then affects the endothelial component and the cells attempt to manufacture more receptor.

The ER is central to estrogen-regulated events. As reported in previous studies, tamoxifen blocks the E2 mediated down-regulation of ER mRNA (Figure 6D) and there is an increase in total ER expression (49) (Figure 6C). Interestingly, the co-administration of brivanib alaninate prevented the tamoxifen induced increase in ER mRNA (Figure 6C) and there was a decrease in total ER expression (Figure 6D). It appears that the administration of an inhibitor of VEGFR-2 can modulate the ER during the anti-tumor process and this is an area worthy of further investigation. Conversely, the expression of VEGFR-2 on the cancer cells in response to estrogen is clearly important to maintain control of tumor growth. These observations further validate the use of a combination of an anti-estrogen and an angiogenesis inhibitor.

In addition to inhibiting VEGFR-2, the inhibitor has also shown activity against FGFR-1 in other tumor models, and is thus useful as a dual inhibitor for angiogenesis (24). In the present study, however, we were unable to detect FGFR-1 in our specific model.

Despite the encouraging results obtained in the present study, several recent reports (50–52) of either the development of resistance to antiangiogenic drugs (50) or enhanced metastatic spread with low dose antiangiogenic drugs (51,52) deserve consideration. Clinical trials have shown that the majority of human tumor types do not respond to inhibitors of integrin as an antiangiogenic strategy. Laboratory models now show (52) that low concentrations of $\alpha_v\beta_3$ and $\alpha_v\beta_5$ inhibitors increase tumor growth via VEGFR-2 trafficking. This promotes endothelial cell migration to VEGF. In related studies, inhibitors of VEGFR can either enhance tumor cell seeding in “metastatic assays” (51) or cause adaptive-evasive responses by tumors with greater malignancy and increased invasiveness (50). Clearly, the complexity of the angiogenic survival signaling pathways present a challenge to seek the clinical relevance of preclinical pharmacology. Nevertheless, in a recent review, Ebos and co-workers (53) contend that it remains unclear whether antiangiogenic therapy will lead to increased invasion or metastases after long or short term treatments. There are more than 40+ adjuvant clinical trials in progress, so the question of the premature tumor resistance caused by low dose antiangiogenesis inhibitors will probably be answered first in the clinical setting(53).

With this concern in mind, we are currently considering an initial short term testing platform in ER positive metastatic breast cancer that has failed exhaustive endocrine therapy (54,55). It is known that apoptosis and tumor regression can be induced by both high or low dose estrogen clinically (56,57), but we propose to use low dose estrogen to reduce thromboembolic events. The therapeutic application of low dose estrogen treatment is a direct translation of laboratory studies over the past 15 years (58,59). By combining a dose escalation schedule of brivanib alaninate, we will be able to monitor tumor response precisely for the 12 week treatment schedule. These preliminary clinical data will guide our future adjuvant applications.

In summary, antiangiogenic agents have been utilized clinically in patients who have breast cancer that is refractory to other agents (44). In these instances, to see a partial clinical benefit, higher doses that are potentially toxic have to be used. The observations that elevations of VEGFA and VEGFR-2 are associated with poor prognosis and response to tamoxifen therapy (48,60) suggests that a strategy to combine anti-hormone treatment with an anti-angiogenic strategy may have merit to test in clinical trials. Based on an increasing laboratory database that implicates an elevation in angiogenic factors in endocrine resistant breast cancer in the presence of tamoxifen (43), we have provided evidence that a combination of tamoxifen plus a low dose dual inhibitor of VEGFR-2 and FGFR-1, brivanib alaninate, effectively controlled tumor growth. The strategy of combining a tyrosine kinase inhibitor of VEGFR-2 has the advantage of reducing toxicity, permitting long-term therapy and therefore compliance to enhance efficacy for adjuvant tamoxifen therapy. Indeed, the strategy of inhibiting angiogenesis, might in fact, improve responsiveness of those ER positive tumors that are refractory to tamoxifen alone. We believe this issue should be addressed in clinical trial.

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Figure 1.

The distribution of the VEGFR-2 receptor (A) and VEGFA (B) in the MCF-7 E2 tumor model. Tumor bearing animals were treated with estradiol (a), estradiol and 2 weeks of 125 µg tamoxifen (b), estradiol and then 2 weeks of estradiol withdrawal (c), and estradiol followed by 2 weeks of estradiol withdrawal and fulvestrant (d). VEGFA and VEGFR-2 expression decreased with estradiol withdrawal. The bars represent 50 micrometers.

2A

2B

2C

2D

Figure 2.

The growth characteristics of MCF-7 E₂ tumors treated with estradiol alone or with estradiol and the lower (0.05 mg/g) and higher doses (0.1mg/g) of brivanib alaninate. There were 5 ovariectomized, athymic mice and 10 tumors per group. The drug treatment resulted in a decreased average CSA of the tumors at the higher dose (0.1mg/g) ($p=0.001$, $\alpha=0.025$), but there was no difference between the group treated with the low dose (0.05 mg/g) of brivanib alaninate and the estrogen only group ($p=0.2$, $\alpha=0.025$). There were no significant differences in animal body weights between groups. H and E staining is shown in panel B and reveals that with increases in the dosing of the drug, there was an increase in the amount of necrotic tissue (*). The bar represents 100 micrometers. Panel C demonstrates that there was no significant change in the total amount of VEGFR-2 expressed by the tumors, but there was a decrease in the phosphorylation pattern of the tumors treated with brivanib alaninate, regardless of the dose given. The presence of ER and its phosphorylated form was indicative of active tumor tissue in all of the samples. Panel D demonstrates analysis by RTPCR. There was a significant increase in VEGFA in the high dose group in comparison to tumors treated with estrogen only ($p=0.02$). There was a small, but significant decrease in ER mRNA in the high dose (0.1mg/g) group ($p=0.04$). VEGF C transcription decreased significantly in tumors treated with the low dose (0.05 mg/g) of brivanib alaninate. Mouse

VEGFR-1 and mouse VEGFR-2 mRNA, which represented the endothelial component of the tumor, significantly decreased in the high dose (0.1mg/g) group ($p=0.02$, $p=.04$).

3A

3B

Figure 3.

The antitumor effects of high dose (0.1 mg/g) brivanib alaninate on the growth of human tumors with acquired resistance to the SERMs raloxifene or tamoxifen. There were no significant differences in animal body weights between groups. Unless stated otherwise, all groups had 5 ovariectomized athymic mice with 10 tumors. **A)** Raloxifene stimulated MCF-7 Ral. Groups were treated with raloxifene (1.5 mg daily by gavage), vehicle, fulvestrant (2 mg SQ 5 days per week), or raloxifene plus brivanib alaninate (0.1 mg/g by gavage). Brivanib alaninate (0.1mg/g) significantly prevented the growth of raloxifene treated tumors ($p < 0.001$, $\alpha = 0.016$). **B).** Raloxifene (1.5 mg daily by gavage) stimulated MCF-7 RAL. Twenty ovariectomized athymic mice were randomized into two groups of 10

mice each with continued raloxifene treatment (total of 17 tumors in the group) or raloxifene plus high dose brivanib alaninate (0.1 mg/g by gavage) (total of 19 tumors in the group). There was a significant decrease in tumor size with brivanib alaninate ($p < 0.001$, $\alpha=0.025$). **C).** Tamoxifen (1.5 mg daily by gavage) stimulated MCF-7 TAM tumors. Athymic, ovariectomized mice were initially placed into 3 groups to receive 1.5 mg tamoxifen (8 mice, 16 tumors), 1.5 mg tamoxifen plus 0.1 mg/g brivanib alaninate (4 mice, 6 tumors) or control vehicle (5 mice, 10 tumors). The group that received tamoxifen was randomized to continue tamoxifen (4 mice, 8 tumors) or receive tamoxifen with 0.1mg/g brivanib alaninate (4 mice, 8 tumors) once the tumors reached an average CSA of 0.5 cm². The VEGFR inhibitor produced significant decreases in tamoxifen stimulated growth rate in early implanted ($p < 0.001$, $\alpha=0.025$) or established ($p < 0.001$, $\alpha=0.025$) tumors. **D).** Treatment of tamoxifen-stimulated (500 µg tamoxifen by gavage daily) EnCa 101 endometrial tumors was continued in two groups of 10 ovariectomized, athymic mice (20 tumors per group for 40 days). One group then received concomitant high dose brivanib alaninate (0.1 mg/g by gavage) for 3 weeks. Tumor volume was significantly decreased in animals treated with brivanib alaninate and tamoxifen compared to tamoxifen alone ($p < 0.001$, $\alpha=0.025$).

Figure 4.

The effect of daily oral tamoxifen dosing on the estradiol stimulated growth of MCF-7 E2 tumors delivered by an implanted 0.3 cm sustained release silastic capsule. There were 5 ovariectomized, athymic mice and 10 tumors per group. There was a dose dependent decrease in estradiol stimulated tumor growth. The tumors did not grow without estradiol. The lowest dose of tamoxifen, 125 µg, suppressed tumor growth by 63%, whereas the higher doses (250 µg and 500 µg) suppressed tumor growth by 75%. There were no significant differences in animal body weights between groups.

Figure 5.

The effect of a combination of tamoxifen (125 µg daily oral dose) and 0.05 mg/g brivanib alaninate on the growth of established estradiol stimulated MCF-7 E₂ tumors. There were 5 ovariectomized, athymic mice and 10 tumors per group. The combination of 125 µg tamoxifen with 0.05 mg/g brivanib alaninate improved the effects of 125 µg tamoxifen ($p < 0.01$, $\alpha = 0.025$) or 0.05 mg/g brivanib alaninate ($p < 0.001$, $\alpha = 0.025$). There were no significant differences in animal body weights between groups.

6A

6B

6C

6D

Figure 6.

Panel A: The efficacy of a combination of 125 µg tamoxifen and 0.05 mg/g brivanib alaninate on the growth of established estradiol stimulated MCF-7 E2 tumors. There were 5 ovariectomized, athymic mice and 10 tumors per group. Tumors were grown to approximately 0.46 cm² and treated with the treatment regimens as indicated. There were no significant differences in animal body weights between groups. However, the decrease in average CSA was significant when comparing the combination treatment to tamoxifen (125 µg) treated tumors ($p=0.01$, $\alpha=0.025$) or those treated with 0.05 mg/g brivanib alaninate ($p=0.007$, $\alpha=0.025$). Panel B: H and E staining demonstrated an increase in necrotic tissue when brivanib alaninate was given alone or with tamoxifen. Once again, the bar represented a 100 micrometer distance. Panel C: Western blot analysis of tumor tissue did not illustrate a decrease in total VEGFR-2, regardless of the treatment group. The addition of brivanib alaninate, decreased the phosphorylation of VEGFR-2. Expression of ER and phosphorylated ER in all tumors, demonstrated the presence of active tumor tissue. Panel D: Relative fold change in the mRNA levels of angiogenic factors in tumors relative to estradiol treatment alone. Mouse VEGFR-1 and mouse VEGFR-2 mRNA increased dramatically in tumors that received the inhibitor ($p=0.001$, $p=0.001$) or the inhibitor plus tamoxifen ($p=0.002$, $p=0.002$). VEGFA mRNA increased in tumors in response to tamoxifen treatment ($p=0.01$) brivanib alaninate treatment ($p=0.001$) and the combination of brivanib alaninate plus tamoxifen ($p=0.002$). VEGFC increased in tamoxifen treated tumors

($p=0.001$) and decreased in tumors treated with brivanib alaninate ($p<0.004$). There was a significant, but small decrease in ER mRNA ($p=0.04$) in tumors treated with the combination of tamoxifen plus brivanib alaninate and an increase in ER mRNA in tamoxifen treated tumors ($p=0.04$).

Figure 7.

Panel A: There is no change in total VEGFR-2 expression by IHC in MCF-7 E2 tumors treated with estradiol (a), estradiol and 2 weeks of 125 µg tamoxifen (b), estradiol and 2 weeks of 0.05mg/g brivanib alaninate (c), or estradiol and 2 weeks of the combination of 125 µg tamoxifen and 0.05 mg/g brivanib alaninate (d). Panel B: By IHC, the VEGFA staining intensity is greatest with 2 weeks of the combination of 125 µg tamoxifen and 0.05 mg/g brivanib alaninate (d). Staining intensity is the same with estradiol (a), estradiol and 2 weeks of 125 µg tamoxifen (b), estradiol, and 2 weeks of 0.05mg/g brivanib alaninate (c). The bars represent 50 micrometers.

Table 1**Primers used for RTPCR**

VEGFA:	fwd: 5`GGGCAGAATCATCACGAAGTG 3` rev: 5`TCAGGGTACTCCTGGAAGATGTC 3`
VEGFB:	fwd: 5`AGCCAGTGTGAATGCAGACCTA 3` rev: 5`AGTCCCAGCCCGGAACAG 3`
VEGFC:	fwd: 5`CCTCAGCAAGACGTTATTTGAAATT 3` rev: 5` TGGCAAACTGATTGTTACTGGTT 3`
VEGFD:	fwd: 5`CGTACATTTCCAAACAGCTCTTTG 3` rev: 5`GGCAAGCACTTACAACCTGTATGA 3`
VEGFR-1:	fwd: 5`TTCTCACAGGATCTAGTTCAGGTTCA 3` rev: 5`CTGCTTCCCCCTGCAT 3`
VEGFR-2:	fwd: 5`CAGAGTGGCAGTGAGCAAAGG 3` rev: 5`TTGTAGGCTCCAGTGTCAATTCC 3`
Mouse VEGFR-1:	fwd: 5`TCCTATCGGCTGTCCATGAAA 3` rev: 5`CCAAATAGCGAGCAGACTTCAA 3`
Mouse VEGFR-2:	fwd: 5`ACCAGCATGGCATCGTGTAC 3` rev: 5`CCTAGCGCAAAGAGACACATTG 3`
Mouse VEGFR-3:	fwd: 5`GTATGAAATTGACCCGTACGAAAA 3` rev: 5` AGGAAATGAGGCTTGAGAGAAGATC 3`