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A phase II neoadjuvant trial of anastrozole, fulvestrant and gefitinib in patients with newly diagnosed estrogen receptor positive breast cancer

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Abstract

Endocrine therapy in patients with breast cancer can be limited by the problem of resistance. Preclinical studies suggest that complete blockade of the estrogen receptor (ER) combined with inhibition of the epidermal growth factor receptor (EGFR) can overcome endocrine resistance. We tested this hypothesis in a phase II neoadjuvant trial of anastrozole and fulvestrant combined with gefitinib in postmenopausal women with newly diagnosed ER-positive breast cancer. After a baseline tumor core biopsy, patients were randomized to receive anastrozole and fulvestrant (AF) or anastrozole, fulvestrant, and gefitinib (AFG) for 3 weeks. After a second biopsy at 3 weeks, all patients received AFG for 4 months and surgery was done if the tumor was operable. The primary endpoint was best clinical response by RECIST criteria and secondary endpoints were toxicity and change in biomarkers. The study closed after 15 patients were enrolled because of slow accrual. Median patient age was 67 years and median clinical tumor size was 7 cm. Four patients had metastatic disease present. Three patients withdrew before response was assessed. In the remaining twelve patients, there were two complete clinical responses (17%), three partial responses (25%), five had stable disease (41%), and two (17%) had progressive disease. Most common adverse events were rash in four patients, diarrhea in four, joint symptoms in three, and abnormal liver function tests in three. There were no grade 4 toxicities and all toxicities were reversible. At 3 weeks, cell proliferation as measured by Ki-67 was significantly reduced in the AFG group (p value= 0.01) with a parallel reduction in the expression of the Cyclin D1 (p value=0.02). RNA microarray data showed a corresponding decrease in the expression of cell cycle genes. These results suggest that AFG was an effective neoadjuvant therapy and consistently reduced proliferation in ER-positive tumors.

Keywords

breast cancer; estrogen receptor; endocrine resistance; EGFR; proliferation

Introduction

The use of adjuvant systemic therapy for breast cancer has resulted in improved patient survival by eliminating micrometastatic disease responsible for disease recurrence and death [1]. Despite the well-documented benefit of adjuvant therapy, it is not effective in all patients, and the success of such therapy can only be judged in retrospect upon disease relapse; a time when breast cancer is nearly always incurable. Currently, there are few reliable methods to predict the success or failure of a particular postoperative treatment and better ways to predict and optimize outcome are needed. Neoadjuvant (preoperative) therapy offers the advantage of directly observing response to treatment and allows sampling of tumor tissue to be examined for molecular changes that may correlate with response or lack thereof. This could potentially identify patients who may benefit from alternative treatment early in the course of their disease.

The role of neoadjuvant chemotherapy was established in pivotal trials which proved its safety and equivalence to adjuvant use [2] and helped identify pathologic complete response as an important surrogate of long-term survival in chemotherapy-treated patients [3]. Despite the benefit of using neoadjuvant chemotherapy in some patients, it is associated with significant toxicity, expense, and many patients do not gain any benefit. In particular, chemotherapy benefit decreases with age [4] and higher ER [5], as well as in tumors with lower proliferation as assessed by multigene assays [6].

With the recent introduction of novel and more potent endocrine agents, such as the third generation aromatase inhibitors (which produce a profound state of estrogen deprivation) [7] and the more complete ER antagonist fulvestrant, which degrades ER [8], there has been a renewed interest in testing a combination of these agents in the neoadjuvant setting. Indeed, aromatase inhibitors were shown to be superior to the selective estrogen receptor modulator (SERM) tamoxifen in the neoadjuvant setting [9, 10] and combining agents with different mechanisms of action hold the promise of more effective inhibition of ER signaling.

Despite the promise of newer endocrine agents, resistance remains a major problem and unraveling the mechanisms of this resistance can lead to improved treatment strategies. We have shown in preclinical breast cancer xenograft models that complete blockade of ER using estrogen deprivation combined with fulvestrant was a more effective strategy than either strategy alone [11, 12] and that using gefitinib (Iressa), which can inhibit both EGFR and HER2, resulted in delayed resistance to endocrine therapy [11, 12]. We therefore designed a neoadjuvant trial using the aromatase inhibitor anastrozole and fulvestrant, in combination with gefitinib, to examine our preclinical hypothesis and to evaluate biologic markers of response and resistance in serial tumor biopsies. Patients were initially randomized to anastrozole and fulvestrant (AF) vs. anastrozole, fulvestrant and gefitinib (AFG) for 3 weeks to examine the differences in biomarker expression between the two groups, and after that, all patients received the 3 drugs for 4 months prior to surgery.

Patients and methods

Patients

Postmenopausal women with previously untreated ER and/or PR positive breast cancer and a WHO performance status of 0–2 were eligible. Patients had to have a primary tumor of 3 cm or greater in size as measured by palpation and not have had a surgical biopsy. Other eligibility criteria included: adequate renal function, defined by a serum creatinine within 3 times the upper limits of normal; adequate liver function, defined by total bilirubin, AST, ALT, and alkaline phosphatase within 3 times the upper limits of normal; adequate bone marrow function defined as a WBC >3.0, PLT > 75,000, Hb >9; no evidence of a bleeding diathesis or prolongation of PT/PTT; and willingness to undergo breast biopsies as required by the study protocol. Patients with diffuse or inflammatory tumors were not eligible for the study. Patients were followed monthly for tolerability and disease evaluation. The study was approved by the institutional review board and all patients signed informed consent.

Study design

This was a single stage, single institution, phase II trial of combined anastrozole (Arimidex), fulvestrant (Faslodex), and gefitinib (Iressa) for four months, to determine the best clinical response in the primary tumor by RECIST criteria. Secondary endpoints were the safety and tolerability of the combination regimen and assessment of biomarker modulation. After a baseline tumor core biopsy on day 1, patients were initially randomized to receive anastrozole 1 mg per oral (PO) daily and fulvestrant 250 mg intramuscular (IM) monthly (AF group), or anastrozole 1 mg PO daily, fulvestrant 250 mg IM monthly and gefitinib 250 mg PO daily (AFG group). After a second biopsy was done on day 21 to allow comparative biomarker analysis between the AF vs. the AFG groups, all patients then received the three drugs (AFG) to complete a total of 4 months from the time of enrollment. Surgery was then done if the tumor was operable (Fig. 1).

Biopsies

Tumor tissue was obtained by core biopsy using a Bard Max Core Biopsy Instrument (#MC1410). The outside needle was 14 gauge (2.1 mm), with a smaller inside cutting needle to obtain the biopsy. The length of the cutting needle penetration was 22 mm and the total length of the needle was 10 cm. Multiple biopsy samples of approximately 2 mm × 10–20 mm were obtained and divided in 10% formalin or immediately frozen in liquid nitrogen. Biopsies were guided by palpation.

Immunohistochemistry (IHC)

There were twelve paired core biopsies collected. Of those, three pairs were removed because at least one sample in the pair had insufficient tumor cells for analysis. Nine paired samples were adequate and available for analysis. Tumor material obtained by core biopsy was fixed in 10% neutral-buffered formalin overnight before processing and paraffin embedding. A 3-micron thick section was examined by hematoxylin and eosin (H&E) staining to verify adequacy of tumor tissue. IHC was performed on 4-micron thick sections from these paraffin blocks. After deparaffinization, heat-induced epitope retrieval was

performed in a pressure cooker using Tris-HCL buffer at a pH of 9.0 for 10 minutes (except for phosphorylated MAP kinase antibody for which Tris-EDTA was at a pH of 8.0). The primary antibodies in this study included: ER 6F11 at 1:200 dilution (Novocastra, UK); PR 1294 at 1:1600 dilution (Dakocytomation, CA); cyclin D1 SP4 at 1:25 dilution (Neomarkers, CA); Ki-67 clone MIB-1 at 1:200 dilution (Dakocytomation, CA); phospho-MAP Kinase polyclonal antibody at 1:80 dilution (Cell signaling, MA); phospho-AKT polyclonal antibody at 1:10 dilution (Cell signaling, MA). Secondary antibodies used were EnVision labeled polymer-HRP (horseradish peroxidase) anti-mouse or anti-rabbit as appropriate. 3, 3'-diaminobenzidine (DAB) chromogen (Dakocytomation, CA) and DAB sparkle Enhancer (Biocare Medical, CA) were used after incubation with primary antibodies at room temperature for 1 hour. Slides were lightly counterstained with hematoxylin. A tissue array with normal tissues of endocervix, endometrium, ovary, breast, appendix, and tonsil was used as positive controls. Mouse or rabbit IgG was used on normal tissues array as negative controls. Slides for ER, PR, cyclin D1, phospho-MAP Kinase, and phospho-AKT were evaluated using the Allred scoring system [13]. For the Ki-67 proliferation index, we counted at least 500 cells and recorded the percentage of cells with positive staining. The pathologist reading the results was blinded to the treatment group (AF vs. AFG arm, or day 1 vs. day 21). Paired t-tests were used to compare pre- vs. post-treatment scores within each arm and graphs were constructed to compare day 1 vs. day 21 results.

Statistical considerations

The primary objective of the study was to determine the best clinical response (CR and PR) after four months of treatment. A neoadjuvant study of the aromatase inhibitor letrozole reported a clinical response rate of 60% in post-menopausal ER+ and/or PR+ women with primary breast cancer [14]. For purposes of sample size requirements of this study, we tested the null hypothesis that clinical response rate is equal to 50% versus the alternative hypothesis that it is equal to 70%. A sample of 50 patients who will have received the three-drug combination as per the treatment plan would allow us to detect a 20% absolute increase in clinical response rate with 82% power based on a two-sided test for a single proportion with significance level set at 5% (NQuery 4.0). We planned to accrue an additional 10 patients for a total of 60 to adjust for drop-outs, non-compliance, and non-evaluable patients due to missing second biopsies. Ultimately, the study was closed after only 15 patients were enrolled because of slow accrual.

All adverse events (AEs) were recorded using the NCI CTCAE v3.0. All AEs were summarized overall and by grade (Grade 1 and Grades 2–3). Biomarker data was summarized on day 1 and day 21, and the difference in measurements between the AF vs. the AFG groups was compared using the paired t-test. All statistical analyses were conducted using SAS 9.1. P-values were two-sided.

Expression Microarrays

Total cellular RNA was extracted from core biopsies which had been pulverized while frozen and then lysed with Trizol reagent (Invitrogen, Carlsbad, CA) followed by Qiagen RNeasy column purification (Qiagen, Valencia, CA). cRNA was synthesized, and hybridized onto Affymetrix U133Plus2.0 chips (www.affymetrix.com) using recommended

procedures for synthesis, hybridization, washing, and staining with streptavidin–phycoerythrin. There were twelve patients with paired biopsy material available for microarray analysis; five in the AF group and seven in the AFG group for a total of 24 chips. One chip from the AFG group did not meet quality control criteria by GCOS/dChip (www.dchip.org) [15] and was removed along with its paired sample, leaving six paired samples from the AFG group. Expressions were estimated using GC RMA model with Biometric Research Branch (BRB)-Array Tools software (<http://linus.nci.nih.gov/BRB-ArrayTools.html>, Simon, R., and Peng, A. 2006). We included in the comparisons 30% most variable probe sets. The sample size of 6 was too small to compare differentially expressed gene sets while maintaining an acceptable low false discovery rate (FDR) for clinical data [16]. To address this issue, BioCarta pathway comparisons with BRB Array Tools were performed [17]. This analysis is based on small subsets of predefined genes and the multiple comparisons problem is less serious in small sample sizes.

Results

Patient and disease characteristics

All 15 patients enrolled had newly-diagnosed, previously untreated breast cancer. Patients were initially randomized to receive AF (n=7) or AFG (n=8) for 21 days and then all patients received the three drug combination and continued on the regimen to complete four months. Median age at diagnosis was 67 years. Fourteen subjects were female and one was a male. 47% of patients were black, 26% Hispanic, 13% white, and 7% Asian (Table 1). Median clinical tumor size by palpation was 7 cm and four patients had gross metastatic disease present (27%). All patients had ER-positive tumors; eleven (73%) were ER-positive/PR-positive and four (27%) were ER-positive/PR-negative. Patients were followed monthly for tolerability and disease evaluation. Three patients withdrew from the study before disease evaluation was made (two before the study medications started for socioeconomic reasons, and one because of Grade 3 joint pain in the first three weeks of treatment).

Safety and tolerability

All thirteen patients who received treatment had some toxicity on therapy. The most common adverse events reported are summarized in table 2. The majority of these adverse events were grade 1. There were five Grade 3 toxicities reported (1 acneiform rash, 1 joint pain- patient withdrew, 2 with elevated liver function tests, and 1 cough-deemed related to metastatic lung disease and unlikely related to study drugs). One of the patients had a both a grade 3 rash and grade 3 elevation of liver function tests, both of these toxicities resolved upon temporarily stopping study treatment. After resumption of treatment, there was no recurrence of toxicity and the patient had a complete clinical response. Overall, there were no grade 4 toxicities reported and all toxicities resolved rapidly with discontinuation of study medications (Table 2).

Response rates

Tumor response was determined by serial clinical measurements of the primary tumor on a monthly basis and the best assessed response was used for the analysis. Of the 12 patients

who were evaluable for response, there were 2 complete responses (17%), 3 partial responses (25%), 5 stable disease (42%), and 2 (17%) progressive disease.

Immunohistochemical analysis

ER, PR, and BCL-2—Of the nine patients with paired tumor samples on day 1 and day 21, four were in the AF group and five in the AFG group. First, we sought to identify changes in ER levels and corresponding differences in levels of PR and Bcl-2, both known to be dependent on ER for expression [18, 19]. In the overall group, there were decreases in the mean scores of ER, PR, and Bcl-2 from baseline to day 21 although the differences were not statistically significant (Table 3). Comparing day 1 and day 21 in the AF group, there was no change in the level of ER expression, while mean PR levels decreased (mean = −2.0), and Bcl-2 slightly increased (mean = 1.0) (Fig. 2). None of these changes, however, were statistically significant (Table 3). Interestingly, in the AFG group there was a decrease in both ER and PR levels (means = −1.2 and −2.8, respectively), with a trend for a decrease in Bcl-2 (mean = −1.0), but none of these changes reached statistical significance (Table 3).

Ki-67 and Cyclin D1—To assess treatment effect on tumor proliferation, we next examined nuclear staining for Ki-67 score and correlated that with cyclin D1, a cell cycle protein product that is also known to be regulated by ER [20] (Fig. 2). In comparing day 1 vs. day 21 in the overall group, there was a significant decrease in mean Ki-67 scores (Mean = −0.24) with a p value of 0.001 (Table 4). Although Ki-67 scores decreased on day 21 in all tumors (9/9, mean = −0.20), this did not reach statistical significance (p = 0.11). In the AFG group, and despite the very small sample size, the decrease in Ki-67 scores (mean = −0.28) was statistically significant with a p value of 0.01 (Table 4). Interestingly, cyclin D1 expression was decreased in the overall group on day 21 (mean = −1.1), and this decrease was statistically significant in the AFG group, with a p value of 0.02 (Table 4).

P-MAPK and p-AKT—To further examine treatment effect on epidermal growth factor receptor (EGFR) signaling, the target for inhibition by gefitinib, we assessed levels of phosphorylated (p) MAPK and AKT by immunohistochemistry, both of which are key downstream signaling molecules in the EGFR pathway and are known to be inhibited by gefitinib [21]. Comparing day 21 vs. day 1, and as predicted by our preclinical model, levels of p-MAPK and p-AKT were decreased using the Allred scoring method (mean = −1.0 and −1.1, respectively) (Fig. 3) although this did not reach statistical significance (Table 4).

Expression Microarray analysis

To further analyze pathway changes in response to treatment, we performed comparison microarray analysis of day 1 vs. day 21 on the frozen paired biopsies obtained. There were twelve paired frozen biopsy samples available; five in the AF group and seven in the AFG group for a total of 24 Affymetrix U133Plus2.0 chips (www.affymetrix.com). Because one chip from the AFG group did not meet quality control criteria (see method section), it was removed with its paired sample, leaving six paired samples from the AFG group. Using the pairwise pathway comparison method, we found no significantly different BioCatra pathways between the day 21 and day 1 samples for patients in the AF treatment group. There were, however, 10 significantly different BioCarta pathways between day 21 and day

1 for the AFG treatment group, including cyclins and cell cycle genes (Table 5). In particular, cyclin D1 from this pathway was significantly downregulated on day 21 vs. day 1 in the AFG group, with a p value of 0.002 (Fold change of day 21 vs. day 1 = 0.3). This change in cell cycle genes correlates with the observed decrease in cyclin D1 protein expression using IHC and the decrease in cell proliferation as reflected by Ki-67 scores.

Discussion

In this study of primary ER-positive breast cancer, we attempted to maximize antitumor effect using complete blockade of ER signaling combined with EGFR inhibition. Despite the small number of patients studied, clinical effect of AFG was consistent with anticipated activity of endocrine therapy in ER-positive breast cancer [14]. Although definitive conclusions about the robustness of clinical effect are clearly limited by the small sample size, there was no observed dramatic antitumor effect for AFG or clear superiority based on the clinical data analysis. This is probably consistent with enrolling unselected patients with ER-positive breast cancer with no validated target for EGFR inhibition by gefitinib. In addition, it is possible that the dose of fulvestrant used in this study (250 mg IM monthly) may have been suboptimal based on most recent data showing more efficacy for higher fulvestrant dosing[22]. Overall, treatment was tolerated with no grade 4 toxicities and all reported drug toxicities resolving rapidly after stopping treatment. Interpretation of toxicity profile may have been somewhat complicated by the relatively advanced stage of disease on presentation in this underserved population, and the threshold for continuing treatment may have been impacted by the socioeconomic status and issues with compliance.

Interestingly, and despite the small number of samples, AFG significantly reduced Ki-67, which is a short term surrogate endpoint for improved relapse free survival in ER-positive antiestrogen-treated breast cancer patients [23]. Cyclin D1, a cell cycle marker that is regulated by both estrogen [20, 24] and EGFR [25], was also significantly reduced by AFG treatment. Cyclin D1 has been shown in preclinical models to be critical for cell cycle progression in antiestrogen-resistant breast cancer [26] and its expression in tumor tissue correlates with risk of relapse in patients with ER-positive breast cancer treated with tamoxifen [27]. Further examination using tumor microarrays showed a significant reduction of cyclin D1 expression, in parallel to the observed changes using IHC. Although p-MAPK and p-AKT were both reduced by gefitinib, the changes were not statistically significant, probably because of the small sample size. Interestingly, Bcl-2- another ER regulated gene product [18]- was also significantly reduced by gefitinib in the AFG group, compared to AF alone, suggesting that EGFR inhibition may inhibit the convergence of growth factor signaling on ER-regulated genes and a potential collaborative antitumor effect between EGFR inhibitors and antiestrogen therapy. A recent neoadjuvant trial using erlotinib, another EGFR inhibitor, similarly showed that short term treatment with this agent significantly reduced tumor proliferation in ER-positive breast cancer, with a significant reduction in phosphorylated ER at Serine-118 [28]. This further suggests the potential for a collaborative impact of EGFR inhibition on ER-driven signaling in clinical breast cancer[29], and supports the concept of preclinical models of inter-pathway crosstalk in endocrine resistance [30].

Other clinical trials of combined EGFR inhibitors with endocrine therapy in the preoperative [31, 32] and metastatic settings [33, 34] were also reported, with mixed results and inconsistent antitumor benefit for the combination. These clinical trial findings contrast sharply with the clarity of the preclinical concept [30] and, interestingly, with similar clinical trials reported for anti-HER2 agents in combination with endocrine therapy [35, 36]. The challenges in conducting clinical trials using novel targeted agents to modulate endocrine resistance may be explained by the heterogeneity of clinical breast cancer as opposed to the more uniform preclinical models of target selection, and the lack of validated predictive markers for newer targeted agents, outside the known role of ER and HER2. Another problem of traditional clinical trial design in targeted therapy is that clinical endpoints take longer to reach, especially knowing that these studies have to compete with chemotherapy trials for patient accrual, and thus are slower to reach their conclusions.

Preoperative clinical trial designs in endocrine resistance have the advantage of rapidly screening for the efficacy of adding a targeted agent to endocrine therapy by evaluating treatment impact on proliferation as a primary endpoint of benefit rather than using a clinical endpoint. Another potential advantage of the preoperative setting is that tumor tissue examined can reveal dynamic molecular changes in response to therapy, and identify pathways that may become *derepressed* and emerge early in response to endocrine therapy through the known reciprocal expression relationship between these pathways and ER [30, 37, 38]. This strategy can provide invaluable and timely information about resistance mechanisms and can potentially identify molecular targets for future clinical trial design.

It is clear that a more robust clinical trial effort in the area of combined targeted and endocrine therapy is needed to study endocrine resistance mechanisms and to improve outcome of ER-positive breast patients. This kind of research effort requires a change in persistent attitudes regarding the continued emphasis on the use of chemotherapy in ER-positive breast cancer, especially in the US, where practice patterns continue to favor use of chemotherapy despite available evidence, which has contributed to the difficulty in conducting non-chemotherapy trials both in neoadjuvant as well as metastatic disease. In conclusion, preoperative clinical trials may be ideally suited to rapidly screen for effective targeted and endocrine therapy combinations through the impact on tumor proliferation as a primary therapeutic endpoint. These types of designs can also help unravel new treatment targets that can emerge early during treatment and that may drive therapeutic resistance. Discovery of these new pathways responsible for resistance can help future design of more individualized clinical trials through better target selection, which may ultimately refine treatment of patients with ER-positive breast cancer.

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Fig. 1. Clinical trial schema. After baseline biopsy, patients were initially randomized to AF vs. AFI for 3 weeks and then a second biopsy was obtained. After 3 weeks all patients received AFI to complete a total of 4 months of preoperative treatment.

Fig. 2.

Graphic comparison within individual patient paired samples between the AF and AFI treatment groups on Day 1 vs. Day 21. Results are shown ER, PR, and Bcl-2 using the Allred scoring method. *Paired t-tests were used to compare pre- vs. post-treatment scores within each arm and the p-values are outlined in table 3.

Fig. 3.

Graphic comparison in individual patient paired samples between the AF and AFI treatment groups (Day 1 vs. Day 21). Biomarkers shown are Ki-67, cyclin D1, p-MAPK, and p-AKT. Ki-67 is represented by percent of nuclear staining, while cyclin D1, p-AKT, and p-MAPK are scored using the Allred method. *Paired t-tests were used to compare pre- vs. post-treatment scores within each arm and the p-values are outlined in table 4.

Table 1

Patient and tumor characteristics by treatment group

	Overall (n=15)	AF (n=7)	AFG (n=8)
		N (%)	N (%)
Race/Ethnicity			
White	2 (13%)	1 (14%)	1 (12%)
Hispanic	4 (26%)	2 (29%)	2 (25%)
Black	7 (47%)	4 (57%)	3 (39%)
Asian	1 (7%)	0 (0%)	1 (12%)
Unknown	1 (7%)	0 (0%)	1 (12%)
Gender			
Female		6 (86%)	8 (100%)
Male		1 (14%)	0 (0%)
Age			
Mean	65.9	63.5	68.0
Median	67.2	64.7	68.3
Range	50 – 87	50 – 76	57 – 87
Baseline Tumor Size (cm)			
Mean	6.9	7.1	6.8
Median	7.0	8.0	5.0
Range	3 – 17	5 – 8	3 – 17
Palpable Nodes, Y/N			
Yes	6 (40%)	5 (71%)	1 (12%)
No	9 (60%)	2 (29%)	7 (88%)
Estrogen Receptor Positive		7(100%)	8 (100%)
Progesterone receptor positive		6 (86%)	5 (63%)
HER2 positive		1 (14%)	0 (0%)

The table reflects all 15 patients who were enrolled.

Table 2

Adverse events

Adverse event	Grade 1	Grade 2	Grade 3	Grade 4	Total
Rash	2	1	1	0	4
Diarrhea	4	0	0	0	4
Elevated LFT's	1	0	2	0	3
Joint pain	2	0	1	0	3
Cough/Dyspnea	1	1	1 ^a	0	3
Abdominal pain	2	0	0	0	2
Fatigue	0	1	0	0	1

^aPatient with grade 3 cough had progressive metastatic disease to the lung and died.

Table 3

Comparison of day 1 vs. day 21 in ER, PR, and Bcl-2 levels by treatment group

		Overall (n=9) Mean (Median) Range	AF (n=4) Mean (Median) Range	AFG (n=5) Mean (Median) Range
ER	Day 1	6.2 (7.0)	6.0 (6.5)	6.4 (7.0)
	Day 21	5.6 (7.0)	6.0 (6.5)	5.2 (7.0)
	Day 21 – Day 1	–0.67 (0.0)	0 (0.0)	–1.2 (–1.0)
			p=1.00	p=0.11
PR	Day 1	2.9 (4.0)	2.0 (2.0)	3.6 (6.0)
	Day 21	0.44 (0.0)	0 (0)	0.8 (0.0)
	Day 21 – Day 1	–2.4 (–2.0)	–2 (–2.0)	–2.8 (–2.0)
			p=0.18	p=0.11
Bcl2	Day 1	5.1 (5.0)	4.8 (5.0)	5.4 (6.0)
	Day 21	5.0 (5.0)	5.8 (5.5)	4.4 (5.0)
	Day 21 – Day 1	–0.11 (0.0)	1.0 (0.5)	–1 .0 (–1.0)
			p=0.25	p=0.09

Paired t-tests were used to compare pre- vs. post-treatment scores within each arm. Levels of expression were assessed using the Allred score method.

Table 4

Comparison of Ki-67 and CD 1, P-MAPK, and P-AKT by treatment group

	Overall (n=9) Mean (Median) Range	AF (n=4) Mean (Median) Range	AFG (n=5) Mean (Median) Range
Ki-67 (% positive cells)			
Day 1	0.50 (0.48)	0.47 (0.47)	0.53 (0.48)
Day 21	0.26 (0.18)	0.27 (0.13)	0.25 (0.20)
Day 21 – Day 1	–0.24 (–0.30)	–0.20 (–0.19)	–0.28 (–0.33)
	P= 0.001	p=0.11	p=0.01
CD1			
Day 1	5.0 (5.0)	3.75 (3.5)	6.0 (7.0)
Day 21	3.9 (4.0)	3.0 (3.5)	4.6 (4.0)
Day 21 – Day 1	–1.1 (–1.0)	–0.75 (–0.5)	–1.4 (–1.0)
		p=0.52	p=0.02
P-MAPK			
Day 1	4.8 (5.0)	4.8 (5.0)	4.8 (5.0)
Day 21	3.8 (4.0)	3.8 (4.5)	3.8 (4.0)
Day 21 – Day 1	–1.0 (0.0)	–1.0 (0.0)	–1.0 (–1.0)
		p=0.51	p=0.23
P-AKT			
Day 1	4.8 (5.0)	5.3 (5.5)	4.4 (5.0)
Day 21	3.7 (4.0)	3.8 (4.0)	3.6 (5.0)
Day 21 – Day 1	–1.1 (0.0)	–1.5 (0.5)	–0.8 (0)
		p=0.40	p=0.66

Paired t-tests were used to compare pre- vs. post-treatment scores within each arm. Levels of expression were assessed using the Allred score method, except for Ki-67 which was assessed by cell counting.

Table 5

BioCarta pathways with significant change by AFG treatment

BioCarta Pathway	Number of probesets	KS Permutation p-value
Glycolysis	18	0.00001
Internal Ribosome Entry	12	0.0007
MTA-3	19	0.003
Ran/Mitotic Spindle	14	0.009
Cyclins and Cell Cycle	25	0.006
Sonic Hedgehog Receptor (SHH)	6	0.001
Apoptosis	10	0.003
mTOR Signaling	42	0.004
Telomeres	29	0.005
IGF1 Pathway	37	0.002