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Aminoflavone Inhibits α6-integrin and Growth of Tamoxifen Resistant Breast Cancer

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by

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Each person whose signature appears below certifies that this dissertation in his/her opinion is adequate, in scope and quality, as a dissertation for the degree Doctor of Philosophy.

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CONTENT

FIGURES

Figures Page

TABLES

Tables Page

ABBREVIATIONS

ER^+	Estrogen receptor positive
ER^-	Estrogen receptor negative
PR	Progesterone receptor
HER2	Human Epidermal Growth Factor Receptor 2
EGFR	Epidermal growth factor receptor
IGFR-1	Insulin-like growth factor 1
FGF2	Fibroblast growth factor 2
TNBCs	Triple negative breast cancers
KI-67	Marker of cell proliferation
PARP1	Poly(ADP-ribose) polymerase 1
AF-2	Activation function-2
ITGA6	Integrin Alpha 6
ITGA4	Integrin Alpha 4
TICs	Tumor- initiating cells
CSCs	Cancer Stem Cells
CTL	Cytotoxic T lymphocyte
CSCTT	Cancer Stem Cells Therapeutic Target Database
EMT	Epithelial to mesenchymal transition
TME	Tumor microenvironment
ALDH	Aldehyde dehydrogenase
CXCR4	C-X-C chemokine receptor type 4
miRNA	microRNA

AhR	Aryl hydrocarbon receptor
ARNT	AhR Nuclear Translocator
HIF-1α	Hypoxia-inducible factor 1α
XRE	Xenobiotic Responsive Element
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
HAs	Halogenated aromatics
ROS	Reactive oxygen species
MRP	Multidrug resistant proteins
CDK	Cyclin-dependent protein kinase
RB	Retinoblastoma
EZH2	Enhancer of zeste homologue 2
HDAC1	Histone deacetylase 1
mBC	Metastatic breast cancer
ABC transporters	ATP-binding cassette transporters
TamR	Tamoxifen resistant
Par MCF-7	Parental MCF-7 cells
TamR MCF-7	Tamoxifen resistant MCF-7 cells
AHR100	AhR ligand-unresponsive MCF-7 breast cancer cells
M05	Spontaneous hormone-dependent mammary mouse model
LM05-E	Epithelial cells derived from tumors of the M05 mouse Model
LM05-Mix	Mixture of fibroblastic and epithelial cells derived from tumors of the M05 mouse model
Fra-1	Fos-related antigen 1
SERM	Selective Estrogen Receptor Modulators

AI	Aromatase Inhibitor
SERD	Selective Estrogen Receptor Down-regulators
SSH	SERM/SERD hybrid
SAhRM	Selective AhR modulators
αNF	Alpha-naphthoflavone
AF	Aminoflavone, 5-amino-2-(4-amino-3-fluorophenyl)-6,8-difluoro-7-methyl-4H-1-benzopyran-4-one
AFP464	Aminoflavone pro-drug
4OHTam	4-Hydroxy-Tamoxifen
DMSO	Dimethyl Sulfoxide
CYP2D6	Cytochrome P450 2D6
TCGA	The Cancer Genome Atlas
RECIST	Response Evaluation Criteria in Solid Tumors
CBR	Clinical benefit rate
TTP	Time to progression
IPA	Ingenuity Pathway Analysis

ABSTRACT OF THE DISSERTATION

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by

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Doctor of Philosophy, Graduate Program in Pharmacology
Loma Linda University, August 2018
Dr. Eileen Brantley, Chairperson

Approximately 40% of estrogen receptor positive (ER^+) breast cancer patients develop resistance to standard of care agent tamoxifen, while ER negative (ER^-) breast cancer patients are intrinsically resistant to tamoxifen. Resistance often promotes metastasis, recurrence and death. Tumor-initiating cells (TICs) represent key contributors to resistance and adhesion protein α6-integrin is a putative TIC biomarker. Investigational agent Aminoflavone (AF) demonstrates efficacy against breast cancer cells irrespective of ER status. Interestingly, we found tamoxifen resistant (TamR) cells and tumors exhibited elevated α6-integrin expression in comparison to their tamoxifen sensitive counterparts. AF effectively disrupted mammospheres enriched for TICs and reduced α6-integrin levels in tamoxifen sensitive and TamR cells. AF further inhibited α6-integrin's pro-cancer signaling in TamR cells. Additionally, AF altered the miRNA expression profile of tamoxifen sensitive and TamR mammosphere-derived cells. Our data suggest that AF inhibits α6-integrin signaling and alters the expression of specific miRNAs to reduce TIC capacity and counteract tamoxifen resistance.

CHAPTER ONE

GENERAL INTRODUCTION

Breast Cancer Remains a Major Global Health Problem

Breast Cancer remains a major global health problem. According to the World Health Organization (WHO), breast cancer remains the 5th leading cause of cancer related deaths globally. The NCI -Surveillance, Epidemiology, and End Results (SEER) Program projected over 266,000 new cases, accounting for ~15% of all new cancer cases in the United States (US) for the year 2018. Of these women, over 40,000 were predicted to die. This averages ≈ 1 death every 13 mins. Interestingly, despite a trend towards an increase in breast cancer diagnosis in the US, the number of deaths has remained relatively steady in recent years, trending towards a decline. This suggests that while we see an annual increase in the number of women diagnosed with breast cancer, more of these women are successfully battling the disease. This speaks to improvements in screening and the development of more advanced therapeutic strategies. On the contrary, despite a small trend towards a reduction in breast cancer mortality in developed countries, WHO reports that the incidence and mortality rate in developing countries is steadily rising. Hence, breast cancer remains a global health problem with which we continue to wrestle.

Figure 1. Breast cancer incidence and mortality estimates for 2018 according to the National Cancer Institute Surveillance Epidemiology and End-Results (SEER) Program.

Clinical Efficacy of Hormone Therapies such as Tamoxifen is Limited by Resistance

The clinical efficacy of hormone therapies such as tamoxifen is limited by resistance, that is, the ability of cancer cells to survive and grow in the presence of these anticancer therapies. Resistance may either be *de novo* or acquired over time. Resistance to therapies often leads to recurrence and metastases which are primary contributors to breast cancer-related deaths(Ahmad 2013). As defined by hormone receptor status, breast cancer is categorized as one of 4 major subtypes: luminal A, luminal B, Human Epidermal Growth Factor Receptor 2 (HER2)-overexpressing, or basal-like (Sioshansi, Huber et al. 2011, Cho 2016). Luminal A breast cancer is estrogen receptor positive (ER^+), progesterone receptor positive (PR^+), HER2 negative ($HER2^-$), and has low levels of the protein Ki-67 (a marker of cellular proliferation). Luminal A cancers tend to be low-grade, have very good prognosis, and respond well to endocrine therapy. Luminal B breast cancers are either $HER2^+$ or $HER2^-$. Luminal B cancers generally express both the ER and PR similar to luminal A. On the other hand, they tend to have high Ki-67 protein levels. This largely explains the higher tumor burden and less favorable prognosis seen in

these cancers compared to the luminal A subtypes. Luminal B cancers are generally treated with endocrine therapy, although adjuvant cytotoxic chemotherapy is also employed in most cases. Together, the ER^+ luminal A and B breast cancer subtypes are the most frequently diagnosed, accounting for at least 70% of all diagnosed cancers (Fumagalli, Wilson et al. 2016). The HER2-overexpressing subtype is ER^- and PR^- but expresses high levels of HER2. These cancers tend to have a worse prognosis compared to the luminal subtype. However, they are often successfully treated with HER2-targeted therapies such as Trastuzumab. The terms triple-negative and basal-like are often used interchangeably to identify a sub-type of breast cancer which lacks ER, PR and HER2 expression. However, triple-negative and basal-like breast cancers are biologically distinct(Alluri and Newman 2014). In addition to lacking the 3 receptor types, the basal subtype is defined by strong expression of basal markers such as cytokeratins 5,6 and 17. Both triple negative and basal-like breast cancers tend to have poor clinical outcomes and are shown to disproportionately affect women of African descent. These cancers also lack targeted therapies. As such, patients with these breast cancer subtypes are primarily treated with cytotoxic chemotherapeutic agents.

Table 1. Breast Cancer Molecular Subtypes

Subtype	***ER***	***PR***	***HER2***	***KI-67***	***Recurrence Risk***	***Therapy***
Luminal A	+	+	-	Low	Low	Endocrine Therapy; Chemotherapy may be used
Luminal B	+	-/low	-/+	High	High	Endocrine Therapy; Chemotherapy for most
HER2 overexpressing	-	-	Overexpressed	n/a	n/a	Anti-HER2; Chemotherapy
Triple negative/Basal-like	-	-	-	n/a	n/a	Chemotherapy; PARP inhibitors

ER-Estrogen Receptor; PR-Progesterone Receptor; HER2-Human Epidermal Growth Factor Receptor 2; KI-67-marker of cell proliferation; PARP1-poly(ADP-ribose) polymerase 1

Most endocrine therapy approaches target estrogen production or the estrogen receptor (ER) itself. These include: selective estrogen receptor modulators (SERMs, e.g., Tamoxifen, Raloxifene), aromatase inhibitors (AIs e.g. Anastrozole), selective estrogen receptor down-regulators (SERDs, e.g. Fulvestrant), and ovarian ablation. Tamoxifen acts as a competitive antagonist binding to the ER and blocking the proliferative effects of estrogen. AIs target the aromatase-enzyme which is responsible for the synthesis of estrogen from androgens/steroid precursors in peripheral tissues such as adipose, muscle and breast. SERDs bind to the ER resulting in destabilization and subsequent proteasomal degradation of the ER protein. Because the ovaries are the primary site of estrogen production in premenopausal women, these therapeutic approaches are primarily coupled with ovarian suppression or ablation which blocks the function of the ovaries in this cohort of women. Ovarian ablation can be achieved surgically via the removal of the ovaries(oophorectomy) or via radiation. Otherwise, ovarian function can be suppressed pharmacologically using drugs such as goserelin (Zoladex®) which is synthetic analogue of the naturally occurring luteinizing hormone-releasing hormone (LHRH; also known as Gonadotropin-releasing hormone).

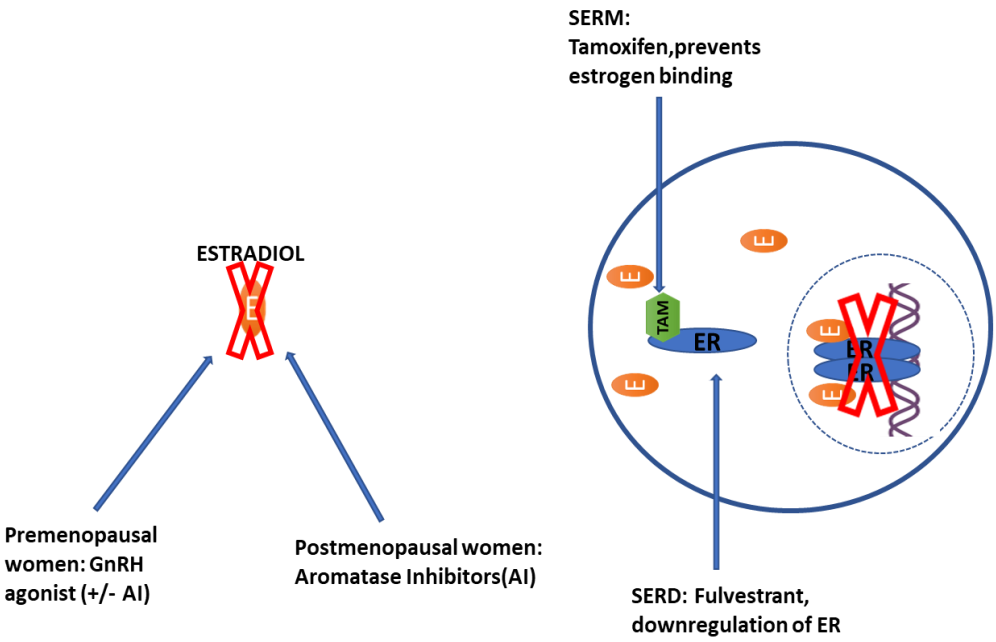

Figure 2. Common Endocrine Therapies for ER^+ Breast Cancer

Despite being one of the most widely used endocrine therapies for ER^+ breast cancers, de novo and acquired resistance to tamoxifen have significantly diminished its clinical efficacy (Tanic, Milovanovic et al. 2012, Ojo, Wei et al. 2015). Multiple mechanisms are likely to contribute to tamoxifen resistance, many of which have not been elucidated (Rondón-Lagos, Villegas et al. 2016). Other endocrine therapies such as the AI Anastrozole are often administered to patients who have developed resistance to tamoxifen. However, resistance to AIs is also common(Ma, Reinert et al. 2015). Hence, there is great need to target alternate mechanisms of breast cancer resistance to successfully thwart this problem and improve patient outcomes.

Proposed Mechanisms of Tamoxifen Resistance

There are several proposed mechanisms believed to contribute to tamoxifen resistance. These include: loss of ER expression and function, altered expression of coactivators or corepressors which play critical roles in ER-mediated gene transcription, growth factor/ER signaling crosstalk, mutations or loss of CYP2D6 which metabolizes tamoxifen, altered regulation of cell death mechanisms such as autophagy and/or apoptosis, the presence of tumor initiating cells (TICs), the aberrant expression of oncogenic and tumor suppressor microRNAs (miRNAs) and the tumor microenvironment(TME).

The ER has been at the forefront of endocrine therapy both as a prognostic marker and a therapeutic target (Osborne and Schiff 2011). Therefore, loss of or modifications to the expression of ER have been linked to reduced tamoxifen sensitivity. Loss of ER expression is believed to be the result of epigenetic changes such as aberrant methylation of the gene promoter (Ottaviano, Issa et al. 1994) as well as, histone deacetylation (Yang, Phillips et al. 2001). Furthermore, most ER^- breast cancers are unresponsive to endocrine therapies(McGuire 1975). Mutations in the ER gene may also render the receptor functionally incompetent despite not markedly affecting receptor expression. It was reported that site-directed mutagenesis of a major co-activator binding site of the ER, the activation function-2 (AF-2) region, decreased ER-dependent transcriptional activation in a mouse model (Mahfoudi, Roulet et al. 1995). Furthermore, a recent study revealed the ability of small molecule inhibitors of the AF2 to target mutant ER driven TamR breast cancer(Singh, Munuganti et al. 2018). In addition to the traditional ERα and β, the

discovery of novel ERs, such as GPER-1/GPR30, offer new potential therapeutic targets in endocrine therapy resistant breast cancer(Molina, Figueroa et al. 2017).

Co-regulatory proteins, namely co-activators and co-repressors are integral to the transcriptional activity of the ER and have been linked to tamoxifen resistance. Co-activators complex with the ER to enhance transcription of target genes, while recruitment of co-repressors decreases gene transcription. Tamoxifen typically acts as an antagonist in breast cancers. However, under certain conditions, tamoxifen may also display agonistic properties. As examples, PGC-1β and SRC1 are co-activators shown to heighten the agonistic activity of tamoxifen (Kressler, Hock et al. 2007). Timeless is a co-activator that has recently been linked to tamoxifen resistance owing to its ability to enhance ERα transcriptional activity of DNA repair genes including PARP1 (Nde, Gimeno et al. 2018).

Cross-talk between the ER and growth factor receptor signaling pathways has also been implicated in tamoxifen resistance. Several reports indicate that overexpression of EGFR or ERBB2/HER2 and associated MAPK activation in ER^+ breast cancer, confers tamoxifen resistance (Kurokawa, Lenferink et al. 2000, Riggins, Schrecengost et al. 2007). Under these conditions, ERK1/2 and AKT appear to be important downstream effectors of this resistance phenotype. Furthermore, insulin-like growth factor receptor 1 (IGFR-1) signaling has also been associated with antiestrogen resistance. IGF-1 regulates endogenous ER expression in breast cancer through transcriptional activation (Lee, Weng et al. 1997). Reciprocally, estrogen signaling can also enhance IGFR-1 signaling via transcriptional upregulation of IGFR-1 and its associated ligands (Umayahara, Kawamori et al. 1994). One mechanism whereby IGF signaling is believed to contribute to

tamoxifen resistance is through the activation of AKT and subsequent phosphorylation of the ER, resulting in ligand-independent activation of ER and evasion of tamoxifen-induced cell death(Campbell, Bhat-Nakshatri et al. 2001).

Cytochrome P450 2D6 (CYP2D6) is necessary for the metabolic conversion of tamoxifen to its active metabolite, endoxifen(Stearns and Rae 2008). Hence, loss of activity of this enzyme has been linked to reduced tamoxifen responsiveness. Certain genetic polymorphisms in the CYP2D6 gene have also been associated with higher risk of disease relapse in tamoxifen treated patients(Goetz, Rae et al. 2005).

Dysregulation of cell death pathways have been linked to tamoxifen resistance. Induction of anti-apoptotic proteins Bcl-2 and Bcl-xL in HER2-overexpressing ER^+ breast cancer cells have been shown to counteract tamoxifen induced apoptosis and confer resistance (Kumar, Mandal et al. 1996). Autophagy (‘self-eating’ or macroautophagy) has also been linked to endocrine therapy resistance. This process allows cells to recycle cellular components under stress conditions to preserve energy. Lysosome-associated membrane protein 3 (LAMP3) is integral in the autophagic process. Elevation of LAMP3 has been observed in tamoxifen resistant cells; knockdown of which lead to significant re-sensitization to tamoxifen (Nagelkerke, Sieuwerts et al. 2014).

Cancer stem cells (CSCs) also known as tumor initiating cells (TICs) represent a small fraction of the overall cellular composition of a tumor. These cells have been increasingly linked to cancer resistance, metastasis and recurrence (Ojo, Wei et al. 2015). This is, in part, due to their ability to evade current treatment approaches, self-renew, and differentiate to form bulk tumor cells (Yang, Xu et al. 2016). Tamoxifen was found to be ineffective against the patient derived TIC population, while treatment combination of

mTOR inhibitors and tamoxifen reduced mammosphere formation (an *in vitro* model with an enriched TIC population) (Karthik, Ma et al. 2015). A variant of the ER, ERα-36 has been shown to promote tamoxifen resistance by driving anti-estrogen selection of TIC/progenitor cells in ER^+ breast cancer (Deng, Yin et al. 2014). However, emerging data suggest phenotypic and genetic heterogeneity exist in the TIC population itself, highlighting the need to identify and effectively target these sub-populations to prevent resistance and relapse (Akrap, Andersson et al. , Visvader and Lindeman).

microRNAs (miRNAs) are a class of small non-coding RNAs which act as post-transcriptional regulators targeting mRNAs for degradation or translational inhibition. Oncogenic miRNAs promote tumor formation by down-regulating tumor suppressor genes thereby stimulating cancer cell proliferation, angiogenesis, and invasion. On the other hand, tumor suppressor miRNAs down-regulate proteins with oncogenic or tumor promoting functions (Shenouda and Alahari 2009). Published findings support a role for both oncogenic and tumor suppressor miRNAs in tamoxifen resistance. Tumor suppressor miR-378a-3p was found to inhibit tamoxifen resistance by reducing expression of its target oncogene GOLT1A(Ikeda, Horie-Inoue et al. 2015). Furthermore, restoration of tumor suppressor miR-375 was shown to reverse tamoxifen resistance and epithelial–mesenchymal transition (EMT)-like properties in breast cancer cells (Ward, Balwierz et al. 2013). Additionally, oncogenic miRNAs such as miRNA-519a have been shown to confer tamoxifen resistance by targeting tumor-suppressor genes in the PI3K signaling pathway including PTEN (Ward, Shukla et al. 2014). A recent publication identified several differentially expressed miRNAs in tamoxifen resistant vs parental cell lines with the combination of two such miRNAs (miR-190b and miR-516a-5p) being

predictive of recurrence in ER^+ breast cancer patients receiving adjuvant tamoxifen therapy (Joshi, Elias et al. 2016).

Emerging data suggest that the tumor microenvironment(TME) also contributes to the development and maintenance of resistant breast cancer. This complex tissue environment consists of interactions between cancer cells and surrounding non-cancerous components including fibroblasts, blood vessel and lymphatic networks, immune and inflammatory cells, signaling molecules and the extracellular matrix (ECM)(Wang, Zhao et al. 2017). Fibroblast growth factor 2 (FGF2) and its receptor FGFR were identified as TME components which promote resistance to anti-estrogens including 4-Hydroxytamoxifen (4-OHTAM) and fulvestrant, as well as, PI3K and MTORC1 inhibitors in ER^+ breast cancer (Shee, Yang et al. 2018). Our lab and others have also identified α6-integrin and its ligand laminin as TME mediators of tamoxifen resistance (Brantley, Callero et al. 2016, Berardi, Raffo et al. 2017).

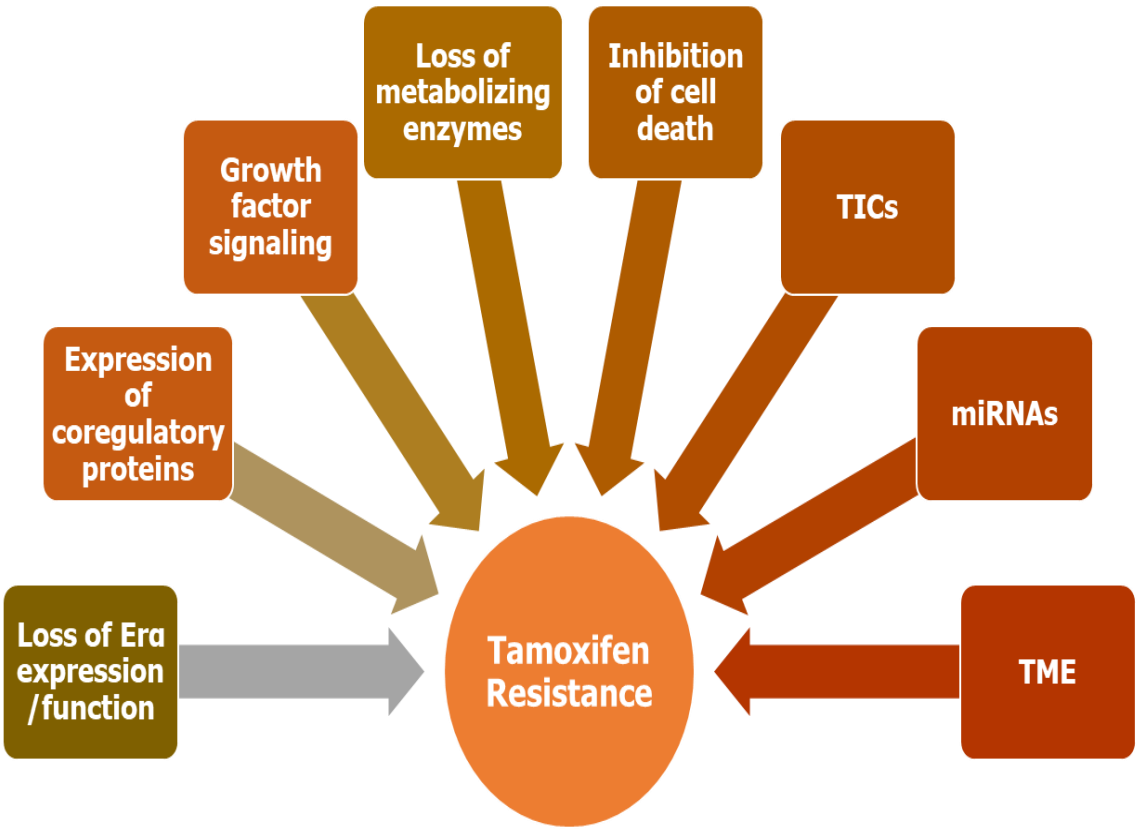

Figure 3. Proposed Mechanisms of Tamoxifen Resistance

As is evident, resistance to endocrine therapies such as tamoxifen involve multiple mechanisms including: aberrations in ER expression and function and its interacting co-modulators, adaptive upregulation of growth factor signaling pathways, decreased efficiency of tamoxifen metabolism, deregulation of cell death mechanisms, increased action of TICs, aberrant miRNA expression and abnormalities in the TME. Elucidation of these underlying molecular mechanisms has led to the testing and development of new therapeutic approaches to overcome resistance. Unfortunately, many of these approaches often fail to translate into actual clinical benefit.

Limitations of Current Approaches to Combat Tamoxifen Resistance

Notwithstanding, there are several approaches to combat tamoxifen resistance that have either been implemented clinically or have been investigated. However, these current approaches are not without their limitations. Mechanisms of combating endocrine therapy resistance include use of an alternate endocrine therapy, chemotherapy or agents that target epigenetic mechanisms, the cell cycle, or survival signaling pathways. It is not uncommon for patients who have progressed or developed resistance to tamoxifen to receive second-line endocrine therapy including non-steroidal AIs (anastrozole, letrozole), steroidal AIs (exemestane) or SERDs (fulvestrant) (Robertson 2001, Osborne, Pippen et al. 2002). Preclinical studies show that fulvestrant is effective at combating tamoxifen resistance. *In vitro* studies demonstrated that tamoxifen resistant cell lines were responsive to fulvestrant (Coopman, Garcia et al. 1994). Furthermore, in a breast tumor MCF-7Ca xenograft model, fulvestrant was superior to tamoxifen in delaying the development of resistance and disease progression, although most tumors eventually developed resistance to fulvestrant (Osborne, Coronado-Heinsohn et al. 1995). Additionally, a phase III clinical trial concluded that fulvestrant showed similar efficacy to anastrozole in patients who had progressed on endocrine therapy such as tamoxifen (Robertson 2001). Despite the apparent superiority of these alternate endocrine therapies when compared to tamoxifen, resistance to these agents frequently ensues (Miller and Larionov 2012, Ciruelos, Pascual et al. 2014).

In clinical practice, chemotherapy is often used as an alternate strategy once endocrine therapy resistance has developed (Zhang, Zhang et al. 2015). However, there is evidence for an overexpression of multidrug resistant proteins (MRPs) in tamoxifen

resistant cells, a mechanism believed to contribute to cross-resistance of tamoxifen resistant cells to chemotherapy (Choi, Yang et al. 2007). In addition to the plethora of toxicities associated with the use of chemotherapeutic agents, studies have also shown that treatment with chemotherapy increases the percentage of highly tumorigenic, tumor initiating $CD44^{hi}/CD24^{-/low}$ population of breast cancer cells(Li, Lewis et al. 2008). This suggests that a sub-population of intrinsically resistant cells emerges in response to chemotherapy or that chemotherapy and other anti-cancer treatments select for cells with this resistant phenotype.

In the cancer environment, the cell cycle machinery is often not well regulated. As such, re-establishing the anomalous changes in cell cycle control seen in resistant cancers may aid in counteracting resistance. Cyclins are a family of proteins important in regulating cell cycle progression. Cyclin D1, a transcriptional target of the ER, has been shown to play a role in estrogen stimulated cell growth (Lukas, Bartkova et al. 1996). Cyclin D1 activates cyclin-dependent protein kinases four and six (CDK4/6) which in turn stimulate cell cycle progression at the G1/S phase via retinoblastoma (RB) inactivation (phosphorylation). Selective CDK4/6 inhibitor, PD-0332991, was shown to inhibit the proliferation of cancer cells in an endocrine therapy resistant model (Thangavel, Dean et al. 2011). Furthermore, bazedoxifene (BZA), a SERM/SERD hybrid (SSH) in combination with Palbociclib, a CDK4/6 inhibitor, inhibited the growth of tamoxifen-resistant breast tumor xenografts, with an associated increase in the duration of treatment response as compared to either treatment alone (Wardell, Ellis et al. 2015). More recently, Enhancer of zeste homologue 2 (EZH2), a histone-lysine N-methyltransferase enzyme which participates in histone methylation and transcriptional

repression was identified as a novel therapeutic target in tamoxifen resistant breast cancer. EZH2 was found to contribute to tamoxifen resistance by repressing expression of the p16 tumor suppressor gene, a CDK inhibitor that results in G1/S phase arrest (Chen, Yao et al. 2018). While the combination of cell cycle inhibitors and endocrine therapies may seem promising at tackling resistance, there is room to speculate that improved growth arrest alone may not lead to lasting clinical improvements.

Epigenetic changes have also been linked to tamoxifen resistance and provides opportunities for combating resistance. Epigenetic modifications refer to heritable changes in gene expression that are independent of changes to the DNA sequence. These changes include DNA methylation, non-coding RNA regulation, and histone modifications. Loss of ER expression has been linked to decreased tamoxifen responsiveness. Mechanisms such as the hypermethylation of the ER promoter and the recruitment of HDAC1 (histone deacetylase 1) which results in deacetylation of the ER promoter are epigenetic changes which reduce ER transcription and expression of the functional receptor protein (Vesuna, Lisok et al. 2012). Furthermore, several preclinical studies suggest that epigenetic modulation of the ER by demethylating agents or HDAC inhibitors enhance the anti-tumor effects of tamoxifen (Jang, Lim et al. 2004, Sharma, Saxena et al. 2006, Legare and Basik 2016). Despite the promising results of preclinical studies combining HDAC inhibitors in tamoxifen resistant models, clinical trials have revealed less impressive results. A phase II study combining the histone deacetylase inhibitor vorinostat with tamoxifen in a cohort of 43 patients who progressed on hormone therapy revealed a relatively low objective response rate of 19% according to the Response Evaluation Criteria in Solid Tumors (RECIST) criteria (Munster, Thurn et al.

2011). In other words, 8 patients demonstrated a partial response to this treatment regimen. It is also important to note that ER expression is often retained and even elevated in instances of tamoxifen resistance (Raha, Thomas et al. 2015). Therefore, ER expression does not always predict tamoxifen responsiveness.

Patients who have acquired resistance to tamoxifen-induced inhibition of ER signaling often demonstrate adaptive upregulation of alternate growth factor signaling pathways. These alternate pathways have been extensively investigated as potential targets in endocrine therapy resistant breast cancer. One of the key growth/survival pathways in ER^+ breast cancer is the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt)/mammalian target of rapamycin (mTOR) pathway. In fact, tamoxifen resistance is associated with aberrant PI3K signaling (Bostner, Karlsson et al. 2013, Shah, Mehta et al. 2014). A phase II clinical trial exploring the efficacy of tamoxifen alone or in combination with mTOR inhibitor Everolimus in AI-resistant metastatic breast cancer (mBC) revealed that clinical benefit rate (CBR), time to progression (TTP) and overall survival was greater with the combination compared to tamoxifen alone (Bachelot, Bourgier et al. 2012). Cross-talk between ER and EGFR has also been linked to endocrine resistance. A randomized phase II trial combining EGFR inhibitor Gefitinib and tamoxifen in ER^+ metastatic breast cancer showed a trend towards benefit with the Gefitinib/tamoxifen combination, with reduced clinical benefit seen with the Gefitinib/AI combination compared to placebo (Osborne, Neven et al. 2011). Attempts to improve endocrine therapy via the use of growth factor pathway inhibitors have proven to be disappointing (Johnston 2015). Resistance to endocrine therapy appears to be a progressive, dynamic process driven by different underlying mechanisms. Hence, since

resistance to targeted therapies often develops, it is critical to identify the dominant pathway(s) that drive the cancer at various stages of the disease. This is further complicated by the need to identify relevant biomarkers predictive of treatment response to individual targeted therapies as well as the cost and toxicities associated with multidrug treatment regimens.

Even more recently, miRNAs have been identified not only as predictive markers but potential therapeutic targets in resistant breast cancers. An antagomir (antimiR) is a small oligonucleotide which binds to specific miRNA thereby preventing the binding of a miRNA to its target mRNA (Krutzfeldt, Rajewsky et al. 2005). An miR mask binds complementarily with a target mRNA sequence thereby preventing the binding of endogenous miRNA, while miR mimics have the same sequence as the endogenous miRNA and so can act as replacements for particular miRNAs such as downregulated tumor suppressor miRNAs (Luqmani and Alam-Eldin 2016). It is well established that a single miRNA can have several mRNA targets which makes them attractive therapeutic targets in a heterogeneous disease such as breast cancer. Nevertheless, this target promiscuity of miRNAs increases susceptibility to unintended effects.

As demonstrated, approved treatment options for tackling endocrine resistance involve sequential hormone therapy or switching one class of endocrine therapy agent for another, as well as the use of chemotherapy. More recently, cell cycle inhibitors have also been included in patient care. Most ongoing clinical trials involve the combination of endocrine therapies with targeted agents including growth factor signaling inhibitors, and inhibitors of epigenetic regulatory molecules such as HDAC inhibitors. Saji and Kimura-Tsuchiya recently proposed a ‘hybrid car model’ to explain the rationale behind co-

targeting of ER signaling and other cell survival pathways (Saji and Kimura-Tsuchiya 2015). This model involves co-targeting the two primary sources of power permitting cancer progression, the gasoline engine (ER) and electric motor (growth factor receptors such as HER2, IGF-1R etc.). The authors proposed simultaneous blockade of these two power sources and related downstream signaling (the transmission system) will circumvent endocrine therapy resistance.

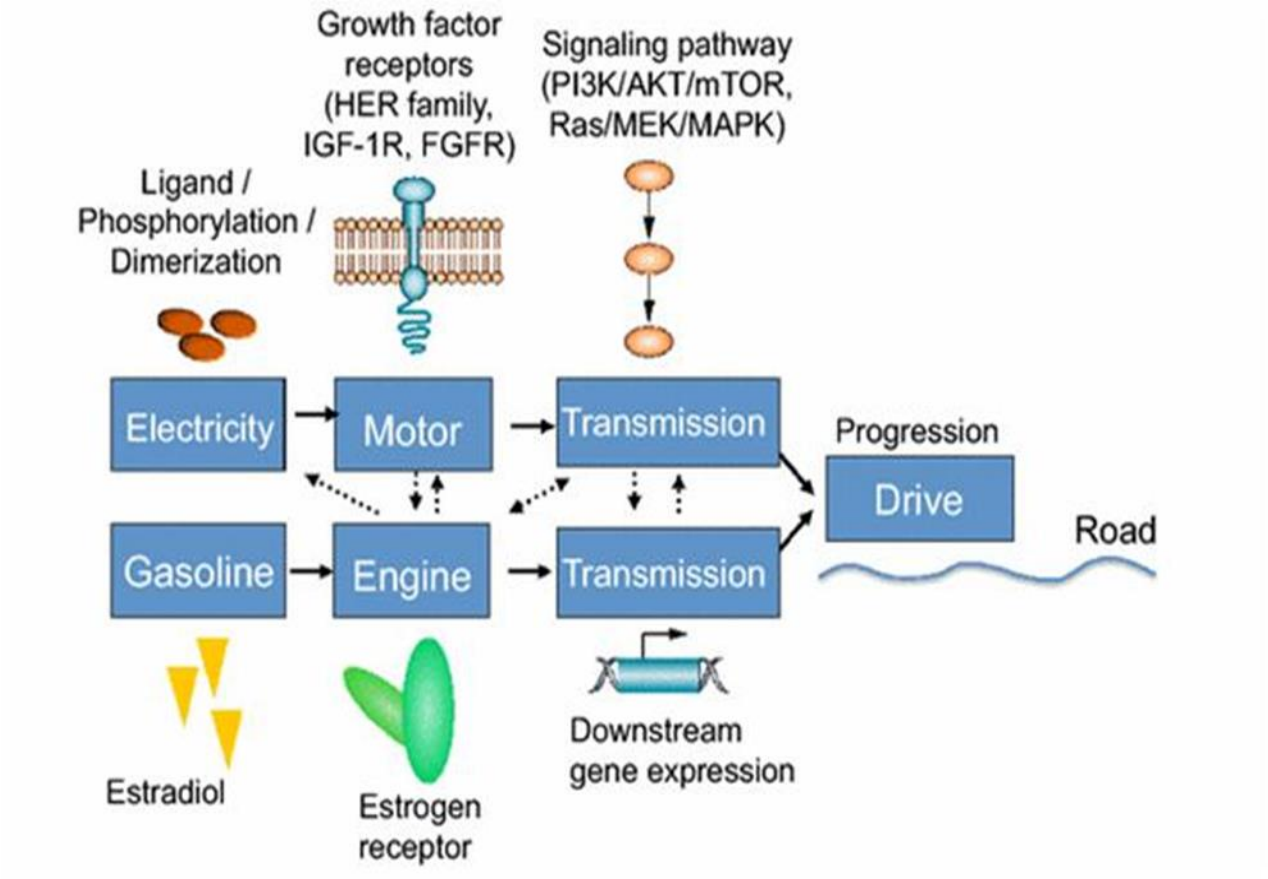

Figure 4. Hybrid car model of endocrine therapy resistance in breast cancer. This hybrid car is powered by a gasoline engine (ER) and an electric motor (growth factor receptors e.g. HER2, IGF-1R etc.). (Saji and Kimura-Tsuchiya 2015)

One key limitation of this model is that it fails to identify the 'driver' of the car. TICs are recognized as key drivers of metastasis and endocrine therapy resistance (Ojo, Wei et al. 2015, Simoes, O'Brien et al. 2015). In addition, pathways or mechanisms contributing to endocrine therapy resistance have the capacity to drive TIC growth and function. For instance, aberrant ER signaling has been shown to promote tamoxifen resistance by increasing endocrine treatment selection or enrichment of the TIC population (Deng, Yin et al. 2014), abnormal growth factor signaling such as HER2, EGFR, and PI3K/PTEN/Akt/mTOR signaling pathways have been linked to TIC survival (McCubrey, Abrams et al. 2015), cyclin dependent kinases promote self-renewal of TICs (Dai, Zhang et al. 2016), and certain HDACs are overexpressed in TICs to support TIC survival (Witt, Lee et al. 2016). Insufficient consideration of TICs may therefore be a key reason many combination therapies only marginally outperform established monotherapies. Furthermore, despite the elucidation of multiple resistance promoting mechanisms, these ideas are largely fragmented.

Considering their central role in the resistance process, TICs may offer a platform on which many of these resistance mechanisms and pathways converge. In fact, it has been suggested that conventional therapeutic measures fail as seen in resistant and recurrent cancers as they are largely ineffective against TICs. Based on this idea, drugs which target both bulk tumor cells and the TICs should more effectively confer long term clinical benefit by eliminating the self-renewing components of the cancer (Ricci-Vitiani, Pagliuca et al. 2008).

Targeting TICs as Part of a Comprehensive Approach to Tackling Tamoxifen Resistance

Considering the increasingly recognized role of TICs in cancer initiation, maintenance, relapse and resistance, several pre-clinical and clinical studies have investigated approaches to target this specific population of cells. Many of these new therapeutic approaches are being considered for use in conjunction with traditional anticancer agents. Proposed methods of eliminating TICs include targeting cell surface or cytoplasmic markers, signal transduction pathways, miRNAs, micro-environmental cues, apoptotic mechanisms, ATP-binding cassette transporters (ABC transporters) and cellular differentiation mechanisms (Dragu, Necula et al. 2015). In fact, an online database, the Cancer Stem Cells Therapeutic Target Database (CSCTT), has recently been established with unique capabilities such as bioinformatic resources for the identification and annotation of potential TIC cell therapeutic targets (Hu, Cong et al. 2017).

Analysis of the various mechanisms contributing to TIC survival reveals the need for a more comprehensive, multi-pronged approach. This approach would ideally include: **1) identifying and targeting molecules specific for TIC maintenance and survival, 2) identifying a class of drugs or agents capable of targeting and eliminating TICS via the inhibition of these key functional molecules, and 3) combining conventional anticancer drugs with TIC-targeted agents to optimize therapy.** This will allow for the eradication of both bulk tumor cells and the self-renewing fractions of the tumor. There are an increasing number of scientists who are in support of TIC-oriented therapy. For instance, L Ricci-Vitiani et. al., argued that conventional therapies fail due to their inability to target the TIC population, allowing these cells to acquire new mutations

which promote a more drug resistant phenotype (Ricci-Vitiani, Pagliuca et al. 2008). They postulated that drugs that kill TICs can successfully treat cancer by inhibiting the self-renewing sub-population of the tumor. The remaining, non-stem cell like components will eventually succumb to normal degenerative fates. We also believe that this approach offers a promising tactic for better management of breast and other cancers and is therefore a very viable means of enhancing patient survival by preventing the onset of therapy resistance and cancer relapse.

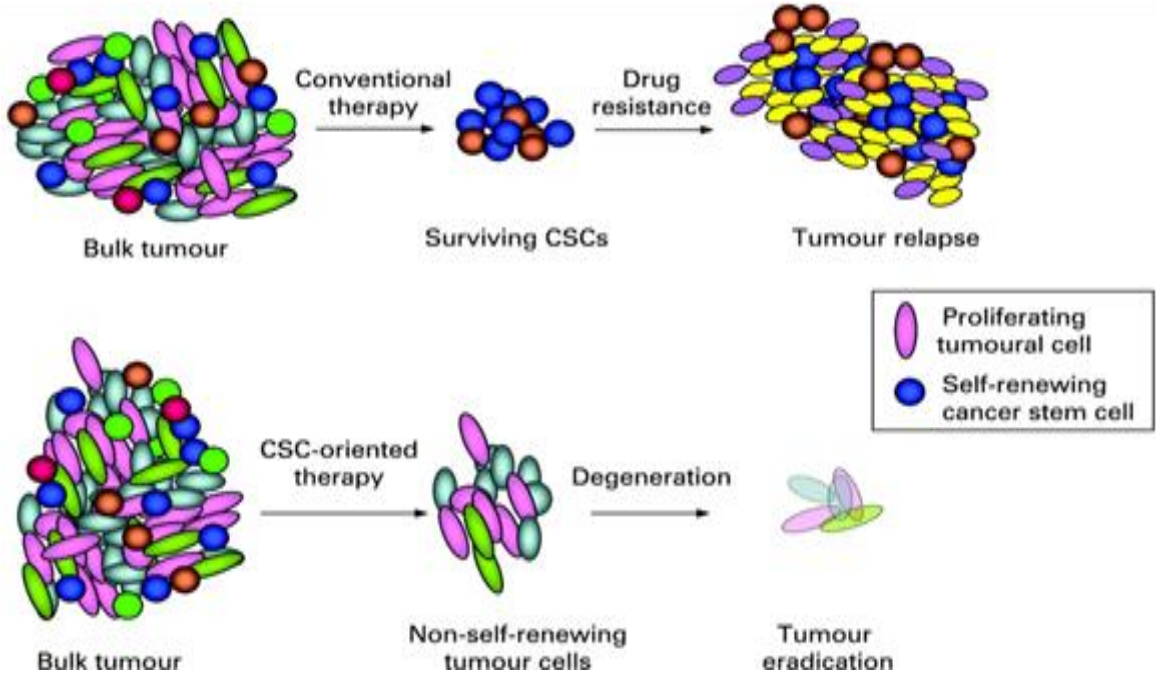

Figure 5. TIC/CSC- Oriented Therapy(Ricci-Vitiani, Pagliuca et al. 2008)

Alpha 6 (α6)-Integrin is a Key Mediator of TIC Growth and Survival and Mediates Tamoxifen Resistance

Integrins may be one such class of molecules that may fit the first criterion in our 'comprehensive approach,' since these key molecules exhibit relevance to TIC maintenance and survival. Integrins are heterodimeric cell surface receptors involved primarily in cell-matrix adhesion along with bidirectional signaling to control many cellular features including migration, proliferation, survival, and differentiation (Giancotti and Ruoslahti 1999). They are the main receptors on mammalian cells for establishing communication between the intracellular environment of the cell and the extracellular matrix, by binding to extracellular matrix components including collagen, fibronectin, and laminin (Alberts B 2002). Integrins are composed of non-covalently linked α and β subunits. Human integrins are formed from at least 9 β and 24 α subunits. This diversity in the number of possible integrins is further increased by alternative mRNA splicing. Despite their role in normal physiological functions, integrins have been identified as important regulators of cancer-related stemness, metastasis, and drug resistance (Seguin, Desgrosellier et al. 2015).

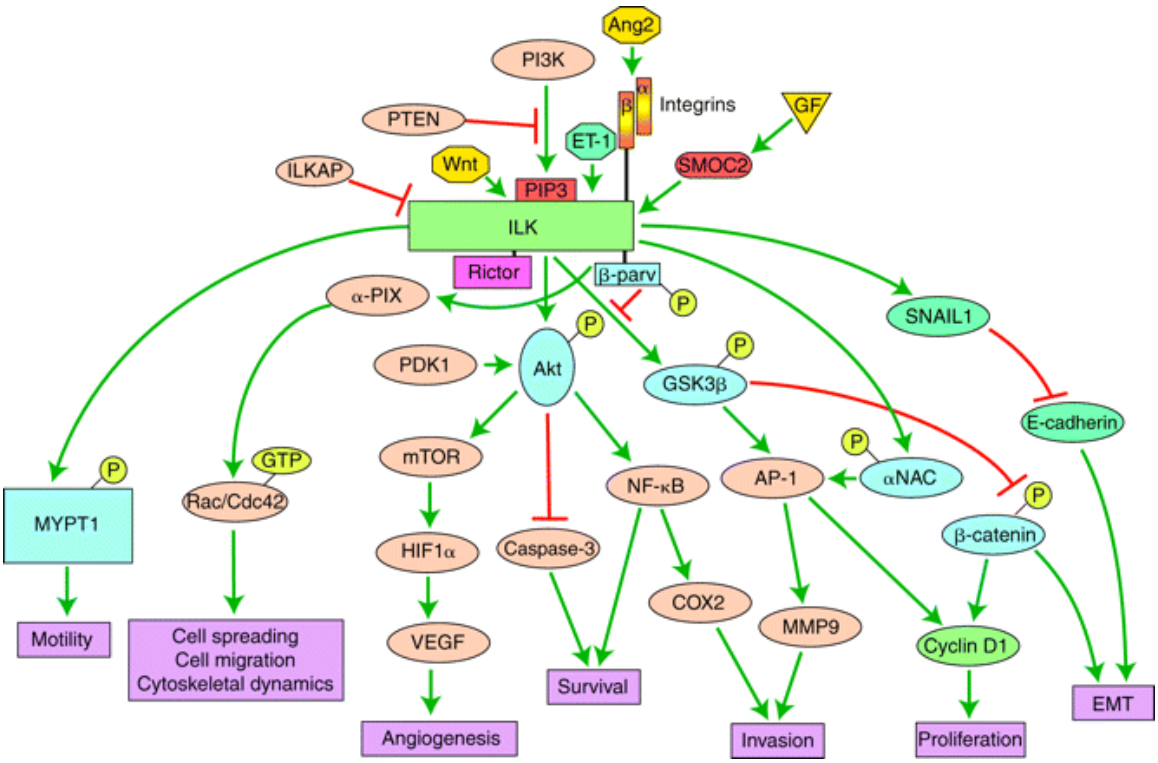

Figure 6. Roles of Integrin Signaling in Cancer. Activation of integrin signaling results in the regulation of downstream effectors which, in turn, modulate cancer promoting processes such as motility, migration and invasion, survival, EMT, proliferation, and angiogenesis(McDonald, Fielding et al. 2008).

One integrin that has risen to notoriety in the breast cancer arena is α6-integrin. α6-integrin exists as two distinct cytoplasmic domain variants, α6A and α6B, formed via alternative mRNA splicing (Goel, Gritsko et al. 2014) and heterodimerizes with either the β1 or β4 subunits to yield α6β1 and α6β4 integrins (Hogervorst, Kuikman et al. 1991). Overexpression of α6-integrin has been correlated with reduced survival and is a proposed prognostic marker in human breast cancer (Friedrichs, Ruiz et al. 1995). α6-integrin has also been recognized as a biomarker for breast and other cancer TICs (Vieira, Ricardo et al. 2012, Ying, Tilghman et al. 2014). Furthermore, α6B has been identified as the variant which defines the mesenchymal population in breast cancer and so is responsible for TIC function (Goel, Gritsko et al. 2014). In addition to its prognostic

value and designation as a cancer stemness marker, there is evidence to support a functional role for α6-integrin in the maintenance and function of TICs (Cariati, Naderi et al. 2008, Lathia, Gallagher et al. 2010), as well as in the promotion of metastasis, aberrant proliferation and invasion of cancer cells (Shaw 2001, Lv, Lv et al. 2013). Activation of the PI3K pathway is one of the main signaling events initiated by α6-integrin in cancer. α6β4 has been shown to activate PI3K signaling and its downstream effectors AKT, RAC and mTOR, during carcinoma progression (Lipscomb and Mercurio 2005). Interestingly, α6-integrin has been shown to enhance resistance to radiotherapy via the PI3K/AKT and MEK/ERK pathways (Hu, Zhou et al. 2016). Furthermore, α6β4/PI3K signaling has been linked to tamoxifen resistance in ERβ1-negative breast carcinomas (Folgiero, Avetrani et al. 2008).

In addition to PI3K signaling, α6-integrn has been associated with other cell survival pathways. Inhibition of α6-integrin/HER2 signaling was shown to reduce breast tumor growth *in vivo* (Gupta and Srivastava 2014). Furthermore, α6-integrin/EGFR cross-talk has been noted in carcinoma progression (Yang, Richardson et al. 2008). α6-integrin has also been shown to play a pro-angiogenic role in tumors with its depletion resulting in a reduction in tumor vascularization and infiltration of pro-angiogenic macrophages (Bouvard, Segaoula et al. 2014).

Not only do tamoxifen resistant cells demonstrate TIC properties (Liu, Zhang et al. 2013), tamoxifen treatment itself selects for cells with self-renewal capacity (Raffo, Berardi et al. 2013). Furthermore, inhibition of pathways crucial to TIC survival, such as the mTOR pathway, hinders tamoxifen induced activation of these cells (Karthik, Ma et al. 2015). Notas and colleagues further demonstrated that exposure of breast cancer cells

to tamoxifen induced the expression of ERα-dependent genes associated with pluripotency as early as 3 hours after initial treatment exposure and therefore proposed a link between this change towards a more stem cell-like phenotype and disease recurrence following tamoxifen therapy in ER^+ breast cancer (Notas, Pelekanou et al. 2015). These researchers further corroborated these findings by showing a similar pattern of induction of pluripotency genes in samples from patients who had relapsed on tamoxifen. In addition, Cottu et.al, found that α6-integrin was elevated in a patient derived luminal breast cancer xenograft model that had acquired resistance to tamoxifen and demonstrated cross-resistance to other hormone therapies (Cottu, Bieche et al. 2014).These studies support the possibility that thwarting the expression of stemness genes such as α6-integrin has the potential to prevent relapse in patients treated with endocrine therapy agents such as tamoxifen.

Notably, the ability of integrins to activate cell survival pathways such as PI3K and promote carcinoma progression involves FAK/Src activation, kinases directly downstream of integrins (Wang, Xiang et al. 2009). Tamoxifen resistance has been linked to integrin-induced FAK/Src activation (Hiscox, Jordan et al. 2007, Yuan, Liu et al. 2015), and inhibition of integrin-mediated FAK/Src activation produces small yet significant sensitization to tamoxifen in ER^+ cancer cells (Cowell, Graham et al. 2006). These observations suggest that altered or prolonged activation of α6-integrin/FAK/Src signaling may contribute to a tamoxifen resistant phenotype by upregulating cell survival pathways such as PI3K/AKT to promote TIC function. A direct link between tamoxifen resistance in ER^+ breast cancer and elevated α6-integrin expression and its related signaling has not been established. However, a recent publication proposed that α6-

integrin ligand laminin confers resistance to tamoxifen in an estrogen-dependent, tamoxifen-sensitive LM05-E breast cancer cell line via α6-integrin-dependent mechanisms (Berardi, Raffo et al. 2017). Based on these observations, this integrin may also be useful as a biomarker for patients less likely to benefit from tamoxifen therapy. As will be discussed later, we found that α6-integrin is overexpressed in tamoxifen resistant (TamR) cells and in tumor samples taken from patients who have relapsed on tamoxifen (*in press*). One can therefore speculate that α6-integrin promotes TIC function and modulates PI3K/AKT signaling to contribute to tamoxifen resistance and eventually tumor relapse.

Potential of AhR Agonist, Aminoflavone (AF), to Thwart α6-Integrin TIC Promoting Properties and Circumvent Tamoxifen Resistance

As discussed earlier, α6-integrin is crucial to TIC maintenance and survival and has been implicated in various cell signaling pathways linked to carcinoma progression and tamoxifen resistance. This makes α6-integrin an ideal target for therapeutic exploration. Therefore, a class of drugs targeting the functional attributes of α6-integrin may be effective at eliminating TICs and reversing or thwarting tamoxifen resistance. One such class of drugs are anti-cancer aryl hydrocarbon receptor (AhR) ligands. Emerging evidence support a role for the AhR in tumorigenesis. The AhR is a ligand activated transcription factor. Upon ligand binding, the AhR behaves like a classical nuclear receptor and translocates into the nucleus, where it heterodimerizes with its binding partner ARNT (AhR Nuclear Translocator). This dimer then binds to Xenobiotic Responsive Elements (XRE) located in the promoter region of target genes. These target

genes include metabolizing enzymes such as cytochrome P450 (CYP) 1A1, CYP1A2, and CYP1B1 important for drug activation (Callero and Loaiza-Pérez 2011).

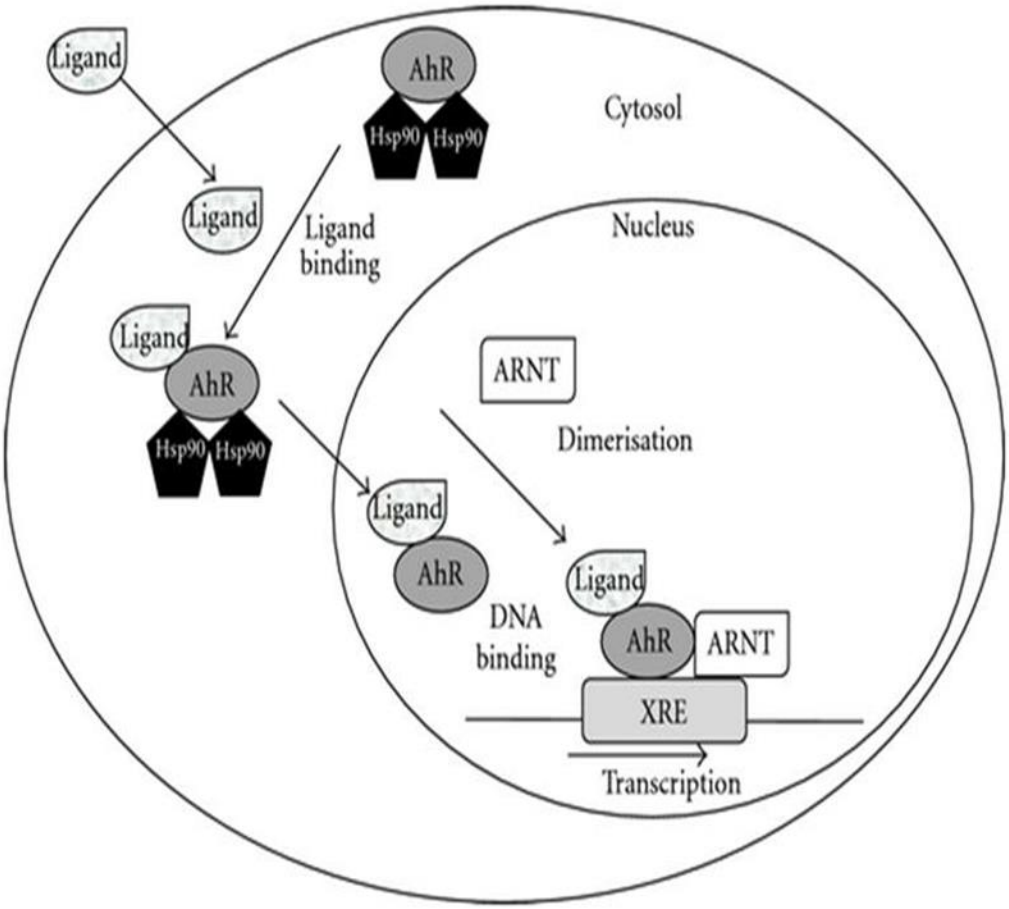

Figure 7. The AhR Signaling Pathway (Callero and Loaiza-Perez 2011)

The AhR was first implicated in cancer as a mediator of the carcinogenic actions of environmental toxins such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and related halogenated aromatics (HAs) (Poland, Glover et al. 1976). However, since then, several other ligands of the AhR have been highlighted for their antitumor actions. As such, the AhR plays a dichotomous role in cancer behaving as a tumor suppressor or tumor

promoter depending on cell context (Safe, Lee et al. 2013). There are several possible mechanisms to account for the apparent inconsistency in the role of the AhR in tumorigenesis. For instance, cell fate can be impacted based on whether AhR signaling activation is transient or constitutive. The AhR is found to be constitutively activated in advanced stage breast and prostate cancers (Powell, Goode et al. 2013, Richmond, Ghotbaddini et al. 2014) suggesting an oncogenic role in such settings. Furthermore, effects of AhR constitutive activation appears to mimic that of more potent, carcinogenic agonists such as TCDD (Diry, Tomkiewicz et al. 2006). On the other hand, compounds with more partial agonistic properties appear to exhibit more tumor suppressor actions. For instance, the AhR agonist flavipin was found to inhibit the migration and invasion of breast cancer cells via miR-212/132 induced suppression of stemness and pro-metastatic gene Sox4. Flavipin was also shown to impede growth of breast cancer cells through inhibition of anti-apoptotic B-cell lymphoma 2 (Bcl2) and α4-integrin (ITGA4) (Hanieh, Mohafez et al. 2016). Similarly, we have found that another AhR agonist Aminoflavone inhibits α6-integrin (Brantley, Callero et al. 2016) and increased the expression of pro-apoptotic gene BAX (*in press*). Furthermore, we have miRNA-sequencing data identifying other miRNAs that may be important in mediating the anticancer actions of Aminoflavone in tamoxifen resistant breast cancer. Another AhR ligand ANI-7 was found to potently and selectively inhibit the growth of breast cancer cell lines compared to normal breast cells, while also inducing cell cycle arrest and DNA damage (Gilbert, De Iuliis et al. 2018). These findings suggest that the downstream effects of AhR activation are largely dependent on the affinity of the ligand for the receptor. More recently, a SERM Raloxifene and its analog were found to induce cell death (apoptosis) in breast

and liver cancer cells via the AhR (Jang, Pearce et al. 2017). Tamoxifen also belongs to this class of compounds and its active metabolite, 4-OHTAM, binds to and activates the AhR receptor in the absence of the ER (DuSell, Nelson et al. 2010). Such findings are fascinating as they suggest that certain SERM derivatives can be developed to function not only as ER inhibitors but as AhR-targeted anticancer agents.

The cellular localization of the AhR may also account for its tissue and cancer specific anticancer or tumor promoting actions. As an example, it was found that elevated levels of AhR in oral squamous cell carcinomas were due to higher levels of AhR in the nucleus compared to adjacent normal tissue where the receptor expression was less and primarily cytosolic (Stanford, Ramirez-Cardenas et al. 2016). In fact, breast cancer cells with AhR restricted to the nucleus were found to be less sensitive to AhR ligands (Callero and Loaiza-Pérez 2011).

Furthermore, tissue specific variations in expression and activity of coactivator and co-repressor proteins of the AhR may dictate differences in response to AhR ligands and account for tissue-specific differences in responses to such ligands (Nguyen, Hoivik et al. 1999, Hankinson 2005). In summary, several factors including the ligand receptor affinity/duration of activation, expression and sub-cellular localization of the AhR, and the action of coregulatory factors dictate whether AhR and its ligands serve a tumor suppressor or tumor promoter role.

AhR ligand, 4H-1-benzopyran-4-one,5-amino-2-(4-amino-3-fluorophenyl)-6,8-difluoro-7-methyl, NSC 686288 (Aminoflavone, AF) is a synthetic compound related to a class of flavonoids naturally found in plants and fungi which possess various biological activities. AF shows potent antitumor activity in both luminal and triple negative breast

cancers(TNBCs) by promoting reactive oxygen species (ROS) formation, oxidative DNA damage and apoptosis (McLean, Soto et al. 2008).

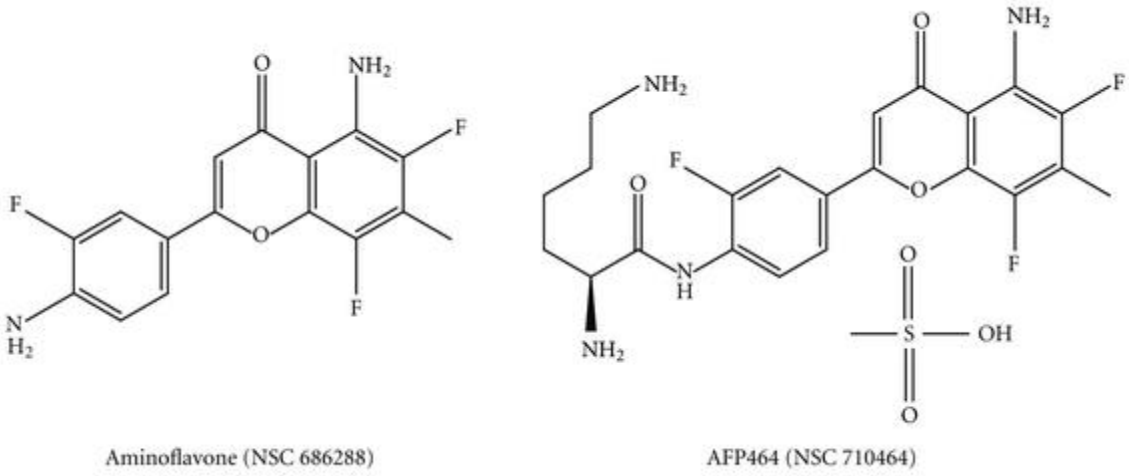

Figure 8. Structure of Aminoflavone and its Pro-drug AFP464 (Callero and Loaiza-Pérez 2011)

There are several pieces of evidence to support a possible role for AF in circumventing tamoxifen resistance. Firstly, breast cancer cells resistant to hormone therapy, including tamoxifen, remain sensitive to the cytotoxic effects of AF (Stark, Burger et al. 2013). Additionally, inhibition of AhR/ER receptor cross-talk by selective AhR modulators (SAhRMs) reduce breast tumor growth (Safe, Qin et al. 1999) with transcriptional reactivation of ER re-sensitizing TNBC to AF (Stark, Burger et al. 2013). Furthermore, we recently showed that AF reduces both bulk tumor growth and the growth of TICs in a tamoxifen sensitive M05 mammary mouse model of breast cancer via the inhibition of α6-integrin (Brantley, Callero et al. 2016). Like AF, another anticancer agent Tranilast, was shown to inhibit breast TICs in an AhR dependent-manner (Prud'homme, Glinka et al. 2010). Tranilast was later found to synergize with

tamoxifen to decrease breast cancer cell viability and proliferation, and further decreased pro-angiogenic markers MMP-9 and VEGF (Darakhshan, Bidmeshkipour et al. 2013).

In accordance with previous publications, we have determined a functional role for α6-integrin in mediating tamoxifen resistance (Berardi, Raffo et al. 2017). This will be discussed in greater detail later in this manuscript. However, in summary we found that tamoxifen increased mammosphere formation and the percentage of cells staining for stemness markers including α6-integrin in sensitive ER^+ cell lines and mouse models. In addition, α6-integrin is elevated in cells that are either innately resistant or have acquired resistance to tamoxifen compared to tamoxifen sensitive controls. This elevation was also seen in tissues from patients who had relapsed on tamoxifen, compared to tamoxifen treatment naïve samples. In addition, cells that have acquired resistance to tamoxifen were sensitive to AF. AF inhibited α6-integrin expression and signaling in TamR cells and knockdown of α6-integrin re-sensitized resistant cells to tamoxifen (*in press*). Our studies and that of others support the idea that tamoxifen may promote its own resistance by selecting for TICs which overexpress stemness markers such as α6-integrin. On the other hand, the ability of AF to reduce α6-integrin expression warrants further exploration to decipher its propensity to thwart tamoxifen resistance via α6-integrin inhibition. In fact, synergism between AF and another endocrine therapy agent, fulvestrant has been demonstrated (Callero and Loaiza-Pérez 2011).

AF also modulates pathways linked to tamoxifen resistance. As previously mentioned, α6-integrin/PI3K/Akt signaling may be altered during the acquisition of tamoxifen resistance which in turn enhances oncogenic properties such as TIC function to further sustain the resistance phenotype. AF may inhibit these resistance-promoting properties.

Micromolar concentrations of AF were shown to reduce Akt (effector molecule downstream of PI3K activation) and induce apoptosis in human breast cancer MCF-7 cells (Meng, Kohn et al. 2007). Based on these observations, we hypothesize that AF inhibits α6-integrin expression and signaling in tamoxifen resistant breast cancer cells to induce cell death and inhibit TICs. We therefore set out to study the role of α6-integrin in promoting tamoxifen resistance and to elucidate the potential of AF to overcome such resistance.

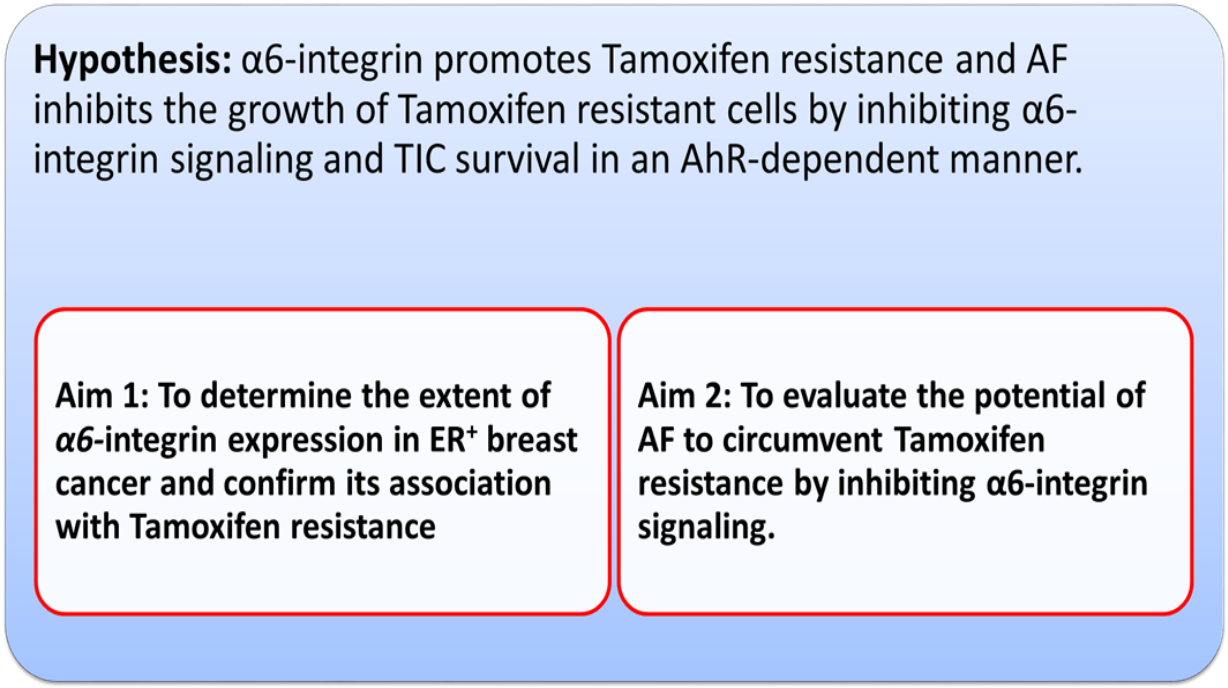

Figure 9. Hypothesis and Specific Aims of Study

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CHAPTER TWO

AHR LIGAND AMINOFLAVONE INHIBITS A6-INTEGRIN EXPRESSION AND BREAST CANCER SPHERE-INITIATING CAPACITY

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Abstract

Traditional chemotherapies debulk tumors but fail to produce long-term clinical remissions due to their inability to eradicate tumor-initiating cells (TICs). This necessitates therapy with activity against the TIC niche. Alpha 6-integrin (α6-integrin) promotes TIC growth. In contrast, aryl hydrocarbon receptor (AhR) signaling activation impedes the formation of mammospheres (clusters of cells enriched for TICs). We investigated the ability of AhR agonist Aminoflavone (AF) and AF pro-drug (AFP464) to disrupt mammospheres derived from breast cancer cells and a M05 mammary mouse model of breast cancer respectively. We further examined the capacity of AF and AFP464 to exhibit anticancer activity and modulate the expression of 'stemness' genes including α6-integrin using immunofluorescence, flow cytometry and qRT-PCR analysis. AF disrupted mammospheres and prevented secondary mammosphere formation. In contrast, AF did not disrupt mammospheres derived from AhR ligand-unresponsive MCF-7 cells. AFP464 treatment suppressed M05 tumor growth and disrupted corresponding mammospheres. AF and AFP464 reduced the expression and percentage of cells that stained for 'stemness' markers including α6-integrin *in vitro* and *in vivo* respectively. These data suggest AFP464 thwarts bulk breast tumor and TIC growth via AhR agonist-mediated α6-integrin inhibition.

Introduction

Despite recent advances in breast cancer therapy, more than 500,000 women die from this disease each year. Chemoresistance and recurrence are contributing factors to this high mortality (Kwon 2013, Mitra, Mishra et al. 2015). Therefore, improvement in breast cancer therapy is critically needed. Tumor-initiating cells (TICs), also known as cancer stem cells, contribute to the emergence of chemoresistance, tumor recurrence, and metastasis (Gong, Yao et al. 2010). Cytoreduction of breast cancer frequently fails to eliminate the TIC population (Morrison, Schmidt et al. 2008) which can self-renew and undergo multi-lineage differentiation (Ghebeh, Sleiman et al. 2013), to drive disease recurrence and chemoresistance. Furthermore, TICs are enriched in mammospheres (Grimshaw, Cooper et al. 2008, Feifei, Mingzhi et al. 2012).

Integrins function as cell adhesion molecules that are vital to signaling pathways that regulate tumor development, migration, and angiogenesis (Marthick and Dickinson 2012, Seguin, Desgrosellier et al. 2015). α6-integrin promotes metastasis (Wang, Shenouda et al. 2011) and represents a putative stemness marker (Marthick and Dickinson 2012, Ghebeh, Sleiman et al. 2013). The overexpression of this gene is associated with aggressive breast cancer and poor prognosis (Friedrichs, Ruiz et al. 1995), as well as, increased mammosphere formation and tumorigenesis (Cariati, Naderi et al. 2008). Of significance, α6-integrin is overexpressed in malignant breast cancer cells compared to normal, non-malignant breast epithelial cells (Keller, Lin et al. 2010, Meyer, Fleming et al. 2010).

Recent studies indicate that aryl hydrocarbon receptor (AhR) signaling impedes mammosphere formation (Prud'homme, Glinka et al. 2010, Zhao, Kanno et al. 2012).

Dysregulation of this pathway is associated with tumor formation, growth and progression (Feng, Cao et al. 2013). Aminoflavone (AF) is an investigational agent and AhR ligand (**Figure 10A**) with potent activity against estrogen receptor positive (ER^+) and certain estrogen receptor negative (ER^-) breast cancer cells (Loaiza-Perez, Kenney et al. 2004, McLean, Soto et al. 2008, Stark, Burger et al. 2013). AF-mediated anticancer activity is linked to reactive oxygen species production, oxidative DNA damage and apoptosis (McLean, Soto et al. 2008), and AF pro-drug AFP464 (**Figure 10A**) has recently undergone evaluation in clinical trials.

The purpose of our study is to investigate the ability of AF and AFP464 to inhibit mammospheres, which harbor an enriched population of TICs. We also seek to determine whether AF modulates the expression of genes associated with TICs such as α6-integrin. We hypothesize that AF reduces TIC capacity of breast cancer cells by thwarting α6-integrin expression in an AhR-dependent fashion. Our data demonstrate that AhR agonists AF and AFP464 readily disrupt mammospheres derived from ER^+ MCF-7 cells and from tumors excised from a Tamoxifen-responsive, ER^+ mammary mouse (M05) model respectively and inhibit α6-integrin expression.

Materials and Methods

Cell Culture and Reagents

Human MCF-7 and MDA-MB-231 tumor breast cancer cell lines were obtained from the Frederick National Laboratory for Cancer Research Division of Cancer Treatment and Diagnosis Tumor Repository (Frederick, MD, USA). AhR ligand-unresponsive MCF-7 breast cancer cells (AHR100 cells) were a kind gift from Dr. Jason

Matthews (University of Toronto, Toronto, Ontario, CA) and their establishment has been detailed elsewhere (Ciolino, Dankwah et al. 2002). Cell culture conditions for the MCF-7 and AHR100 cells have been described previously (McLean, Soto et al. 2008). LM05-E and LM05-Mix cells were cultured in DMEM/F12 medium as previously described (Pontiggia, Sampayo et al. 2012). 5-amino-2-(4-amino-3-fluorophenyl)-6,8-difluoro-7-methyl-4H-1-benzopyran-4-one (Aminoflavone, AF) was obtained from the "The NCI/DTP Open Chemical Repository" (http://dtp.cancer.gov, Frederick, MD) at the Frederick National Laboratory for Cancer Research. Aminoflavone pro-drug AFP464 was obtained from Tigris Pharmaceuticals (Bonita Springs, FL, USA). Stock solutions of AF, Tamoxifen and 4-hydroxy-Tamoxifen (4OHTam) were dissolved in dimethyl sulfoxide (DMSO). The AFP464 stock was dissolved in dextrose water. All stocks were stored protected from light at -20^{o}C until use.

Determination of Cancer Cell Viability

LM05 cells were isolated from tumors that developed spontaneously in a Balb/c mouse which were further transplanted into syngeneic mice to constitute the M05 mouse mammary model of breast cancer (Pontiggia, Sampayo et al. 2012). The cells were maintained in a single cell suspension of primarily epithelial (LM05-E) or a mixture of fibroblastic and epithelial cells (LM05-Mix) as previously described (Pontiggia, Sampayo et al. 2012). LM05-Mix or LM05-E cells were cultured in 96 well plates prior to treatment with varying concentrations of AF and analyzed using the MTS assay as described previously (Callero, Suarez et al. 2012). Alternatively, cytotoxicity of AF in MCF-7 cells was evaluated using the Alamar Blue assay as previously described

(McLean, Soto et al. 2008). MCF-7 derived mammospheres treated with AF were harvested and trypsinized as previously described before cytotoxicity was determined using the Alamar Blue assay (Walzl, Unger et al. 2014).

In Vitro Culture of Mammospheres

MCF-7 and AHR100 cells were used to generate mammospheres using the MammoCult™ Human Medium Kit (Stem Cell Technologies, Vancouver, BC, Canada). For second generation mammosphere culturing, first generation mammospheres were harvested via trypsinization at 37°C, and mechanically dispersed by gentle pipetting. Single cell suspensions were confirmed microscopically, and cells counted and resuspended in fresh MammoCult™ medium. In either instance, after 5 d, mammospheres were exposed to given treatments and visualized using an IX-71 Olympus microscope (relief contrast mode) or collected and prepared for flow cytometry analysis, semi-quantitative or quantitative reverse transcription (RT) PCR analysis.

RNA Extraction, Semi-quantitative RT-PCR and qPCR Analyses

Total RNA was isolated from MCF-7 and AHR100 cells as well as their corresponding mammospheres post treatment using the Quick-RNA MiniPrep Kit (Zymo Research, Irvine, CA, USA) according to manufacturer instructions. Semi-quantitative RT-PCR analysis was performed as previously described (van Riggelen, Buchwalter et al. 2005) and the relative amounts of GAPDH, fos-related antigen-1 (Fra-1), c-myc, α3, α5, α6, β1 and β4-integrin mRNAs determined. Primers for genes are listed in **Table 2**. Quantitative real-time PCR analysis was also performed using a CFX-96 PCR instrument

(Bio-Rad, Hercules, CA). Primers for genes indicated above were obtained from Integrated DNA Technologies (Coralville, IA, USA).

Table 2. Primers used for semi-quantitative RT-PCR assays

α-3 Integrin: 246 bp
Forward: GCC TGC CAA GCT AAT GAG AC
Reverse: AGA AGC TTT GTA GCC GGT GA
α-5 Integrin: 185 bp
Forward: AGC CTC AGA AGG AGG AGG AC
Reverse: GGT TAA TGG GGT GAT TGG TG
α-6 Integrin: 824 bp
Forward: GTG TTG CCA ACC AGA ATG GCT CGC
Reverse: CAG TCA CTC GAA CCT GAG TGC CTG C
β-1 Integrin: 194 bp
Forward: CCC TTG CAC AAG TGA ACA GA
Reverse: ACA TTC CTC CAGCCA ATC AG
β-4 Integrin: 183 bp
Forward: AGT GAA GAG CTG CAC GGA GT
Reverse: GGT GGT GTC AAT CTG GGT CT
c-myc: 478 bp
Forward: TAC CCT CTC AAC GAC AGC AG
Reverse: TCT TGA CAT TCT CCT CGG TG
Fra-1: 211 bp
Forward: GCG CCT AGG CCT TGT ATC TCC CTT TCC CC
Reverse: CCG CTC GAG GCG AGG AGG GTT GGA GAG CC
GAPDH: 460 bp
Forward: TGG ATA TTG TTG CCA TCA ATG ACC
Reverse: GAT GGC ATG GAC TGT GGT CAT G

Immunofluorescence Assay

Cells were fixed in 4% formalin in PBS and permeabilized with 0.1% Triton X-100 in PBS as previously described (Pontiggia, Rodriguez et al. 2009). Briefly, fixed cells were blocked before overnight incubation with anti-AhR rabbit polyclonal antibody (sc-5579, Santa Cruz Biotechnology [Santa Cruz, CA, USA]; 1:100 in PBS) followed by incubation with goat anti-mouse Alexa 488-conjugated secondary antibody (A-11008, Life Technologies [Carlsbad, CA, USA]; 1:1000, 2 h). Cells were then incubated with 1:5000 propidium iodide (2 mg/ml; Sigma St. Louis, MO, USA) and mounted onto glass slides using fluoromount (BDH Laboratory Supplies, Poole, Dorset, UK) following storage at 4°C in the dark. Stained cells were visualized on a fluorescence microscope using a Plan-Apochromat 40x 0.95 objective and images were processed and analyzed with Nikon C1-EZ package, version 2.20.

Flow Cytometry

Treated MCF-7 derived mammospheres were harvested and resuspended in PBS following staining to detect CD24-PE, CD44-FITC, and CD49f (α6 integrin)-APC (eBioscience, San Diego, CA, USA). The FACSCalibur (BD Bioscience, San Jose, CA, USA) was used to analyze the cells by flow cytometry. Alternatively, cells in suspension derived from the M05 tumor were labeled under optimized conditions as follows: 1:100 for CD29-FITC, 1:300 for CD24-APC and 1:7 for LIN-PE antibodies (BioLegend, San Diego, CA, USA) for 1 h on ice before flow cytometry analysis (PASIII, PARTEC, Munich, Germany).

Mice

Inbred 2–4 month-old BALB/c female mice were obtained from the Animal Care Division at the Instituto de Oncologia ‘‘Angel H. Roffo’’. Animal care and manipulation were in agreement with institutional guidelines and the Guide for the Care and Use of Laboratory Animals [27]. The M05 mouse mammary tumor model is described elsewhere (Simian, Manzur et al. 2009). Tumors in mice were measured twice a week with a Vernier caliper in two planes (length and width). Tumor surface was calculated using the formula: (length x width^2)/2. Tumor measurements were determined relative to size at time zero. For animal dosing, AFP464 was prepared as a smooth suspension in saline containing 0.05% Tween 80. Each experiment contained a vehicle control group (n = 20) treated in parallel with the AFP464-treated groups. AFP464 was evaluated at two dose levels (n = 6 per dose) intraperitoneally. The treatment schedule followed once daily dosing for a total of 5 d (QD × 5), with the first treatment given when the average size of the tumors was about 1 cm^2. Median tumor surfaces were used to calculate the tumor growth rate (slope of the curve “median tumor surface vs. time”). The experiment was performed three times with similar results.

Immunohistochemistry

Specimens were fixed in 10% formalin, dehydrated and embedded in paraffin. Sections were stained with hematoxylin for 6 min and counterstained with eosin for 30s followed by visualization with a Nikon Eclipse E400 microscope.

M05 Tumor Cell Suspension Preparation and Mammosphere Formation Assay

M05 tumors were minced and digested in digestion media as previously described in detail (Raffo, Berardi et al. 2013). In brief, M05 tumor-derived cells were grown in suspension in 6-well low attachment culture plates (Greiner Bio-One, Koln, Germany) at a density of 10,000–15,000 viable cells/ml. Resulting mammospheres were counted after 5–8 d in culture using a Nikon eclipse TE2000-S inverted microscope.

Statistical Analysis

Differences between groups were analyzed using one-way ANOVA with Tukey's test or the Tukey-Kramer multiple comparison test for evaluating three or more groups. To compare two groups, the unpaired Student's t test with Welch's correction was used. For *in vivo* assays, statistical significance was determined using two-way ANOVA. Statistical analysis was performed using GraphPad Prism 4.0, Graph Pad software, Inc. San Diego, California, USA, www.graphpad.com. Differences were considered significant at $p < 0.05$.

Results

AF Exhibits Cytotoxicity, Disrupts Mammospheres Derived from MCF-7 Cells and Impedes Secondary Mammosphere Formation

Previous data indicate that AhR activation represses mammosphere formation in MCF-7 cells (Zhao, Kanno et al. 2012). Additionally, while tamoxifen facilitates the selection of cells which favor mammosphere formation (Raffo, Berardi et al. 2013), the non-toxic AhR agonist Tranilast readily disrupts mammospheres (Prud'homme, Glinka et

al. 2010). We therefore sought to determine whether AF, an AhR agonist, could also disrupt mammospheres and to compare the relative cytotoxicity of AF in mammospheres relative to monolayers. We first exposed MCF-7 monolayers and MCF-7 derived mammospheres to media containing 0.1% DMSO (control) or AF (1-10,000 nM) for 48 h before evaluating cytotoxicity using the Alamar Blue assay. We found that AF potently suppressed both MCF-7 monolayer growth (IC_{50} = 123 nM) and MCF-7 derived mammosphere growth (IC_{50} = 196 nM) (**Figure 10B**). We then exposed MCF-7 derived mammospheres to media containing 0.1% DMSO (control), tamoxifen (1 μM), 4OHTam (1 μM) or AF (1 μM) for 48 h and visualized mammosphere disruption using the IX71 Olympus microscope (relief contrast mode). AF disrupted mammospheres while neither tamoxifen nor 4OHTam did so (**Figure 10C**). We found that another antitumor AhR agonist 2-(4-amino-3-methylphenyl)-5-fluorobenzothiazole (Hutchinson, Chua et al. 2001) also disrupted mammospheres (data not shown). We next evaluated whether AF suppresses the generation of secondary mammospheres, a characteristic suggestive of self-renewal capacity. We mechanically disrupted mammospheres and cultured single cells to promote mammosphere re-formation (secondary mammospheres). Cells derived from mammospheres previously exposed to AF did not form secondary mammospheres, unlike those exposed to 0.1% DMSO or tamoxifen (**Figure 10D**).

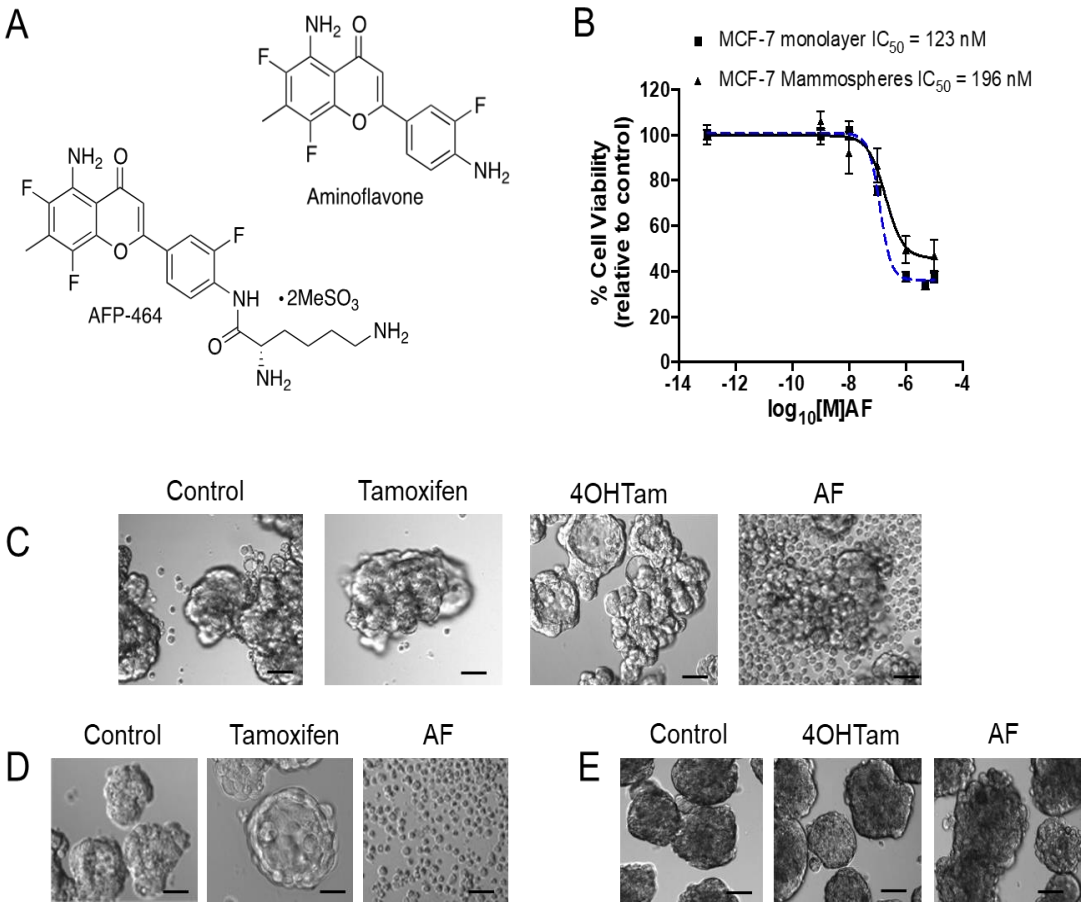

Figure 10. **AF, Tamoxifen and 4-OHTam differentially impact mammosphere formation.** (A) Structures of AF and AFP464 (B) MCF-7 cells and MCF-7 cell-derived mammospheres were treated with media containing 0.1% DMSO or AF (0.001-10 μM) for 48 h after which cytotoxicity was assessed using the Alamar Blue assay in accordance with Materials and Methods. Data represent the average of two independent experiments performed with at least four replicates. Images of (C) MCF-7 derived mammospheres, (D) secondary mammospheres and (E) AHR100 derived mammospheres captured using the Olympus IX-71 microscope (100× magnification) following treatment as described in Materials and Methods. Scale bar = 50 μm.

Mammospheres contain a higher percentage of TICs compared to 2D cell monolayers (Barbieri, Wurth et al. 2012). Consistent with previous studies, we detected an increase in the percentage of MCF-7 cells that stained positive for $CD44^{+}/CD24^{-/low}$ in mammospheres compared to 2D monolayers (**Figure 11A**). A further increase was detected under hypoxia as determined by relief contrast mode microscopy and flow

cytometry (**Figure 11A**). Using fluorescence microscopy, we identified Aldefluor™ positive cells (which stain green) within mammospheres (**Figure 11B**). MCF-7 mammospheres in the presence of hypoxia showed the greatest induction of the OCT-4 gene, a key stemness biomarker (**Figure 11C**). Both tamoxifen and 4OHTam increased the percentage of cells in mammospheres that stained for $CD44^{+}/CD24^{-/low}$, an effect substantially reduced by AF (**Figure 11D**). This suggests AF has the capacity to suppress the growth of cells with TIC capacity.

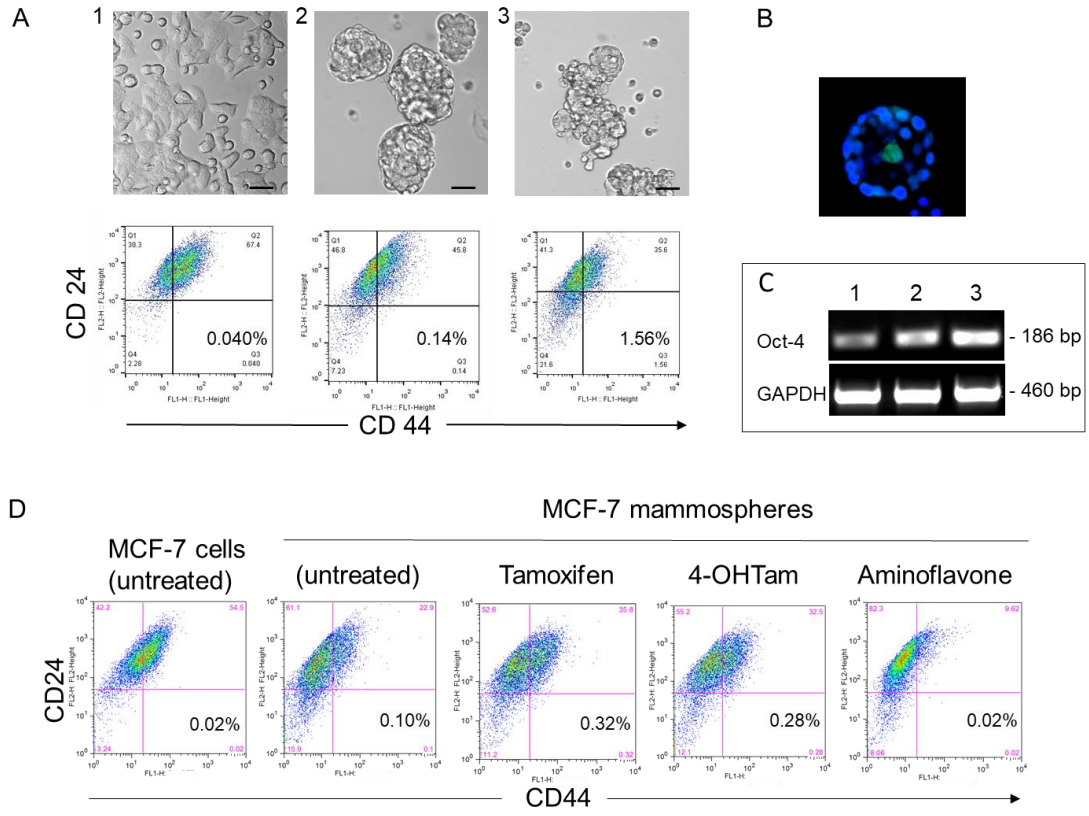

Figure 11. **MCF-7-derived mammospheres show an increase in $CD44^{+}/CD24^{-/low}$ levels in the presence of hypoxia.** (A) 2D monolayer of MCF-7 cells cultured under normoxia (1), MCF-7derived mammospheres obtained from 7 d culture under normoxia (20.8% O_2) (2), or hypoxia (1% O_2) (3). Scale bar = 50 μm. Lower panel indicates the percentage of cells staining $CD44^{+}/CD24^{-/low}$ under conditions 1-3 by flow cytometry analysis. (B) Mammosphere revealing cells staining positive for Aldefluor (green), counterstained with Hoechst 33342 (blue). (C) RT-PCR for Oct-4 (D) flow cytometry analysis indicating percentage of cells within mammospheres that stains for $CD44^{+}/CD24^{-/low}$.

AF Lacks the Ability to Disrupt AHR100-derived Mammospheres

Since activation of the AhR signaling pathway has been shown to impair mammosphere formation (Prud'homme, Glinka et al. 2010, Zhao, Kanno et al. 2012), we evaluated whether AF could disrupt mammospheres derived from AHR100 cells, an AhR ligand unresponsive variant of MCF-7 cells. AF failed to disrupt AHR100-derived

mammospheres (**Figure 10E**) suggesting AF disrupts mammospheres in an AhR-dependent fashion.

AF Modulates α6-Integrin, c-Myc and Fra-1 Expression in MCF-7-Derived Mammospheres.

We next investigated the mechanism by which AF disrupts mammospheres. Integrins contribute to tumorigenesis (Timmer, Oosterhuis et al. 1994, Gonzalez, Gonzales et al. 2002, Alexander 2008, Taddei, Deugnier et al. 2008). In particular, α6-integrin promotes mammosphere formation and tumorigenicity in MCF-7 cells (Cariati, Naderi et al. 2008). Among the panel of integrins examined, AF only inhibited the expression of α6-integrin (**Figure 12A**). AF-mediated suppression of α6-integrin was also demonstrated using flow cytometry (**figure 13A**). C-myc promotes breast tumorigenesis (Nass and Dickson 1997). Since the α6-integrin promoter contains a c-myc binding site (Nishida, Kitazawa et al. 1997), we examined c-myc expression levels. Fos-related antigen 1 (Fra-1) is a component of the AP-1 transcription factor shown to promote chemo-sensitization and TIC inhibition (Lu, Chen et al. 2012). Moreover, the α6-integrin promoter contains an AP-1 binding site (**Figure 12B**). Therefore, we evaluated α6-integrin, c-myc and Fra-1 gene expression in mammospheres following exposure to AF or tamoxifen (**Figure 13B, C**). Interestingly, AF readily abolished α6-integrin while tamoxifen increased its expression. Tamoxifen diminished while AF abolished c-myc expression. AF induced while tamoxifen suppressed Fra-1 expression. AF was unable to significantly inhibit α6-integrin expression in AHR100 cells (2D) or in AHR100 derived mammospheres and this implies AF-mediated cytotoxicity and

disruption of mammospheres predominately occurs in an AhR-dependent fashion (**Figure 13D**). Taken together, tamoxifen and AF differentially modulate genes to cause differences in cell fate, with tamoxifen promoting mammosphere formation (Raffo, Berardi et al. 2013) and AF disrupting mammosphere formation.

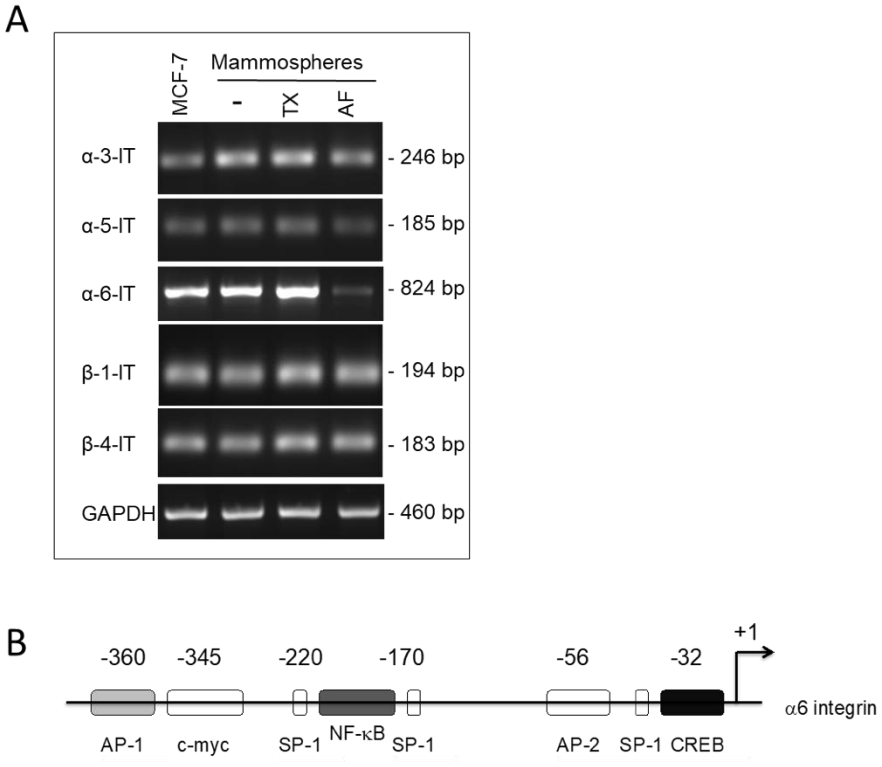

Figure 12. **AF abolishes α6-integrin expression yet does not impact the expression of other members of the integrin family.** (A) RNA was isolated from MCF-7 cells grown as 2D cell monolayers, vehicle treated mammospheres (control), or mammospheres treated with 1 μM tamoxifen (TX), or 1 μM AF for 48 h. RT-PCR was performed for the integrin genes indicated. (B) Schematic representation of α6-Integrin promoter region with plausible binding sites for transcription factors adapted from (Nishida, Kitazawa et al. 1997).

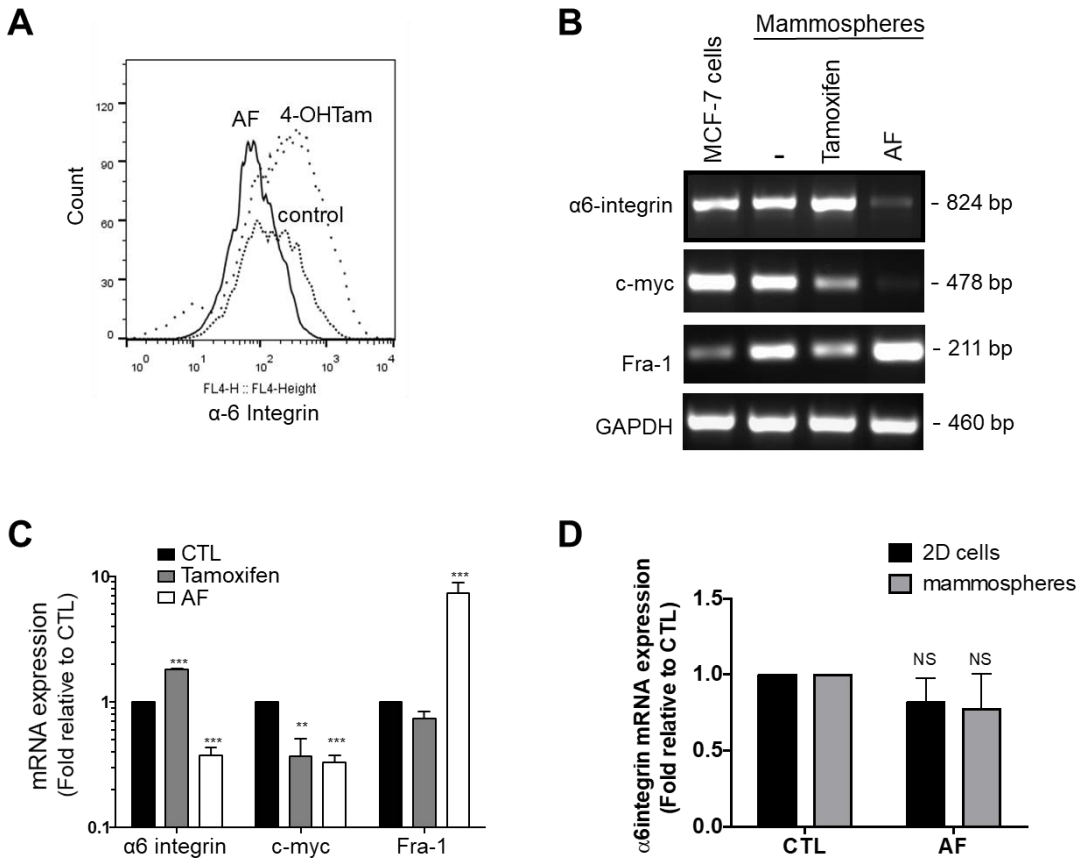

Figure 13. **AF and Tamoxifen differentially modulate gene expression in mammospheres.** (A) Flow cytometry analysis was performed on cells from MCF-7 derived mammospheres exposed to control (0.1% DMSO), 1 μM 4OHTam or 1μM AF for 48 h. (B) RNA was isolated from mammospheres exposed to 0.1% DMSO, 1 μM tamoxifen or 1 μM AF for 48 h. Semi-quantitative RT-PCR was performed to detect α6-integrin, c-myc and Fra-1 gene expression. (C) RT-qPCR analysis of samples exposed to 0.1% DMSO, 1 μM tamoxifen or 1μM AF for 48 h was performed as outlined in Materials and Methods for genes described in B. (D) AHR100 cells (2D) or AHR100-derived mammospheres were exposed to 0.1% DMSO or 1 μM AF for 48 harvested and analyzed for α6-integrin expression using RT-qPCR. The values represent the average of three independent experiments. NS indicates not significant or ** $p < 0.01$ and *** $p < 0.001$ compared to vehicle-exposed cells.

AF Inhibits α6-Integrin Levels in MCF-7 but not MDA-MB-231 Breast Cancer Cells

We evaluated CD24/CD44 levels and endogenous α6-integrin expression in MDA-MB-231 breast cancer cells which are known to exhibit a mesenchymal phenotype and compared the expression levels to those in MCF-7 cells. We found that MDA-MB-231 cells exhibited a substantially higher percentage of cells that stain for $CD44^{hi}/CD24^{low/-}$ (**Figure 14A**). In addition, our data show MDA-MB-231 cells show 20-fold higher levels of α6-integrin in comparison to MCF-7 cells. While AF suppresses α6-integrin levels in MCF-7 cells, AF was unable to impact α6-integrin levels in MDA-MB-231 cells (**Figure 14B**).

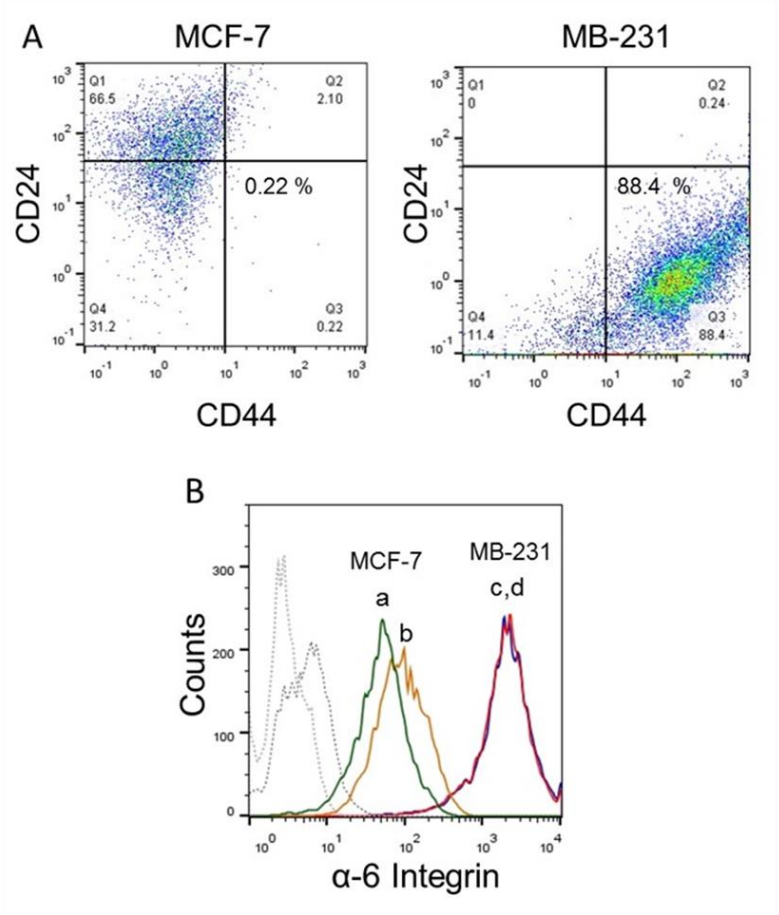

Figure 14. **AF is unable to inhibit α6-integrin expression in MDA-MB-231 breast cancer cells.** (A) MCF-7 and MDA-MB-231 (M-231) breast cancer cells were stained to assess the population of cells that were $CD44^{+}/CD24^{-/low}$ using flow cytometry (B) MCF-7 and M-231 cells were exposed to media containing 0.1% DMSO (designated b and d) or 1 μM AF (designated a and c) for 48 h before flow cytometry analysis was performed to assess α6-integrin expression.

AF Displays Cytotoxicity and Promotes AhR Translocation from the Cytosol to the Nucleus of LM05 Cells

AF was previously found to demonstrate potent anticancer activity in MCF-7 breast cancer cells (Loaiza-Perez, Kenney et al. 2004, McLean, Soto et al. 2008, Callero and Loaiza-Perez 2011, Stark, Burger et al. 2013). T47D ER^{+} breast cancer cells also

exhibit sensitivity to AF (McLean, Soto et al. 2008, Callero and Loaiza-Perez 2011). We therefore sought to evaluate whether breast cancer cells derived from a spontaneous mouse model cultured to contain epithelial or a mixture of fibroblastic and epithelial cells would also display sensitivity to AF. LM05-E (epithelial) and LM05-Mix (epithelial and fibroblastic) cells respectively were exposed to varying concentrations of AF. Previously, LM05-E demonstrated greater sensitivity to tamoxifen than LM05-Mix cells (Pontiggia, Rodriguez et al. 2009, Pontiggia, Sampayo et al. 2012, Raffo, Pontiggia et al. 2015) suggesting that the tumor microenvironment may contribute to tamoxifen resistance. Alternatively, LMO5-Mix cells may have undergone epithelial to mesenchymal transition (EMT) since they contain a subset of cells with mesenchymal characteristics unlike LMO5-E cells. We found that LM05-E cells were more responsive to AF than LM05-mix cells (**Figure 15A**). This suggests that a tumor microenvironment enriched with fibroblastic cells is less susceptible to the anticancer actions of AF, similar to what was observed in tamoxifen-exposed cells (Pontiggia, Rodriguez et al. 2009, Pontiggia, Sampayo et al. 2012).

AF promotes AhR nuclear translocation where it binds to the xenobiotic response element to activate the transcription of cytochrome P450 genes in responsive breast cancer cells (Loaiza-Perez, Kenney et al. 2004). Similarly, we found that AF promoted AhR translocation in LM05-E breast cancer cells more so than LM05-Mix cells (**Figure 15B**). We used AhR inhibitor α-Naphthoflavone (αNF) to confirm AhR signaling dependence in AF-mediated AhR translocation. These data suggest that AF promotes AhR nuclear translocation in breast cancer cells possessing an epithelial phenotype.

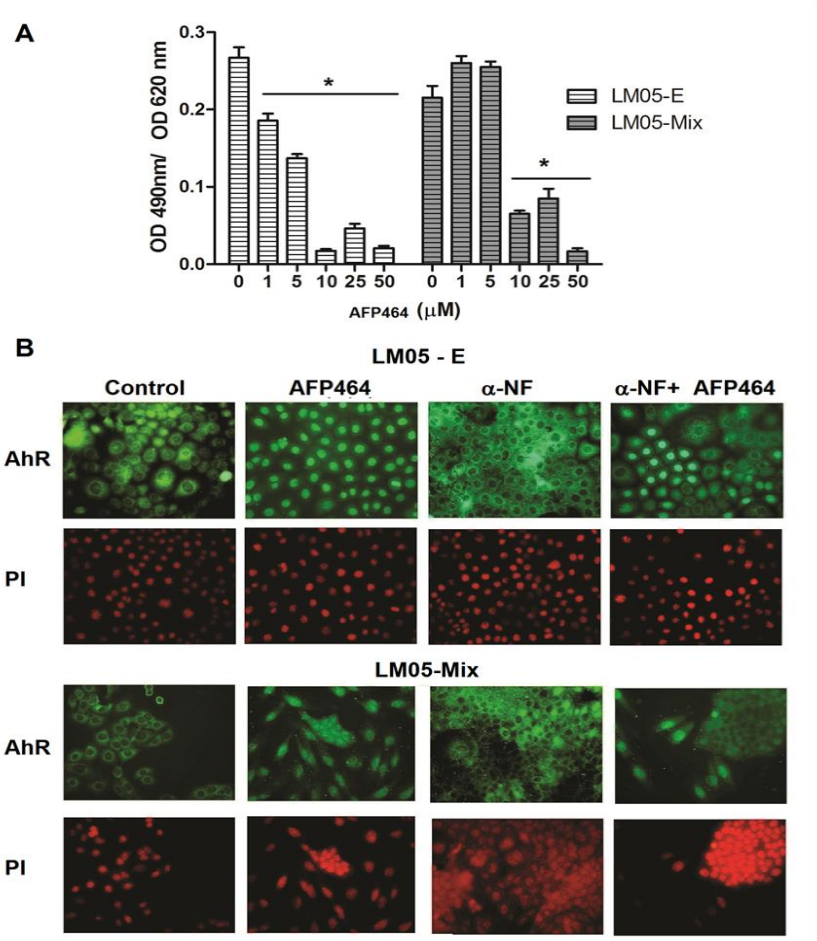

Figure 15. **AFP464 exhibits cytotoxicity in LM05 epithelial cells in an AhR-dependent fashion. (**A) LM05-E and LM05-Mix cells were incubated with AFP464 for 5 d. Cellular viability was evaluated by the MTS assay. The values represent the average of three independent experiments (N = 9), * $p < 0.05$ compared to vehicle-exposed cells. (B) LM05-E and LM05-Mix cells were grown on coverslips and treated for 1 h with 1 μM alpha-naphthoflavone (α-NF) or 20 μM AFP464 with or without 1 h pretreatment with α-NF. Pretreatment with α-NF was followed by 1 h co-treatment of AFP464 and α-NF. Cells were then fixed and incubated with primary anti-AhR antibody, goat anti-mouse Alexa 488-conjugated secondary antibody (green) and propidium iodide (PI, red).

AFP464 Decreases M05 Mouse Breast Tumor Growth Rate In Vivo

To determine the responsiveness of M05 tumors to AFP464, we inoculated female virgin syngeneic mice with M05 tumor cells as described in materials and methods. Once the average size of tumors reached 1 cm^2, we treated mice with AFP464 [1.2 mg/kg

or 12 mg/kg] or vehicle (**Figure 16A,B**). AFP464 (12 mg/kg) treatment yielded sustained, significant inhibition of M05 tumor growth. In contrast, no appreciable growth inhibition was observed in animals exposed to 1.2 mg/kg AFP464.

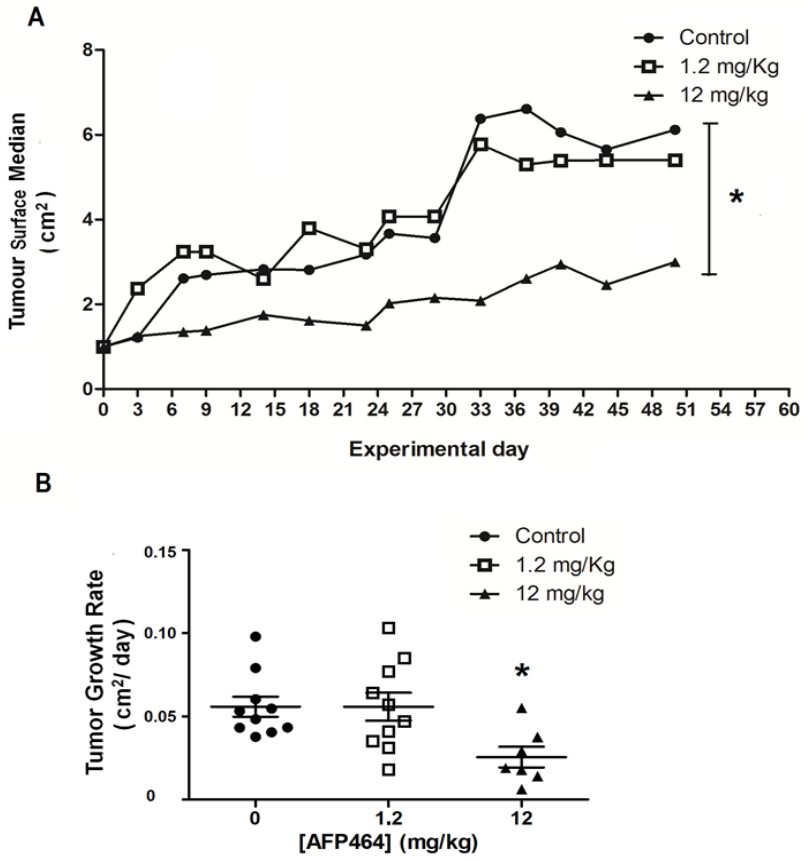

Figure 16. **AFP464 decreases M05 mouse breast tumor growth rate *in vivo***. Mice were inoculated with M05 tumor cells. When the tumors reached an average size of 1 cm^2, the mice were randomly divided into 3 groups and treated with vehicle or AFP464 (1.2 mg/kg, *i.p.*, QD × 5 or 12 mg/kg, i.p., QD × 5) as outlined in Materials and Methods. Data are presented with respect to tumor surface median (A) or tumor growth rate relative to control at time 0 (B). Data are the mean of at least three independent experiments (N = 6). * $P < 0.5$ compared to tumors in untreated animals.

AFP464 Induces a Less Invasive Phenotype in M05 Tumors

Anticancer agents frequently alter breast tumor phenotype while suppressing tumor growth (Hebbard, Cecena et al. 2011, Takehara, Hoshino et al. 2011, Fiorillo, Verre et al. 2015). Histopathological analyses of M05 tumors revealed a semi-differentiated adenocarcinoma with papillary differentiation. **Figure 17** shows two different cell populations: epithelial and stromal. Tumors derived from AFP464-treated animals, possessed a circumscribed collection of mucin lake clusters or acini of floating tumor cells. A greater number of mucinous type cells, residing predominantly in the epithelial zones, were observed in tumors from AFP464-treated animals as compared to untreated animals. Mucinous breast carcinomas are less prone to metastases and carry a more favorable prognosis compared to other carcinomas (Yu, Deng et al. 2010). Our data suggest AFP464 promotes mucin lake formation resulting in a less invasive breast cancer phenotype.

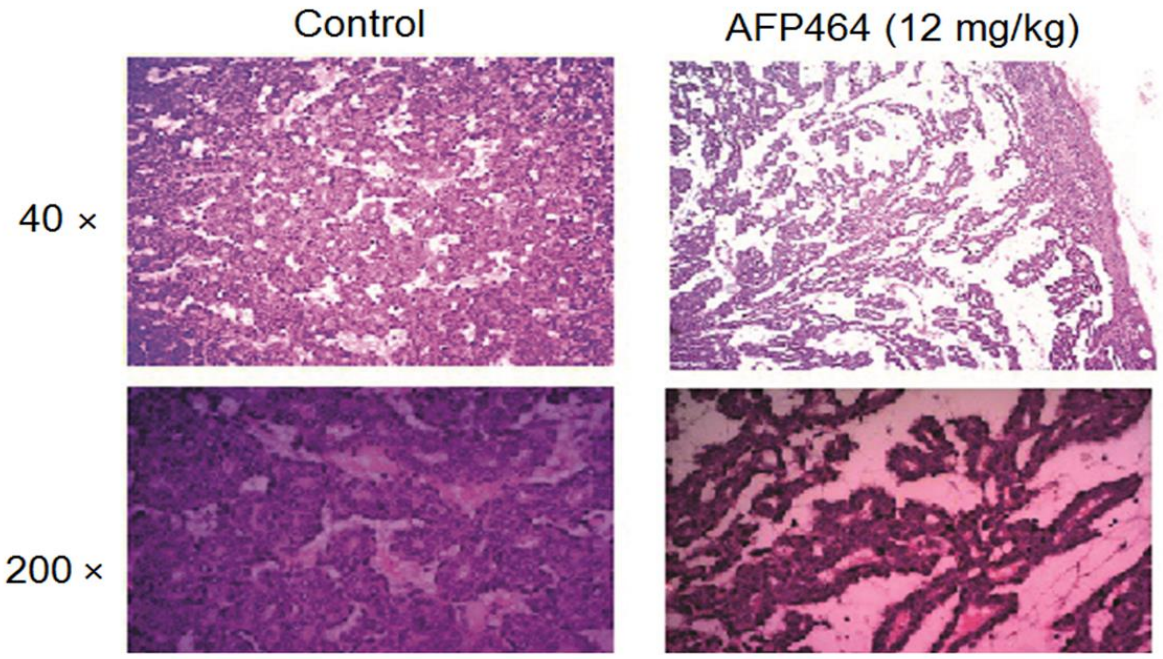

Figure 17. **AFP464 alters M05 breast tumor phenotype to resemble a less invasive subtype**. Tumors excised from mice exposed to either vehicle or 12 mg/kg AFP464 were sectioned and stained with hematoxylin and eosin as described in materials and methods before being visualized microscopically (40× and 200×).

AFP464 Decreases Mammosphere Forming Capacity of M05 Mouse Breast Tumors

Chemotherapy and radiotherapy often increase the frequency of cells with stem cell properties (Croker and Allan 2012). Since the estrogen-dependent M05 mouse model exhibits sensitivity to AFP464, we investigated the capacity for AFP464 to diminish TIC capacity within M05 tumors. Mice bearing M05 tumors were treated with AFP464 once tumors reached an average size of 1 cm^2 as previously described (Simian, Manzur et al. 2009). Tumors were then removed and prepared as single cell suspensions for mammosphere formation. AFP464 (12 mg/kg) effectively decreased M05-tumor derived mammosphere formation (**Figure 18A**). This suggests AFP464 suppresses self-renewal capacity within the tumor microenvironment.

AFP464 Decreases Cells with Stemness Characteristics within the M05 Tumor

We next sought to determine whether AFP464 diminishes the percentage of tumor cells that stain for markers of stemness. Cells with stemness properties derived from the mouse mammary gland tumor readily stain for Lin (-)/$CD29^{h}$/$CD24^{+}$ (Shackleton, Vaillant et al. 2006, Zhang, Behbod et al. 2008). AFP464 significantly decreased this population of cells at 12 mg/kg (**Figure 18B-C**) AFP464 (12 mg/kg) also significantly decreased α6-integrin positive cells (**Figure 18D**). These data suggest AFP464 suppresses breast cancer stem cell-like characteristics *in vivo*.

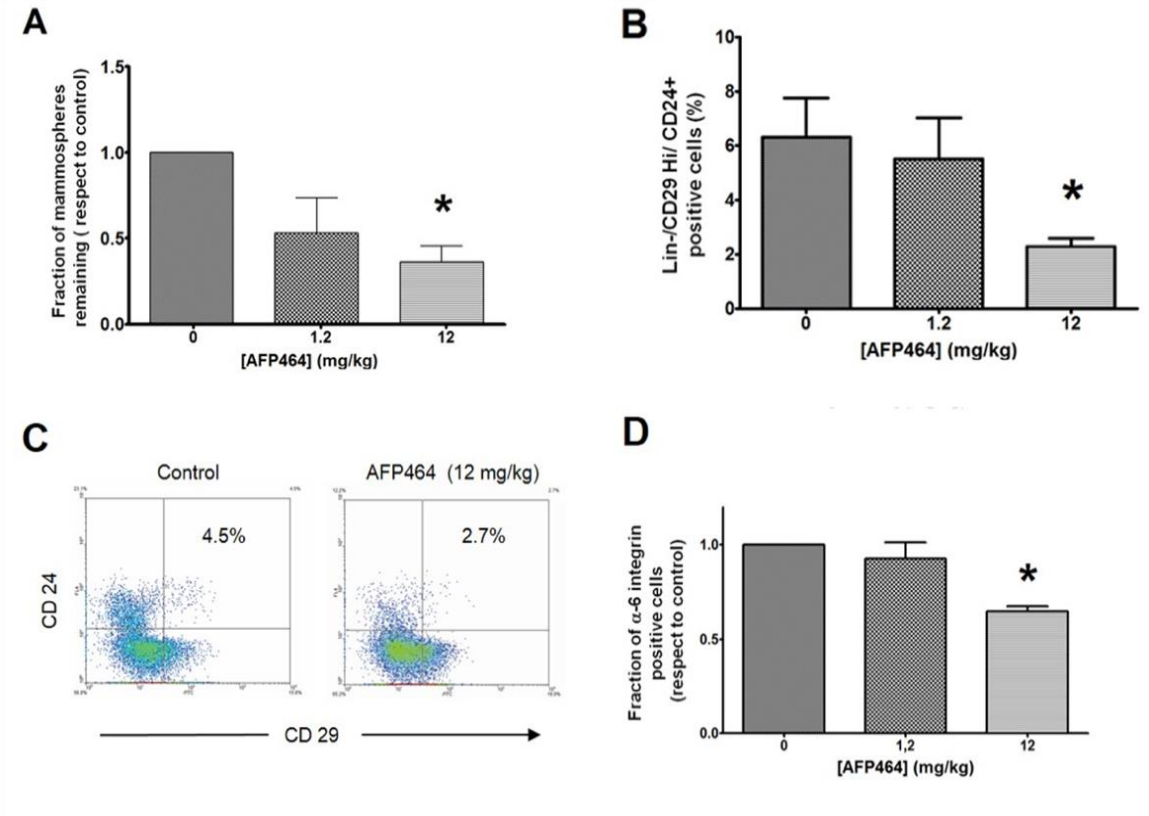

Figure 18. **AFP464 decreases the number of cells with mammosphere forming capacity and stem cell-like phenotype within the M05 tumor.** Quantification of mammospheres derived from tumors grown in M05 mice treated with either vehicle (control) or AFP464 as detailed previously in Materials and Methods. Cells derived from tumors in animals exposed to AFP464 or vehicle were stained to assess the population of cells that were (B, C) Lin (-)/CD29h/CD24(+) or (D) CD49f+ (α6-integrin+) as determined by flow cytometry. Data are the mean of at least three independent experiments (N = 6). * $P < 0.5$ compared to mammosphere number in untreated animals.

Discussion

Tumor initiating cells (TICs) resist the anticancer actions of chemotherapeutic and targeted anticancer agents (Li, Lewis et al. 2008, Creighton, Li et al. 2009). This creates a rationale for combination therapy involving small molecules that target TICs and established anticancer agents (Chai, Zhou et al. 2013, Liu, Kumar et al. 2013, Soo, Ng et al. 2015). AF and AFP464 elicit anticancer actions *in vitro* and *in vivo* respectively following AhR signaling activation (Kuffel, Schroeder et al. 2002, McLean, Soto et al. 2008, Meng, Meng et al. 2008, Callero, Suarez et al. 2012, Stark, Burger et al. 2013). Our data suggest that AFP464 suppresses bulk tumor growth and cells with TIC characteristics in an AhR-dependent fashion by thwarting α6-integrin expression. Blocking α6-integrin function has been previously shown to arrest the progression of metastatic prostate cancer (Landowski, Gard et al. 2014). Therefore, inhibiting α6-integrin expression may serve as a general mechanism by which agents suppress tumor progression and metastasis in a variety of malignancies.

AhR activation represses mammosphere formation and disrupts mammospheres (Prud'homme, Glinka et al. 2010, Zhao, Kanno et al. 2012). We sought to examine the capacity for AF and AFP464 to disrupt mammospheres and to identify plausible mechanism(s). We show that AF and AFP464 disrupt mammospheres derived from both *in vitro* and *in vivo* models respectively. Interestingly, AhR signaling exhibits a dual function in stem cell maintenance in breast tumors. Ligand-independent constitutive AhR activation tends to promote rather than suppress mammosphere formation (Zhao, Ohara et al. 2013). However, AhR agonist AF readily suppressed mammospheres similar to what was detected with another non-toxic AhR agonist (Prud'homme, Glinka et al. 2010).

Integrins regulate numerous cellular processes including proliferation and self-renewal (Li, Zhang et al. 2005, Hayashi, Furue et al. 2007, Cattavarayane, Palovuori et al. 2015). In particular, α6-integrin is overexpressed in breast cancer to promote breast tumorigenesis and metastases (Cariati, Naderi et al. 2008). α6-integrin overexpression is linked to reduced breast cancer patient survival (Friedrichs, Ruiz et al. 1995).

Although the ability of AF to disrupt mammospheres appears to be mediated at least in part via α6-integrin suppression, it is very plausible that AF modulates other genes to disrupt mammospheres and exert its cytotoxic actions. Recently, we found that AF disrupts mammospheres derived from Tamoxifen-resistant MCF-7 cells and these mammospheres express α6-integrin at levels 3-fold greater than what is observed with mammospheres derived from parental MCF-7 cells (data not shown). We also found that AF completely lacks the ability to inhibit α6-integrin expression levels in MDA-MB-231 breast cancer cells which we found express α6-integrin levels 20-fold higher than MCF-7 cells (**Figure 14**). MDA-MB-231 cells are completely unresponsive to the cytotoxic actions of AF (McLean, Soto et al. 2008, Callero and Loaiza-Perez 2011). These data suggest that AF-mediated α6-integrin inhibition at least contributes to the ability of AF to disrupt mammospheres as opposed to a simple by-stander effect since the actions of AF appear to be diminished once α6-integrin levels in cancer cells exceed a certain threshold.

We found that tamoxifen increased α6-integrin expression (**Figure 13B,C**). This is consistent with findings that reveal tamoxifen increases the propensity of cells to form mammospheres and the expression of stemness genes (Raffo, Berardi et al. 2013). Raffo and colleagues also demonstrated that tamoxifen decreases the epithelial marker E-cadherin in M05 mouse mammary tumors. These and our findings suggest that

tamoxifen promotes the growth of breast TICs and EMT (Hiscox, Jiang et al. 2006). Though TIC capacity occurs independently of EMT (Xie, Ji et al. 2014), it is plausible that tamoxifen diminishes its own effectiveness after triggering either of these processes.

Fra-1 has been shown to promote malignant progression via cytoplasmic accumulation (Song, Song et al. 2006). However, Fra-1 has also been found to drive stem cells out of dormancy to promote chemo-sensitization (Lu, Chen et al. 2012). Fra-1 up-regulation may therefore constitute a facet of AF's ability to suppress breast TIC growth. Tamoxifen's inhibition of Fra-1 expression in this context is consistent with its propensity to promote stemness. Additional studies are needed to delineate the role of AF-mediated up-regulation of Fra-1 in TIC growth suppression and its potential role in sensitizing TICs to the anticancer actions of current therapies.

AF was better able to inhibit c-myc expression in MCF-7 derived mammospheres than Tamoxifen. AF also prevented the formation of secondary mammospheres highlighting its inhibition of self-renewal capacity. It is plausible that differences in the ability of tamoxifen and AF to disrupt mammospheres pertain to differences in their ability to modulate genes that regulate TIC behavior.

Our data further support previous findings suggesting tamoxifen may promote its own resistance by up-regulating stemness genes (Raffo, Berardi et al. 2013) including α6-integrin. This propensity to promote TIC capacity and ensuing resistance likely contributes to Tamoxifen-mediated relapse. This provides a rationale to determine whether AFP464 counteracts stemness-associated properties to increase the efficacy of tamoxifen and other endocrine therapies.

AFP464 suppressed the growth of tumors in the M05 model consistent with a previous study revealing AF's anti-tumor actions in athymic mice bearing human breast cancer xenografts (Stark, Burger et al. 2013). AFP464 appears to alter tumor morphology to a mucinous phenotype (**Figure 17**). Pure mucinous breast cancer carries a more favorable prognosis than mucinous breast cancer mixed with other invasive subtypes (Jambal, Badtke et al. 2013). Mucins exhibit tumor suppressor activity in colorectal cancer (Velcich, Yang et al. 2002) and a reduction in mucin lakes in breast tumors corresponds to a more aggressive state (Adsay, Merati et al. 2003). It is plausible that AFP464 suppresses breast tumor progression by promoting a less aggressive phenotype.

AFP464 has recently been evaluated in clinical trials for the treatment of solid tumors; yet to the best of our knowledge, our study is the first to suggest its ability to suppress TIC growth. Additional studies are needed to confirm the ability of AFP464 to inhibit TIC growth since no 'perfect' *in vitro* assays exist to assess indices of stemness. Furthermore, AF demonstrates activity against certain ER^- breast cancer cell lines and tumors, and thus may also have activity against their corresponding TICs.

In conclusion, our data provide a rationale for the continued development of AFP464 as an agent to enhance the therapeutic management of breast cancer. We found that AFP464 not only reduces bulk tumor similar to other P450 pro-drugs (Swanson, Njar et al. 2010), but also appears to target cells with stem cell-like properties, at least in part by abolishing α6-integrin expression. In contrast, tamoxifen appears to increase stemness properties in breast cancer cells. This raises questions concerning its overall clinical efficacy. However, the promising actions of AFP464 against both bulk tumor cells and

mammospheres indicate that combination therapy approaches involving AFP464 and endocrine therapy should improve clinical outcomes for breast cancer patients.

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CHAPTER THREE

AHR LIGAND AMINOFLAVONE SUPPRESSES A6-INTEGRIN-SRC-AKT SIGNALING TO ATTENUATE TAMOXIFEN RESISTANCE IN BREAST CANCER CELLS

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Abstract

More than 40% of patients with luminal breast cancer treated with endocrine therapy agent tamoxifen demonstrate resistance. Emerging evidence suggests tumor initiating cells (TICs) and aberrant activation of Src and Akt signaling drive tamoxifen resistance and relapse. We previously demonstrated that aryl hydrocarbon receptor (AhR) ligand Aminoflavone (AF) inhibits the expression of TIC gene α6-integrin and disrupts mammospheres derived from tamoxifen-sensitive breast cancer cells. In the current study, we hypothesize that tamoxifen resistant (TamR) cells exhibit higher levels of α6-integrin than tamoxifen sensitive cells and that AF inhibits the growth of TamR cells by suppressing α6-integrin-Src-Akt signaling. In support of our hypothesis, TamR cells and associated mammospheres were found to exhibit elevated α6-integrin expression compared to their tamoxifen sensitive counterparts. Furthermore, tumor sections from patients who relapsed on tamoxifen showed enhanced α6-integrin expression. Gene expression profiling from the TCGA database further revealed that basal-like breast cancer samples, known to be largely unresponsive to tamoxifen, demonstrated higher α6-integrin levels than luminal breast cancer samples. Importantly, AF reduced TamR cell viability and disrupted TamR mammospheres while concomitantly reducing α6-integrin mRNA and protein levels. In addition, AF and siRNA against α6-integrin blocked tamoxifen-stimulated proliferation of TamR MCF-7 cells and further sensitized these cells to tamoxifen. Moreover, AF reduced Src and Akt signaling activation in TamR MCF-7 cells. Our findings suggest elevated α6-integrin expression is associated with tamoxifen resistance and AF suppresses α6-integrin-Src-Akt signaling activation to confer activity against TamR breast cancer.

Introduction

Breast cancer is the most commonly diagnosed cancer in women worldwide. Resistance to therapies often results in metastasis which leads to recurrence and breast cancer mortality (Ahmad 2013). Estrogen receptor positive (ER^+) breast cancer is the most frequently diagnosed breast cancer subtype. Tamoxifen is widely used to treat ER^+ breast cancer although the emergence of resistance significantly diminishes its clinical efficacy (Tanic, Milovanovic et al. 2012). Tumor initiating cells (TICs) are key contributors to tamoxifen resistance owing to their ability to evade treatment and self-renew to produce recurrent tumors (Ojo, Wei et al. 2015). Tamoxifen treatment itself has been shown to select for cells with self-renewal capacity (Raffo, Berardi et al. 2013). As such, elimination of TICs is crucial to circumvent tamoxifen resistance and confer long-term clinical benefit (Ricci-Vitiani, Pagliuca et al. 2008, Gruber, Scheidt et al. 2017).

Integrins have been identified as important regulators of tumor initiation or cancer stemness and drug resistance (Seguin, Desgrosellier et al. 2015). In particular, α6-integrin is important for TIC maintenance and function (Lathia, Gallagher et al. 2010). Indeed, elevated α6-integrin expression in breast tumor tissues has been associated with poor overall survival among patients (Friedrichs, Ruiz et al. 1995). We recently demonstrated that, contrary to tamoxifen, aryl hydrocarbon receptor (AhR) ligand Aminoflavone (AF) inhibits α6-integrin expression to suppress TIC proliferation in ER^+ breast cancer models and though α6-integrin often partners with β1 and β4 integrins, AF did not markedly alter the expression of these integrins (Brantley, Callero et al. 2016). Although another non-toxic AhR ligand Tranilast has been shown to synergize with Tamoxifen *in vitro* (Darakhshan, Bidmeshkipour et al. 2013) and inhibit TIC proliferation (Prud'homme,

Glinka et al. 2010), our recent study was the first to link α6-integrin with AhR ligand-mediated suppression of TIC proliferation (Brantley, Callero et al. 2016). Thus far, factors that contribute to TIC survival in TamR cancers have not been fully elucidated.

Though endocrine therapy resistance has been associated with elevated expression of AhR target genes cytochrome P450s 1A1 and 1B1, elevated expression of these genes did not mediate resistance to endocrine therapy agent fulvestrant (Brockdorff, Skouv et al. 2000). Interestingly, fulvestrant induces AhR signaling to suggest cross-talk interactions occur between ER and AhR signaling pathways. McDonnell and colleagues previously demonstrated the ability of 4-hydroxy-tamoxifen (4OHTam), an active tamoxifen metabolite, to induce AhR target genes in the absence of estrogen (DuSell, Nelson et al. 2010). Safe and colleagues previously reported that AhR agonists, in certain contexts, block estradiol-mediated mammary tumor growth via AhR-ER crosstalk mechanisms (Safe and McDougal 2002). In addition, small molecules that activate AhR signaling were found to inhibit cancer cell invasion and metastases in breast cancer cells including basal-like subtypes known to resist endocrine therapy (Hall, Barhoover et al. 2010, Jin, Lee et al. 2014). Moreover, AhR ligand Aminoflavone demonstrates the potential to activate AhR signaling yet demonstrates potent and selective anticancer activity in certain breast cancer cell lines and corresponding tumors (Loaiza-Pérez, Kenney et al. 2004).

The purpose of this study is to examine an association between α6-integrin expression and tamoxifen resistance and to determine whether AF demonstrates anticancer activity in TamR cells by targeting the α6-integrin-Src-Akt signaling axis. AF has undergone extensive preclinical development and has been evaluated in clinical trials

for efficacy against solid tumors. However, the ability for AF to demonstrate efficacy in TamR cells of varying molecular subtypes and the potential mechanism(s) of such anticancer actions has not been fully explored. A better understanding of the molecular targets, such as α6-integrin, that contribute to tamoxifen resistance provides an avenue to identify biomarkers useful in recognizing patients less likely to benefit from endocrine therapy.

Materials and Methods

Cell Culture and Reagents

Human Parental MCF-7 and T47D (Par MCF-7, Par T47D) and MCF-7 and T47D tamoxifen resistant (TamR MCF-7 and TamR T47D) cells are of the luminal A breast cancer subtype and were developed and maintained as previously described (Morrison, Fu et al. 2014, Fu, Jeselsohn et al. 2016). Parental MCF-7 cells were originally obtained from Dr. Marc Lippman (National Cancer Institute, Bethesda, MD) while the parental T47D (ATCC cat# HTB-133, RRID:CVCL_0553) cells were originally obtained from the American Type Culture Collection (ATCC). Luminal B ZR-75-30 (ATCC cat# CRL-1504, RRID:CVCL_1661) cells were a kind gift from Dr. Daisy De Leon (Loma Linda University Health School of Medicine, Loma Linda, CA) though they were originally obtained from ATCC. Luminal B BT-474 (ATCC cat# HTB-20, RRID:CVCL_0179) cells were obtained from the American Type Culture Collection (ATCC). All cell lines were either authenticated once tamoxifen resistance was established or using STR DNA profiling. ZR-75-30 breast cancer cells were cultured in RPMI-1640 medium containing 10% FBS (Hyclone, Logan, UT), supplemented with 2 mM glutamine and penicillin and streptomycin antibiotics (Mediatech, Herndon, VA). BT-474 cells were cultured in

ATCC Hybri-Care Medium, reconstituted in 1 L cell-culture-grade water and supplemented with 1.5 g/L sodium bicarbonate, 10% FBS and 2 mM glutamine and penicillin and streptomycin antibiotics. The α6-integrin blocking antibody GoH3 (clone NKI-GoH3) was obtained from Millipore (cat# MAB1378; Temecula, CA, RRID:AB_1121-794). 5-amino-2-(4-amino-3-fluorophenyl)-6,8-difluoro-7-methyl-4H-1-benzopyran-4-one (Aminoflavone, AF) was obtained from the "The NCI/DTP Open Chemical Repository" (http://dtp.cancer.gov, Frederick, MD) at the Frederick National Laboratory for Cancer Research. 4-hydroxy-tamoxifen (4OHTam) was obtained from Sigma-Aldrich (St. Louis, MO). Stock solutions of AF and 4OHTam were dissolved in dimethyl sulfoxide (DMSO). All stocks were stored protected from light at −20°C until use.

Determination of Cancer Cell Viability

We evaluated the ability of AF to inhibit the growth of breast cancer cells with varying degrees of sensitivity to Tamoxifen. Briefly, MCF-7 cells and T47D cells (Par and TamR), as well as BT-474 and ZR-75-30 cells were cultured in their respective media as mentioned above and plated in 96 well plates. Approximately 24 h later, cells were treated with AF (0.1 nM-10,000 nM), 4OHTam or 0.1% DMSO for 72 h for all cell lines except BT-474 and ZR-75-30 cells which received treatment for 120 h. Cytotoxicity was determined using the Alamar Blue assay as previously described elsewhere (McLean, Soto et al. 2008). Otherwise, cells were grown in suspension as mammospheres as described in accordance with the Mammosphere assay (described below), exposed to AF or 4OHTam followed by harvesting and disruption in trypsin by

thorough mixing. The resulting individual cell suspensions were transferred to a 96 well plate and the Alamar Blue Assay was performed as previously described (Brantley, Callero et al. 2016). To determine whether α6-integrin mediates responsiveness of 4OHTam in TamR cells, TamR monolayers were exposed to blocking antibody GoH3 (1 or 10 μg/ml) for 3 d (TamR MCF-7) or for 5 d (BT-474 cells) alone or in combination with either 4OHTam or AF. Cells were otherwise transfected with a pool of siRNAs against α6-integrin as described below. Cell viability was then determined as described above.

siRNA Transfection

siRNA and transfection reagents were obtained from GE Dharmacon (Lafayette, Colorado, US). Positive control siRNA (ON-TARGETplus Cyclophilin B Control Pool (Human), cat#D-001820-10-05), negative control siRNA (ON-TARGETplus Non-targeting Pool, cat# D-001810-10-05), test siRNA (ON-TARGETplus Human ITGA6 (3655) siRNA - SMARTpool, cat#L-007214-00-0005) were resuspended in RNase free water and aliquoted for short-term storage at -20^{0}C prior to use. TamR MCF-7 cells were diluted in antibiotic-free complete medium to achieve a plating density of 60-80% confluency in either 96 or 6 well plates followed by incubation at 37°C with 5% CO_2 overnight. Transfection medium was prepared according to the manufacturer's instructions. Cells were transfected with 25nM control siRNAs or 10nM ITGA6 siRNA for 24 h followed by an additional 24 h incubation in complete media. Transfection efficiency was verified using quantitative PCR (qPCR). Conditions with target mRNA

knockdown of > 80% as well as > 80 % cell viability were used in subsequent studies (**Figure 19**).

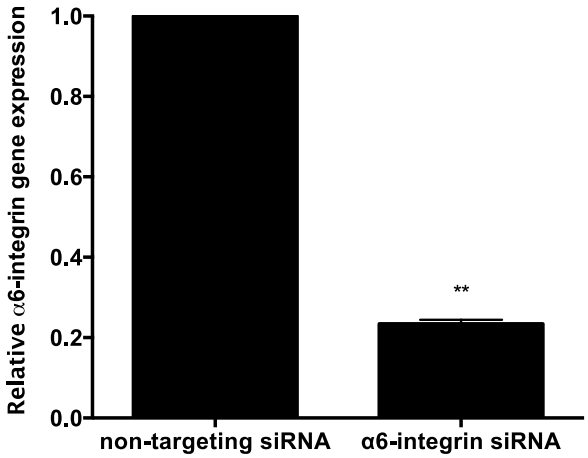


Figure 19. **Confirmation of α6-integrin silencing.** TamR MCF-7 breast cancer cells were transfected with a pool of siRNAs against α6-integrin. Efficiency of knockdown was determined using qPCR and western blotting. Silencing of greater than 75% was achieved in transfected cells. ** $P < 0.0001$, statistically significant difference as compared with non-targeting siRNA.

Mammosphere Assay

Cells were cultured in suspension as mammospheres using the MammoCult™ Human Medium Kit (Stem Cell Technologies, Vancouver, BC, Canada). Mammospheres were cultured for 5 days in Falcon 6-well non-treated polystyrene plates (product# 351146) before being exposed to respective treatments. Mammospheres were visualized using an IX-71 Olympus microscope (relief contrast mode) and pictures taken before and after treatment. Additionally, mammospheres were counted manually or collected and prepared for Alamar Blue™, semi-quantitative or qPCR analyses as described previously (Brantley, Callero et al. 2016).

RNA Extraction, Semi-quantitative RT-PCR, and qPCR Analyses

Total RNA was isolated from Par MCF-7, TamR MCF-7, BT-474, and ZR-75-30 cells (grown in monolayers) or as Par MCF-7, TamR MCF-7, ZR-75-30 and BT-474 mammospheres using either the Quick-RNA MiniPrep Kit (Zymo Research, Irvine, CA, USA) or miRNeasy Mini Kit (Qiagen, Germantown, MD, USA) in accordance with the manufacturers' instructions. cDNA was prepared using an iScript Advanced cDNA synthesis kit (BioRad, Richmond, CA). Semi-quantitative PCR was conducted as detailed elsewhere (van Riggelen, Buchwalter et al. 2005) to determine the relative expression of α6-integrin variant A (875 bp) and variant B (745 bp) in mammospheres. Primers used for semi-quantitative PCR have been described elsewhere and were as follows: α6-integrin- Forward: 5'-CTA ACG GAG TCT CAC AAC TC-3', Reverse: 5'-AGT TAA AAC TGT AGG TTC G-3' and GAPDH: 460 bp**,** Forward: 5'-TGG ATA TTG TTG CCA TCA ATG ACC-3' and Reverse: 5'-GAT GGC ATG GAC TGT GGT CAT G-3' (Dydensborg, Teller et al. 2009). QPCR analysis was also performed using a CFX-96 PCR instrument (Bio-Rad, Hercules, CA). PCR products were obtained using the following primers from Qiagen (Germantown, MD): human ITGA6, human BAX, human GAPDH, and human RPLP0.

Western Blot Analysis

Cells were seeded at 3-4x10^6 cells per plate (100 mm) and allowed to attach. Cells were then serum starved for approximately 24 h before treatment with 1 μM AF or 0.1% DMSO for 8, 24 or 48h. In some instances, cells were treated with GoH3 blocking antibody. Following treatment, the cells were harvested on ice by scraping, washed twice

with cold PBS before adding CelLytic™ M lysis buffer (Sigma, St. Louis, MO) supplemented with protease and phosphatase inhibitors. Protein concentration was determined using the BCA™ Protein Assay Kit (Prod#23250, ThermoScientific, Rockford, IL), according to the manufacturer's instructions. For Western blot analysis, proteins were resolved on 4–12% NuPage® Bis-Tris Mini Gels at a constant voltage of 200V. Gels were then blotted onto PVDF membranes using the iBlot® 7-Minute Blotting System (ThermoScientific, Rockford, IL). The membranes were blocked for 1h in blocking buffer consisting of 5% non-fat dry milk in 1X TBST at room temperature. The membranes were then incubated with primary antibody overnight at 4^0C with gentle rocking. The primary antibodies used were phospho-Src (Tyr527) (Cell Signaling Technology [CST] cat#2105, RRID:AB_10829463), phospho-Akt (Ser473) (CST cat#9271, RRID:AB_329825), phospho-Akt (Thr308) (CST cat#9275, RRID:AB_32928), Integrin α6 (CST cat#3750, RRID:AB_2249263), total Akt (CST cat#9272, RRID:AB_329827), Src (36D10) Rabbit mAb (CST cat#2109, RRID:AB_2106059) purchased from Cell Signaling Technology (Danvers, MA). Monoclonal anti-β-actin antibody (cat#A2228, RRID:AB_476697) was purchased from Sigma-Aldrich. Membranes were incubated with an ant-rabbit IgG, HRP-linked secondary antibody (CST cat#7074, RRID:AB_2099233) from Cell Signaling Technology or goat anti-mouse IgG-HRP (cat# sc-2005) from Santa Cruz Biotechnology (Dallas, Texas) for 1 h at room temperature. Protein detection was then done using the SuperSignal West Dura Extended Duration Substrate enhanced chemiluminescence detection system (ThermoFisher Scientific, Rockford, IL).

Tumor Specimens & Immunohistochemistry

Fourteen breast tumor specimens were retrieved from patients who relapsed on endocrine therapy in accordance with an IRB approved protocol from the Loma Linda University ethics committee. Three of the patients experienced relapse following treatment with Tamoxifen. All patients provided informed consent. Formalin-fixed paraffin embedded (FFPE) tissues were cut into 4μm sections and α6-integrin expression was detected using an EXPOSE Mouse and Rabbit-specific HRP/DAB detection IHC kit (Abcam, Cambridge, MA) in accordance with manufacturer's recommendations. FFPE cancer tissue sections were deparaffinized by baking overnight at 56^0C, followed by xylene treatment. Tissue sections were then immediately rehydrated in graded concentrations (100% to 70%) of ethanol. Antigen retrieval was then performed via microwaving in citrate buffer (6.0 pH) for 10 minutes. Endogenous peroxidase activity was blocked via the application of a Hydrogen Peroxide Block. Non-specific staining was also blocked using a Protein Block. This was followed by overnight incubation with a rabbit polyclonal antibody to α6-integrin (ab133386, Abcam; Cambridge, MA). Thereafter, the sections were exposed to a Mouse Specifying Reagent and a Goat anti-rabbit HRP conjugate for 15 minutes and 1h respectively. Tissue sections were then stained using a DAB Chromogen and Substrate mixture, followed by counterstaining with hematoxylin. Positive and negative controls included normal lymph node tissue sections (ab4350, Abcam) and thyroid carcinoma tissue sections, known to express our target α6-integrin, incubated with or without primary antibody respectively (data not shown). Stained tissue sections were visualized via light microscopy. A pathologist

(LD) blinded to the sample identity manually quantified all stains. Stains were scored as 1 (weak), 2 (moderate) or 3 (strong) to describe relative α6-integrin expression.

Molecular and Histological Assessment of Tumor Subtypes

Using RNA sequencing data derived from The Cancer Genome Atlas (TCGA) (Cancer Genome Atlas 2012)[RRID:SCR_003193], we evaluated α6-integrin expression in patient tumors stratified based on molecular subtypes, which were determined by the Pam 50 gene set. The molecular subtypes include: basal-like, luminal A, luminal B and Her2 enriched. In brief, these subtypes are defined based on the expression levels of specific hormone receptors (Estrogen Receptor (ER), Progesterone Receptor (PR) and v-erb-b2 erythroblastic leukemia viral oncogene homolog 2 (ERBB2 or HER2). The presence of ER defines the Luminal subtypes and the absence of HER2 amplification distinguishes Luminal A from Luminal B. The absence of all three receptors in tumors further characterized with EGFR and ck5/6 expression, are selected as 'Basal-like'.

Statistical Analysis

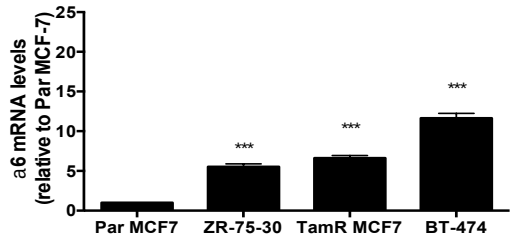
Differences between groups were analyzed using one-way ANOVA with Tukey's test or the Tukey–Kramer multiple comparison tests for evaluating three or more groups. To compare two groups, the unpaired Student's *t*-test with Welch's correction was used. Statistical significance was determined using two-way ANOVA. Statistical analysis was performed using GraphPad Prism 4.0, Graph Pad software, Inc. San Diego, California, USA, www.graphpad.com. Differences were considered significant at $p < 0.05$.

Results

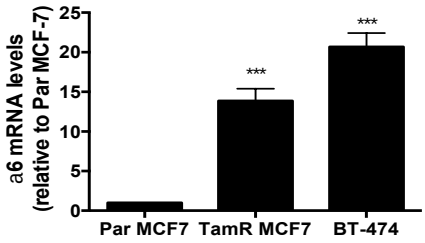
Elevated Levels of α6-Integrin are Found in Cells and Patient Tumors that Are TamR

Overexpression of α6-integrin has been shown to promote breast cancer resistance to radiotherapy (Hu, Zhou et al. 2016). To determine whether α6-integrin expression is associated with tamoxifen resistance in ER^+ breast cancer, we measured the expression of α6-integrin in a panel of tamoxifen resistant breast cancer cells including TamR MCF-7, BT-474 and ZR-75-30 cells in comparison to Par MCF-7 cells. We found that basal α6-integrin mRNA levels were significantly elevated in these cells compared to Par MCF-7 cells (**Figure 20A**). Furthermore, α6-integrin expression levels were higher in TamR MCF-7 and BT-474 mammospheres compared to Par MCF-7 mammospheres (**Figure 20B**). We also found elevated α6-integrin protein expression levels among TamR MCF-7, BT-474 and ZR-75-30 breast cancer cells compared to Par MCF-7 cells (**Figure 20C**). Immunohistochemistry data from a representative patient revealed that treatment naïve tumor tissue sections stained positive for α6-integrin expression. However, once patients relapse on Tamoxifen, α6-integrin expression intensifies (**Figure 20D**). Positive staining was also evident among tissue sections taken from bone metastases (data not shown). Furthermore, α6-integrin expression levels were significantly higher in tumor samples of the basal-like molecular subtype than the luminal A, luminal B or Her2 enriched subtypes (**Figure 20E**) and basal-like tumors are known to exhibit resistance to tamoxifen. Taken together, our data suggest that α6-integrin overexpression is associated with Tamoxifen resistance.

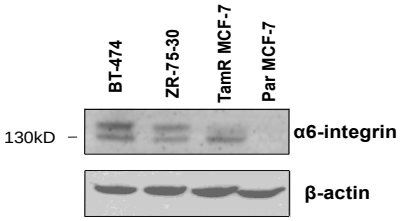
A


a6 mRNA levels (relative to Par MCF-7)
25
20
15
10
5
0
***
***
***
Par MCF7
ZR-75-30
TamR MCF7
BT-474

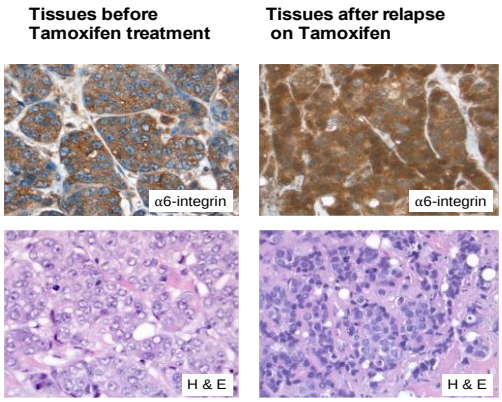
B


a6 mRNA levels (relative to Par MCF-7)
25
20
15
10
5
0
***
***
Par MCF7
TamR MCF7
BT-474

C


BT-474
ZR-75-30
TamR MCF-7
Par MCF-7
130kD
α6-integrin
β-actin

D


Tissues before Tamoxifen treatment
Tissues after relapse on Tamoxifen
α6-integrin
α6-integrin
H & E
H & E

E

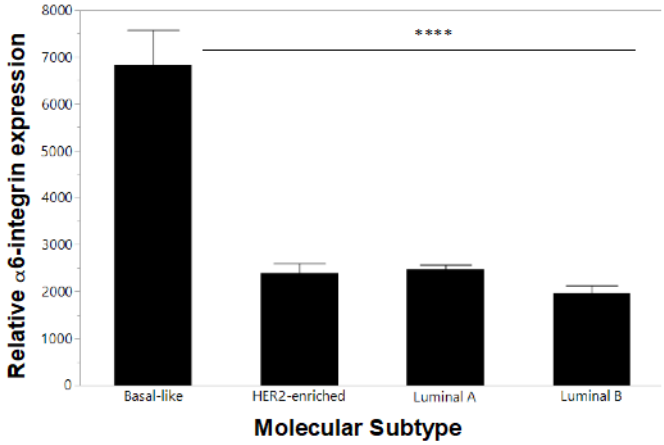

Relative α6-integrin expression
8000
7000
6000
5000
4000
3000
2000
1000
0
****
Basal-like
HER2-enriched
Luminal A
Luminal B
Molecular Subtype

Figure 20. **α6-integrin expression in Tamoxifen-resistant breast cancer cells and breast tumor tissues**. (A) Endogenous α6-integrin mRNA expression was evaluated in Par MCF-7, TamR MCF-7, ZR-75-30 and BT-474 cells and (B) in Par MCF-7, TamR MCF-7 and BT-474 mammospheres. Data represent the mean of at least 3 independent experiments. Bars, SEM. Significantly different at ***P < 0.001 in comparison to Par MCF-7 cells or mammospheres. (C) Western blot revealing relative α6-integrin protein expression in Par MCF-7, TamR MCF-7, ZR-75-30 and BT-474 cells. (D) Representative α6-integrin IHC stains for treatment naïve patient tumor tissues (left) and patient tumor tissues following relapse on tamoxifen (right). Magnification 40X. (E) Bar graph depicting α6-integrin mRNA expression levels (Pam50 gene set) from different breast tumor types derived from the TCGA database. Bars, SD. Significantly different as denoted **** P < 0.0001 when comparing basal-like subtypes with luminal A, luminal B and Her2-amplified subtypes.

Aminoflavone Inhibits ER$^+$ TamR Cell Proliferation and Disrupts ER$^+$ TamR Mammospheres

We previously showed that AFP464 (AF pro-drug) and AF disrupt mammospheres derived from *in vitro* and *in vivo* models via α6-integrin suppression (Brantley, Callero et al. 2016). Therefore, we sought to determine whether AF inhibits the proliferation of TamR cells and disrupts TamR mammospheres. Interestingly, the luminal A T47D cells (both Par and TamR) and to a lesser extent luminal A, MCF-7 cells (both Par and TamR) exhibited a biphasic dose response following treatment with AF while this effect was not apparent in the luminal B ZR-75-30 or BT-474 cells (**Figure 21A**). With the exception of the TamR T47D cells (IC_{50} ~ 1 μM), all cells demonstrated responsiveness to AF at sub-micromolar concentrations, with TamR MCF-7 cells showing the most sensitivity to AF (**Figure 21A**). In support of other studies indicating the tendency for Her2/neu enriched cells to resist tamoxifen (Chen, Wang et al. 2008), we found that BT-474 and ZR-75-30 cells were unresponsive to tamoxifen (data not shown). Notably, TamR MCF-7 cells were not only insensitive to tamoxifen but demonstrated an increase in viability following tamoxifen exposure, while AF treatment prevented tamoxifen-induced TamR cell proliferation as seen by increased cell viability (**Figure 21B**). In keeping with our observations, it has been reported that ER$^+$ tumors that have acquired resistance to tamoxifen often demonstrate tamoxifen-stimulated proliferation while retaining ER expression (Chang and Fan 2013). AF helped to restore sensitivity to tamoxifen in TamR MCF-7 and BT-474 cells (**Figure 20 B,C**). We previously demonstrated that AF impedes mammosphere formation in MCF-7 cells sensitive to tamoxifen (Brantley, Callero et al. 2016). In the current study, we found that AF

disrupted mammospheres derived from TamR MCF-7, BT-474 and ZR-75-30 cells (**Figure 21D**). AF was also able to reduce the number of mammospheres formed by the TamR MCF-7 cells (**Figure 21E**). Due to size differences between untreated mammospheres and fragmented, AF exposed mammospheres, manual count appeared to show an increase in the number of AF exposed BT-474 mammospheres compared to control (data not shown). An accurate count on ZR-75-30 mammospheres was not readily achievable as these cells, at best, formed very loose mammospheres and were completely disrupted following AF treatment. Thus, determining actual mammosphere number was not readily feasible. However, using the Alamar Blue^{TM} assay, we found AF reduced cell viability of TamR MCF-7, ZR-75-30 and BT-474 mammospheres (**Figure 21F**). Our data suggest that AF inhibits TamR cell viability, impedes tamoxifen-induced TamR MCF-7 cell proliferation and disrupts TamR mammospheres.

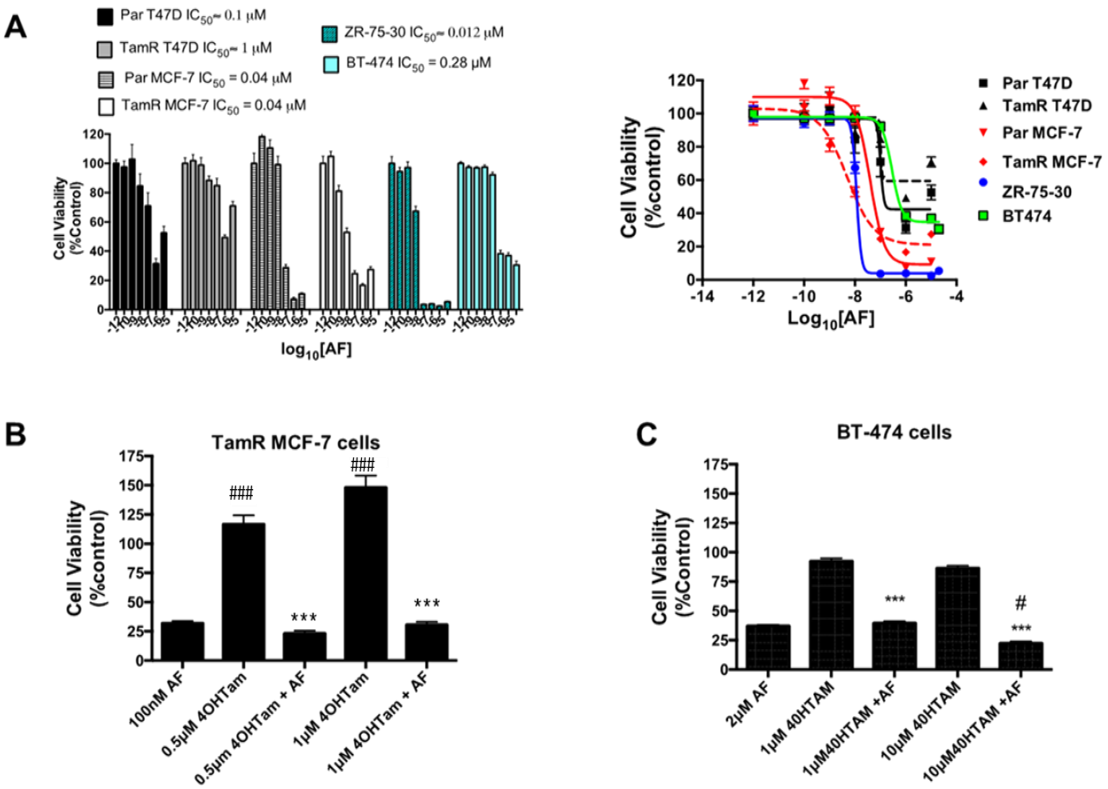

A
Par T47D IC50~ 0.1 µM
TamR T47D IC50~ 1 µM
Par MCF-7 IC50 = 0.04 µM
TamR MCF-7 IC50 = 0.04 µM
ZR-75-30 IC50~ 0.012 µM
BT-474 IC50 = 0.28 µM
Cell Viability (%Control)
log10[AF]
Par T47D
TamR T47D
Par MCF-7
TamR MCF-7
ZR-75-30
BT474
Cell Viability (%control)
Log10[AF]
B
TamR MCF-7 cells
Cell Viability (%control)
100nM AF
0.5µM 4OHTam
0.5µm 4OHTam + AF
1µM 4OHTam
1µM 4OHTam + AF
C
BT-474 cells
Cell Viability (%Control)
2µM AF
1µM 40HTAM
1µM40HTAM +AF
10µM 40HTAM
10µM40HTAM +AF
D
TamR MCF-7
BT474
ZR-75-30
CTL
AF
E
Mammosphere Count
CTL
AF
F
TamR MCF-7 mammospheres
ZR-75-30 mammospheres
BT474 mammospheres
% Mammosphere Cell Viability
CTL
AF

Figure 21. **Determination of AF-mediated anticancer activity in Tamoxifen-resistant breast cancer cells and mammospheres.** (**A**) Parental MCF-7 (Par MCF-7), Parental T47D (Par T47D), TamR MCF-7, TamR T47D, BT-474 and ZR-75-30 cells were exposed to AF (0.1-10000 nM) up to 5 d before analysis via the Alamar Blue™ assay in accordance with Materials and Methods. Data represent the mean of at least 4 independent experiments using at least quadruplicate samples for each concentration (**B**) TamR MCF-7 cells were exposed to AF, 4-hydroxytamoxifen(4OHTam) or AF and 4OHTam in combination before using the Alamar Blue™ assay as described in detail in Materials and Methods. Statistically significant at $^{###}P < 0.001$ in comparison to control (0.1% DMSO) or ***P < 0.001 in comparison to 4OHTam alone. (**C**) BT-474 cells were exposed to AF, 4OHTam or AF and 4OHTam in combination before using the Alamar Blue™ assay as described in detail in Materials and Methods. Statistically significant at ***P < 0.001 in comparison to cells treated with 4OHTam alone. (**D**) Mammospheres derived from TamR MCF-7, BT-474 and ZR-75-30 cells were treated with 0.1% DMSO (control, CTL) or AF in accordance with Materials and Methods before imaging using relief contrast microscopy. Scale bar = 50 μm. (**E**) TamR MCF-7 mammospheres were treated with CTL or AF (1 μM, 48h) and then counted in accordance with Materials and Methods. (**F**) The cell viability of mammospheres derived from TamR cells was determined following treatment with CTL or AF (2 μM for BT-474 cells, 1 μM for TamR MCF-7 cells and 100 nM for ZR-75-30 cells) for 48 h. Viability was determined in accordance with Materials and Methods.

Blocking α6-Integrin Expression and Function Inhibits 4OHTam-induced TamR Cell Proliferation and Enhances the Anticancer Efficacy of AF

We previously revealed that cells that substantially overexpress α6-integrin are rescued from the cytotoxic effects of AF (Brantley, Callero et al. 2016). To determine whether α6-integrin contributes to driving the resistance phenotype in TamR cells, we used a functional blocking antibody in select studies. In addition, we used a pool of siRNAs against α6-integrin. We used 100 nM AF rather than 1 μM due to the longer incubations times and to better determine whether AF in combination with other treatments would lead to an enhancement in anticancer activity as compared to AF alone. Blocking antibody GoH3 enhanced the anticancer activity of tamoxifen and AF in Par MCF-7 cells and in TamR cells (**Figure 22A-C**). Suppressing α6-integrin's function or silencing α6-integrin reduced the cell viability of TamR cells, prevented the 4OHTam-induced proliferation, and enhanced responsiveness of these cells to 4OHTam (**Figure 22B,D**). As expected, the effects on cell proliferation were a bit more pronounced with AF and siRNA against α6-integrin as compared to the blocking antibody since the blocking antibody is unable to negate the downstream effects (e.g., cell proliferation) while AF and α6-integrin siRNA are able to. Furthermore, blocking both the function and expression of α6-integrin enhanced the cytotoxic effects of AF against TamR cells (**Figure 22B,D**). Notably, the TamR MCF-7 cells were more responsive to the GoH3 treatment alone compared to the Par MCF-7 cells suggesting greater reliance on α6-integrin by these resistant cells for survival. These data suggest α6-integrin is important in the survival of TamR cells, particularly tamoxifen-induced cell proliferation, and contributes to AF-mediated anticancer actions.

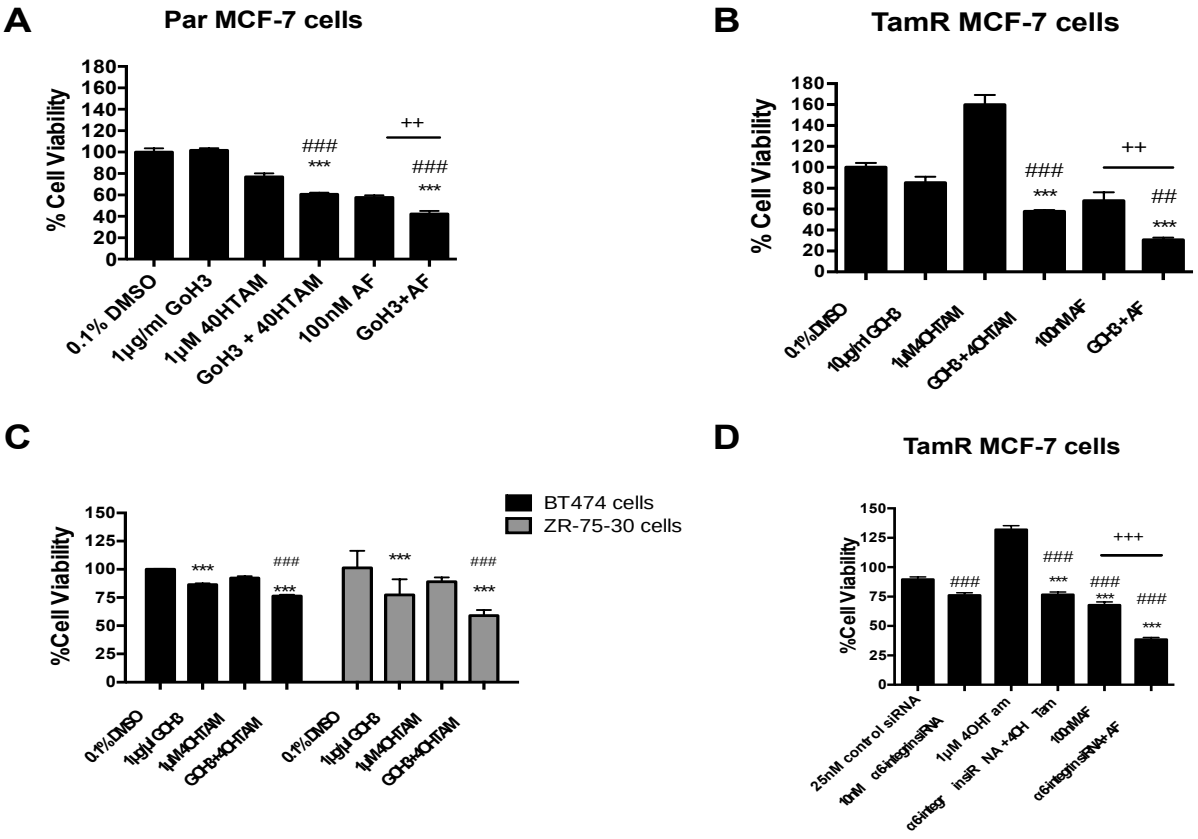


Figure 22. **Impact of AF and α6-integrin suppression on the responsiveness of breast cancer cells to Tamoxifen.** (**A**) Par MCF-7 cells were treated with DMSO (control), 4OHTam, α6-integrin blocking antibody GoH3, AF or GoH3 in combination with 4OHTam or AF before cell viability was assessed using the Alamar Blue™ assay as described in Materials and Methods. Statistically significant at ###P < 0.001 in comparison to DMSO (control) exposed. Statistically significant at ***P < 0.001 in comparison to 4OHTam alone and statistically significant at ++ P = 0.002, where indicated. (**B**) TamR MCF-7 cells were exposed to 4OHTam**,** α6-integrin blocking antibody GoH3, AF or GoH3 in combination with 4OHTam or AF before cell viability was assessed using the Alamar Blue™ assay as described in Materials and Methods. Statistically significant at ###P < 0.001 or ##P < 0.01 in comparison to 0.1% DMSO (control) exposed. Statistically significant at ***P < 0.001 in comparison to 4OHTam alone. Statistically significant at ++ P = 0.01, where indicated. (**C**) BT-474 and ZR-75-30 cells were exposed to GoH3, 4OHTam or the combination for up to 5 days before the Alamar Blue™ assay was used in accordance with Materials and Methods. Statistically significant at ***P < 0.001 in comparison to DMSO (control) exposed cells. Bars, SEM. Statistically significant at ### P < 0.001 in comparison to 4OHTam alone. (**D**) TamR MCF-7 cells were transfected with a pool of siRNAs against α6-integrin or non-targeting siRNAs. Transfected cells were exposed to 4OHTam or AF alone. Cell viability was determined using the Alamar Blue™ assay as described in the Materials and Methods. Statistically significant at ###P < 0.001 in comparison to DMSO (control) exposed. Statistically significant at ***P < 0.001 in comparison to 4OHTam alone. Statistically significant at +++P < 0.001, where indicated.

Aminoflavone Inhibits α6-Integrin Expression, α6-Integrin-Src-Akt Signaling Activation and Induces BAX Expression in TamR Cells

We found that AF reduced the expression of both cytoplasmic variants of α6-integrin (α6A and α6B) in TamR MCF-7 mammospheres (**Figure 23A**). AF also reduced α6-integrin gene expression in TamR MCF-7 mammospheres (**Figure 23B**). AF treatment was also found to significantly reduce α6-integrin expression in TamR MCF-7 and BT-474 cells (**Figure 23C**). However, AF was unable to inhibit α6-integrin expression in ZR-75-30 cells, despite their sensitivity to this agent (data not shown) which suggests that ZR-75-30 cells demonstrate sensitivity to AF via α6-integrin-independent mechanisms. It is interesting to note that ZR-75-30 cells lack progesterone receptor (PR) expression while BT-474 cells express the PR and this may account for some of the differences seen in viability and α6-integrin expression inhibition in these cells following AF treatment. AF decreased α6-integrin protein expression in TamR MCF-7 breast cancer cells (**Figure 23D**).

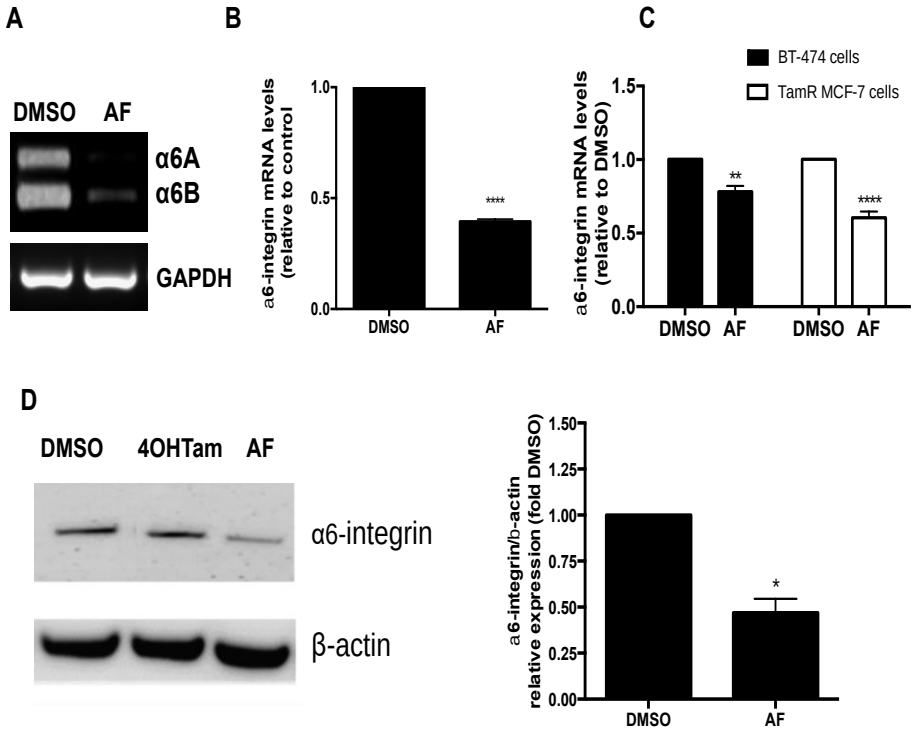


Figure 23. **AF suppresses α6-integrin expression in Tamoxifen-resistant breast cancer cells.** (**A**) Semi-quantitative PCR analysis was performed to evaluate the expression of A and B isoform variants of α6-integrin in TamR MCF-7 mammospheres exposed to CTL (0.1% DMSO) or 1 μM AF for 48h. (**B**) Tam MCF-7 mammospheres were treated with 0.1% DMSO or 1 μM AF for 48h before qPCR analyses were performed to evaluate α6-integrin expression. Data represent the mean of at least 5 independent experiments performed in quadruplicate. Bars, SEM. Statistically significant at ****P < 0.0001 in comparison to 0.1% DMSO. (**C**) BT-474 and TamR MCF-7 cells were exposed to 0.1% DMSO or 2 μM AF for 120h and CTL 0.1% DMSO or 1 μM AF for 48h respectively before qPCR analyses were performed to evaluate α6-integrin expression. Data represent the mean of at least 5 independent experiments performed in quadruplicate. Bars, SEM. Statistically significant at **P < 0.01 or ****P < 0.0001 in comparison to 0.1% DMSO. (**D**) TamR MCF-7 cells were treated with 0.1% DMSO, 1 μM 4OHTam or 1 μM AF for 48 h before being analyzed for α6-integrin protein expression using Western blotting in accordance with Materials and Methods. Bars, SEM. Statistically significant at *P < 0.05 in comparison to DMSO.

α6-integrin signaling events that are crucial in cancer progression include α6-FAK/Src activation of the PI3K-Akt pathway (Kim, Choi et al. 2009). To assess whether down-regulation of α6-integrin lead to a reduction in Src and Akt signaling, we assessed levels of phosphorylated Src (p-Src) and Akt (p-AKT). AF caused an increase in pAkt (ser 473) that was inhibited by the α6-integrin blocking antibody GoH3 (**Figure 24A**). We observed a more pronounced increase in pAkt (ser 473) expression in Par MCF-7 cells following AF treatment (data not shown) that is consistent with a previous study using MCF-7 cells (Meng, Kohn et al. 2007). GoH3 treatment caused no appreciable change in pAkt (ser 473) phosphorylation at either time point in TamR MCF-7 cells (**Figure 24A**). Both AF and GoH3 reduced pAkt (thr 308) levels in TamR MCF-7 cells at both time points while GoH3 enhanced the ability of AF to reduce pAkt (thr 308) activation after 24 h of treatment (**Figure 24B**). AF and GoH3 increased phosphorylation at the Src inactivation site, Tyr527, in TamR MCF-7 cells as early as 8h (**Figure 24C**). This phosphorylation was sustained up to 24 h of treatment (**Figure 24C**), though GoH3 was unable to enhance AF-mediated inhibition of Src signaling at either time point causing a paradoxical decrease after 24 h of combined treatment (**Figure 24C**). Taken together, AF caused a net decrease in Akt and Src signaling activation.

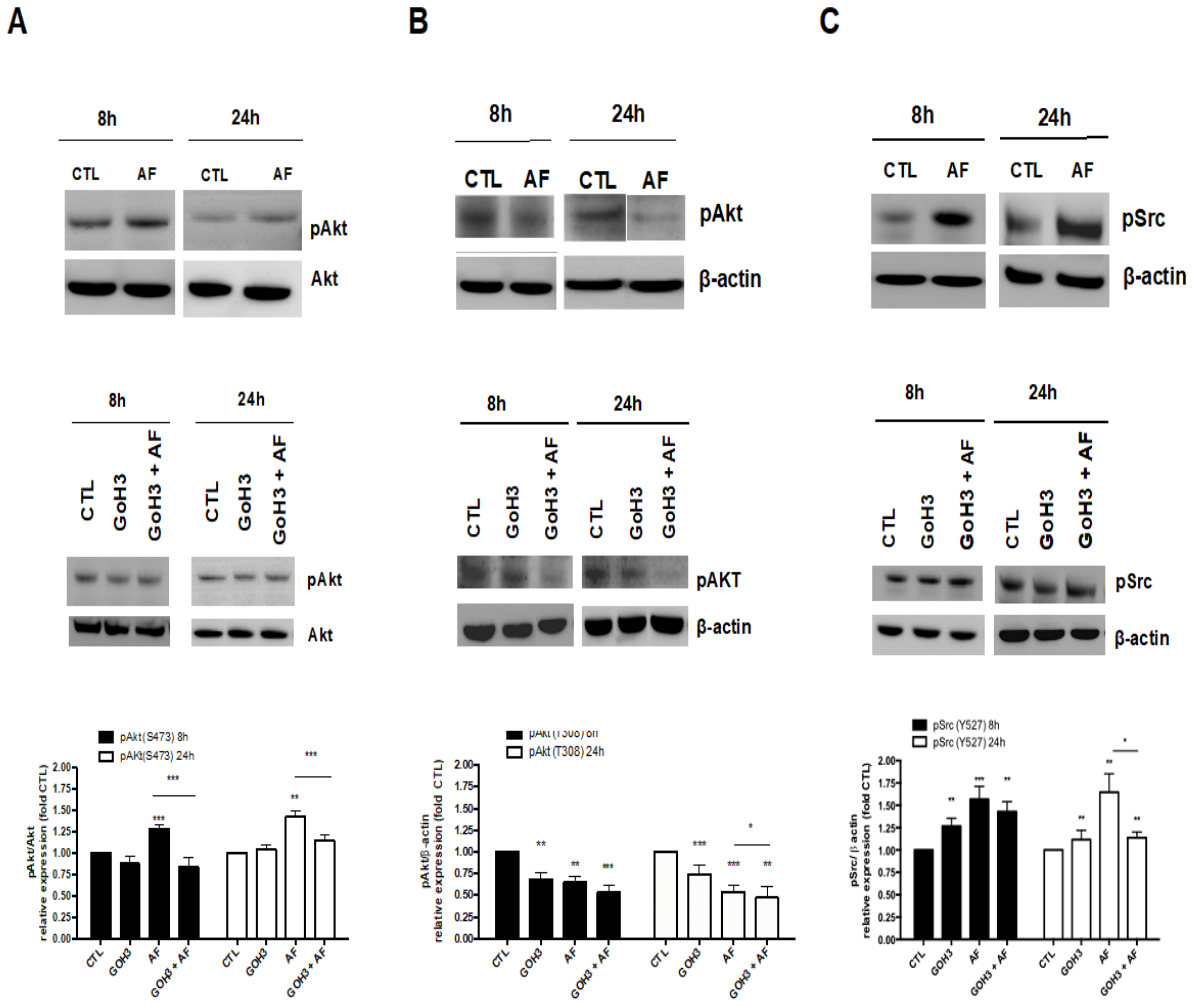


Figure 24. **AF modulates Akt and Src signaling in Tamoxifen-resistant breast cancer cells.** **(A-B)** TamR MCF-7 cells were exposed to media only or media containing 0.01% DMSO (CTL), 1 μM AF, 1μg/ml GoH3, or AF + GoH3 in combination for 8 and 24 h before Akt phosphorylation was assessed using Western blot analyses in accordance with Materials and Methods. **(C)** TamR MCF-7 cells were exposed to media only or media containing 0.01% DMSO (CTL), 1 μM AF, 1μg/ml GoH3, or AF + GoH3 in combination for 8 and 24 h before Src phosphorylation was assessed using Western blot analyses in accordance with Materials and Methods. Data represent the mean of at least 3 independent experiments. Bars, SEM. Statistically significant at *P < 0.05, **P < 0.01 or ***P < 0.001 in comparison to CTL or where indicated.

Integrin-mediated cell survival has been linked to the regulation of the pro-apoptotic gene BAX and integrin signaling appears to block BAX-induced apoptosis by preventing BAX translocation to the mitochondria (Gilmore, Metcalfe et al. 2000).We previously demonstrated the ability of AF to induce apoptosis in sensitive breast cancer cells as evidenced by PARP cleavage and caspase 9 activation (McLean, Soto et al. 2008). We therefore evaluated the expression of BAX following AF treatment in Par and TamR MCF-7 cells. We found that AF significantly increased BAX expression in both Par and TamR MCF-7 cells (**Figure 25A-B**). Our data suggest that AF inhibits Src and Akt signaling activation to initiate TamR cell death via BAX induction and to suppress TamR cell proliferation (**Figure 26**).

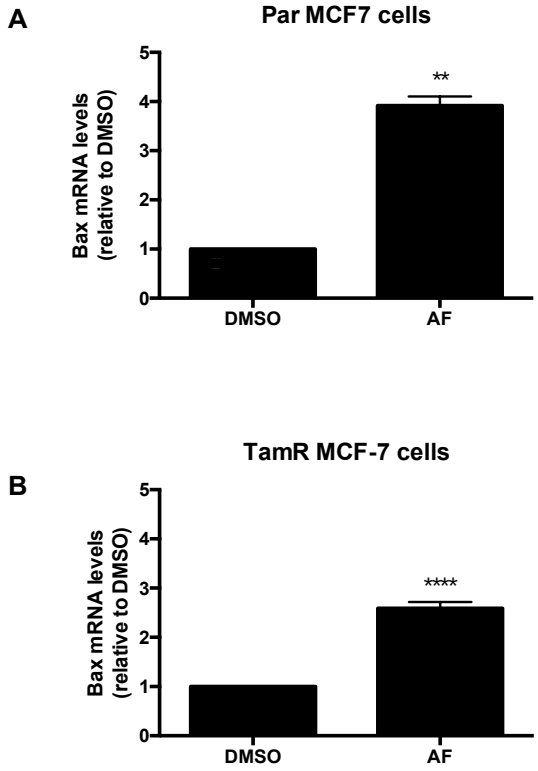


Figure 25. **AF induces the expression of pro-apoptotic gene Bax in Tamoxifen-sensitive and Tamoxifen-resistant breast cancer cells.** (**A**) Par and (**B**) TamR MCF-7 cells were exposed to CTL or 1 μM AF for 48 h before qPCR analysis was employed to detect Bax mRNA expression. Data represent the mean of at least 3 independent experiments. Bars, SEM. Statistically significant at **P < 0.01 or ****P < 0.0001 in comparison to DMSO.

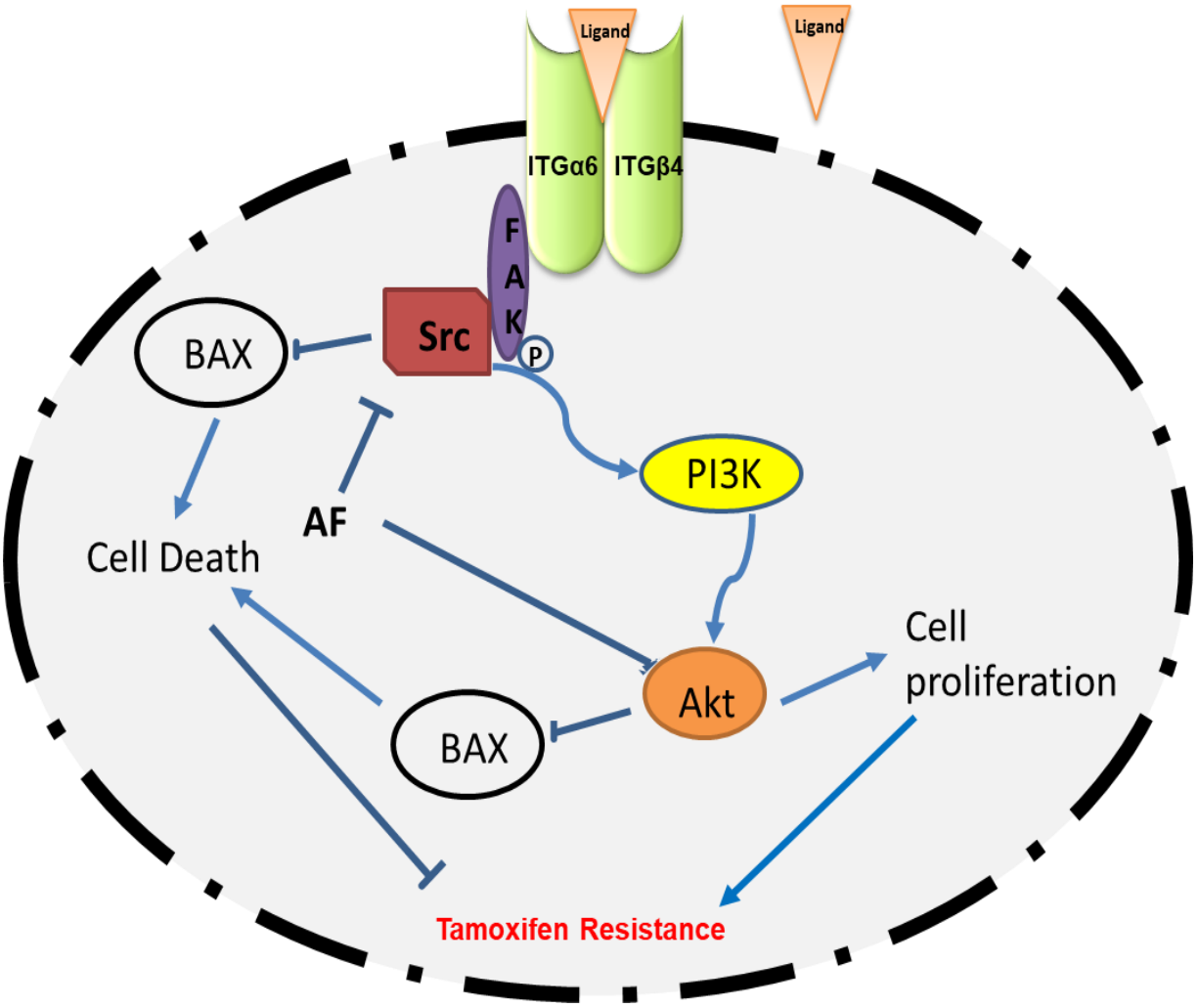


Figure 26. **Schematic depiction of proposed mechanism by which AF confers anticancer actions in TamR breast cancer cells.** Ligands such as laminin bind to the α6/β4 integrin heterodimer to stimulate FAK/Src activation. This activation in turn stimulates cell-survival pathways such as the PI3K/Akt pathway, which increases cell proliferation and inhibits cell death to promote tamoxifen resistance. On the contrary, AF inhibits α6 integrin/Src/Akt signaling to overcome resistance.

Discussion

Tamoxifen resistance frequently leads to relapse, metastases and death. It is therefore imperative to develop effective therapeutic agents to combat tamoxifen resistance. In this study, we discovered that AhR ligand AF inhibits the proliferation of TamR cells at least in part by reducing α6-integrin expression and inhibiting activation of down-stream Src and Akt signaling pathways. Our findings and that of others also suggest that elevated α6-integrin expression is linked to tamoxifen resistance.

Although AhR signaling activation has been shown to promote tumorigenesis, emerging evidence indicates that certain AhR agonists exhibit anti-invasive and anti-metastatic actions (Hall, Barhoover et al. 2010, Prud'homme, Glinka et al. 2010, Hanieh, Mohafez et al. 2016). AF selectively and potently inhibits the growth of cancer cells and tumors with no appreciable toxicity to non-malignant cells (Loaiza-Pérez, Kenney et al. 2004, McLean, Soto et al. 2008). Non-toxic AhR agonists such as AF and Tranilast behave like partial AhR agonists which often oppose the tumor promoting actions of toxic, full AhR agonists similar to AhR antagonists. Small molecule AhR antagonists have been shown to inhibit the progenitor population within TamR cells *in vitro* and *in vivo* (Dubrovska, Hartung et al. 2012).

Cells with higher levels of α6-integrin expression such as the BT-474 cells were less sensitive to the cytotoxic actions of AF and this supports our earlier observation that breast cancer cells with very high α6-integrin expression resist the cytotoxic actions of AF (**Figure. 21**) (Brantley, Callero et al. 2016). There is likely a threshold of α6-integrin expression that when exceeded, renders cells resistant to AF (Brantley, Callero et al. 2016). In the current study, TamR cells also demonstrated varying levels of sensitivity to

AF due to differences in their molecular makeup. Synergism has been reported between AF and fulvestrant, in ER^+ breast cancer cells (Shelton, Sausville et al. 2007). Importantly, fulvestrant is a standard of care agent used to treat patients who have relapsed on tamoxifen.

The ability of α6-integrin blockade to enhance AF efficacy in TamR cells suggests further benefit is plausible from combining α6-integrin blocking agents with anti-cancer AhR agonists to treat TamR breast cancer. Furthermore, tamoxifen in combination with other AhR agonists such as the selective aryl hydrocarbon receptor modulator, 6-methyl-1,3,8-trichlorodibenzofuran (6-MCDF) has previously shown remarkable efficacy in mouse models of breast cancer that show responsiveness to tamoxifen (McDougal, Wormke et al. 2001). Interestingly, 6-MCDF decreased levels of ERα through proteasomal degradation. Thus, AhR ligands have potential to demonstrate efficacy in the treatment of breast cancer including subtypes that are resistant to endocrine therapy.

ER expression does not entirely define the anticancer efficacy of AF. For instance, certain basal-like breast cancer cells such as MDA-MB-468 are highly sensitive to AF (Brinkman, Wu et al. 2014), yet treatment with histone deacetylase inhibitor vorinostat is necessary to sensitize basal-like MDA-MB-231 breast cancer cells to AF via ER reactivation (Stark, Burger et al. 2013). Responsiveness to AF appears to rely in part on the ability of this small molecule to induce AhR-mediated signaling activation and to suppress α6-integrin-mediated signaling pathways.

Our data suggest that elevated α6-integrin expression is linked to tamoxifen resistance and sustains the proliferation and survival of tamoxifen resistant cells. Notably,

AF reduced the expression of both cytoplasmic splice variants of α6-integrin (α6A and α6B) in TamR MCF-7 mammospheres (**Figure 23A**). Importantly, α6B expression defines the mesenchymal population in breast cancer that is necessary for TIC function (Goel, Gritsko et al. 2014). Our findings are consistent with previous reports that revealed elevated α6-integrin expression of more than 3-fold in patient-derived ER^+ breast cancer xenografts with acquired resistance to tamoxifen (Cottu, Bieche et al. 2014). Furthermore, α6-integrin expression was comparatively higher in mammosphere-derived cells than cells from 2D cell culture (monolayers). This finding is consistent with what we found previously (Brantley, Callero et al. 2016). Indeed, mammospheres are known to enrich for TICs (Saadin, Burke et al. 2013). Though our patient sample size was small in the IHC study (**Figure 20**), the trend toward elevated α6-integrin expression in patients who relapsed on tamoxifen was further demonstrated in basal-like tumors (tamoxifen unresponsive) in comparison to other tumor types from the TCGA database involving a much larger cohort of patients. Nonetheless, the above-mentioned findings suggest that elevated levels of α6-integrin are associated with tamoxifen resistance and α6-integrin may be valuable as a predictive biomarker of tamoxifen responsiveness.

TICs have been shown to play a key role in the development of resistance to tamoxifen (Bostner, Karlsson et al. 2013). In fact, tamoxifen treatment itself has been shown to select for cells with self-renewal capacity and promote mammosphere formation (Raffo, Berardi et al. 2013). A recent study showed that α6-integrin ligand laminin conferred resistance to tamoxifen in an estrogen-dependent, tamoxifen-sensitive LM05-E breast cancer cell line via α6-integrin (Berardi, Raffo et al. 2016). These observations support the hypothesis that tamoxifen may promote its own resistance by

up-regulating α6-integrin levels and other TIC-related pathways and genes. Tamoxifen can also act as an ER agonist in breast cancer cells to promote Tamoxifen resistance. In keeping with our observations, it has been reported that ER^+ tumors that have acquired resistance to tamoxifen may either be unresponsive to this agent or demonstrate tamoxifen stimulated growth while retaining ER expression (Chang 2012). Reduced expression of co-repressors observed in tamoxifen resistance, results in stabilization of the agonist confirmation of the ERα, thereby allowing ERα activation by tamoxifen. (Chakraborty and Biswas 2014). This may explain why tamoxifen stimulates proliferation in certain resistant cells.

Integrins have been shown to activate cell survival pathways such as PI3K to promote cancer cell proliferation and cell death via downstream FAK/Src signaling activation (Kim, Choi et al. 2009). In particular, α6-integrin primarily activates PI3K signaling to promote cancer cell migration, invasion, and survival (Lipscomb and Mercurio 2005). In the current study, we found that increased α6-integrin expression correlated with an overall increase in Src-Akt signaling since we found TamR cells exhibited not only increased α6-integrin expression, but elevations in Akt phosphorylation (**Figure 20C**, **Figure 27**). Additionally, AF effectively suppressed α6-integrin expression and this lead to an overall decrease Src-Akt signaling. Thus, Src-Akt signaling is decreased after α6-integrin expression is suppressed.

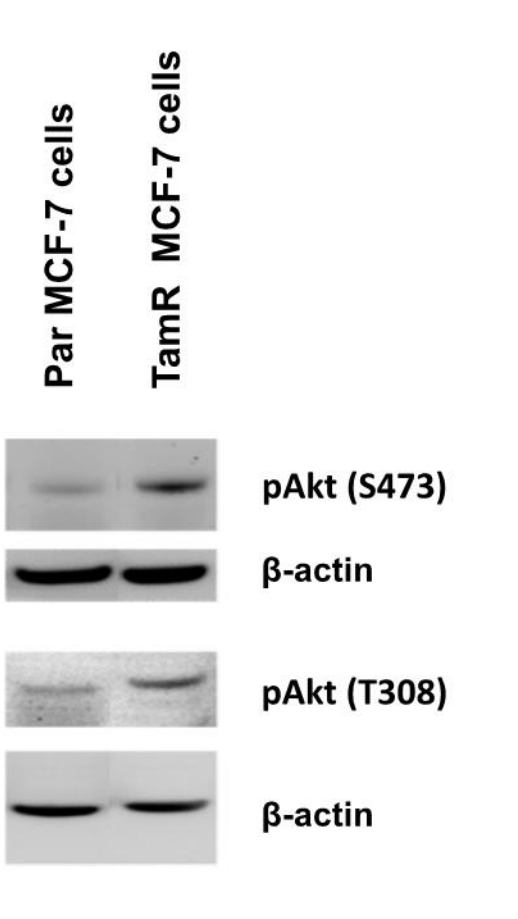


Figure 27. **Basal phosphorylation of Akt in Par and TamR MCF-7 breast cancer cells**.

AF phosphorylated Src at Tyr527 in TamR MCF-7 cells as early as 8h and this phosphorylation was sustained for at least 24h (**Figure 24C**). GoH3 also promoted this phosphorylation as well, though GoH3 combined with AF did not enhance this effect (**Figure 24C**). Phosphorylation of p-Src(Tyr527) results in Src inactivation through interaction with the SH2 domain and protein folding which makes Src inaccessible to substrates (Frame 2002). Interestingly, acquired tamoxifen resistance leads to integrin-induced FAK/Src activation; inhibition of integrin-mediated FAK/Src/Akt activation was found to produce small yet significant sensitization to tamoxifen (Cowell, Graham et al.

2006). Taken together, our findings indicate AF suppresses Src activation in TamR MCF-7 cells.

AF increased pAkt(ser473) in Par MCF-7 cells (data not shown) consistent with a previous report which showed that sub-micromolar concentrations of AF caused S phase arrest when these cells were treated up to 8 h (Meng, Kohn et al. 2007). AF increased Akt activation in Par MCF-7 cells to a greater extent than in TamR MCF-7 cells and interestingly the α6 integrin blocking antibody GoH3 inhibited AF-mediated increases in Akt activation in TamR MCF-7 cells (**Figure 24A**). We concur with Pommier and colleagues that our findings suggest that activation of Akt might reflect a cellular defense mechanism to AF-mediated DNA damage. It is, therefore, possible that this switch from Akt inactivation to activation with 1μM AF used in the current study may represent an initial apoptotic response followed by cell cycle arrest in response to DNA damage caused by more prolonged exposure. Indeed, AF induces oxidative DNA damage and S-phase arrest in triple negative MDA-MB-468 cells (McLean, Soto et al. 2008).

Phosphorylation of Thr308 in the activation loop of the kinase domain and Ser473 in the C-terminal regulatory domain is needed for full activation of Akt, with Thr308 phosphorylation playing the dominant role in Akt activation (Song, Ouyang et al. 2005, Vincent, Elder et al. 2011). Furthermore, Akt phosphorylation at these two sites occurs independently of each other (Alessi, Andjelkovic et al. 1996) with PDK1 phosphorylating Akt at Thr308 and mTORC2 phosphorylating Akt at Ser473. Therefore, since AF significantly reduced Thr308 phosphorylation, we can conclude that this AhR ligand decreased overall Akt kinase activity in TamR MCF-7 cells, an effect that was enhanced by GoH3 following 24 h of co-treatment (**Figure 24B**). AF has targets other than α6-

integrin that may contribute to its ability to inhibit Src-Akt signaling activation. For instance, β-naphthoflavone, another AhR agonist with *in vivo* anti-tumor activity, was found to inhibit PI3K/Akt signaling in MCF-7cells in an AhR-dependent manner (Wang, Xu et al. 2014). On the other hand, GoH3 specifically blocks the function of α6-integrin and thus AF and GoH3 have the potential to inhibit Src-Akt signaling by related as well as distinct mechanisms.

Activated Akt and Src resulting from integrin signaling and concomitant inhibition of pro-apoptotic BAX activity opposes cell death (Bouchard, Harnois et al. 2008, Shishido, Bonig et al. 2014). These observations support our findings that AF inhibits α6-integrin/Src/Akt signaling and induces BAX expression to promote TamR MCF-7 cell death. Additionally, AF suppresses the proliferation of TamR MCF-7 cells by suppressing Thr308 Akt phosphorylation. In our study, both Par and TamR MCF-7 cells showed increased α6-integrin/Src/Akt signaling though TamR cells exhibited this enhanced signaling to a greater extent (**Figure 20C and figure 27**). Thus, Src-Akt inhibition in TamR and Par MCF-7 cells likely occurs via similar means and the greater level of BAX induction observed in Par MCF-7 cells compared to TamR MCF-7 cells concurs with the enhanced ability of AF to suppress α6-integrin expression in these cells. It is quite plausible that when these cells are untreated, BAX translocation to the mitochondria is suppressed. We speculate that following AF treatment, α6-integrin/Src/Akt signaling becomes inhibited to enable BAX translocation irrespective of tamoxifen responsiveness. This may explain why BAX induction was observed in both cell lines after AF treatment. Taken together, our data suggest that BAX translocation is readily restored following AF-mediated α6-integrin/Src/Akt signaling blockade.

In conclusion, our data suggest AF inhibits α6-integrin-Src-Akt signaling to induce apoptosis, reduce cell proliferation and counteract tamoxifen resistance in ER^+ breast cancer cells. More in-depth studies are needed to conclusively determine whether α6-integrin plays a causal role in tamoxifen resistance as has been recently determined for TIC genes OCT-4 and SOX-9 (Bhatt, Stender et al. 2016, Jeselsohn, Cornwell et al. 2017). Our findings do suggest that AhR ligands such as AF have the potential to help combat tamoxifen resistance to ultimately improve clinical outcomes for patients who have relapsed on tamoxifen. Other AhR ligands such as anti-allergy agent Tranilast disrupt mammospheres (Prud'homme, Glinka et al. 2010). We recently determined that related AhR ligand, 5F 203 suppresses α6-integrin expression and disrupts mammospheres (data not shown). To the best of our knowledge, our report is the first to demonstrate the ability of AhR ligands to reverse tamoxifen resistance by attenuating α6-integrin-Src-Akt signaling. Our study provides a rationale for evaluating α6-integrin as a potential biomarker for tamoxifen resistance and to more appropriately stratify luminal breast cancer patients that would ultimately benefit from endocrine therapy in combination with AhR ligands such as AF.

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CHAPTER FOUR

AHR LIGAND AMINOFLAVONE REGULATES miRNA EXPRESSION IN A TAMOXIFEN RESISTANT TUMOR INITIATING CELL MODEL

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The work presented in this chapter has not been published.

Abstract

Currently, there are no published findings exploring the ability of AF to modulate miRNA expression in breast cancer and especially in a tamoxifen resistant breast cancer model. We previously found that AF demonstrates efficacy against the TIC population in tamoxifen resistant breast cancer by downregulating α6-integrin expression. Furthermore, miRNAs are key regulators of TIC growth and function. As such, we explored the ability of AF to regulate miRNA expression in an *in vitro* TIC model (mammospheres enriched for TICs) derived from tamoxifen sensitive and resistant cells. To facilitate this, we conducted miRNA-sequencing, a Next Generation Sequencing (NGS) research tool for high-throughput miRNA expression analysis. We hypothesized that certain tumor suppressor miRNAs are downregulated in tamoxifen resistant (TamR) mammospheres compared to tamoxifen sensitive mammospheres and that AF treatment would restore the expression of these tumor-suppressor miRNAs to thwart α6-integrin expression and tamoxifen resistance. Our results revealed 366 differentially expressed small non-coding RNAs (ncRNA) including miRNAs and PIWI-interacting RNAs (piRNAs) in tamoxifen resistant mammospheres relative to tamoxifen sensitive mammospheres. Furthermore, AF differentially regulated small ncRNAs in both tamoxifen sensitive and resistant mammospheres. In total, AF treatment altered the expression of 341 distinct ncRNAs in TamR mammospheres and 315 in tamoxifen sensitive mammospheres. Ingenuity Pathway Analysis (IPA) of differentially expressed miRNAs revealed five top networks with cancer-related functions that were impacted. These networks were associated with cellular development, growth, proliferation, cell death and survival. Disease and function analysis of the highest-scoring networks using the IPA showed a correlation with

invasive ductal breast carcinoma. In conclusion, AF treatment altered the miRNA expression profile in tamoxifen sensitive and resistant mammospheres. Future studies will be conducted to correlate the miRNA expression data with novel mechanisms of tamoxifen resistance, as well as, AF's anticancer activity.

Introduction

RNA molecules that are not translated into proteins are referred to as non-coding RNAs (ncRNAs). miRNAs are small ncRNAs, approximately 18-25 nucleotides in length, which account for the majority of small RNAs in somatic cells (Bertoli, Cava et al. 2015). Other small ncRNAs include small interfering RNAs (siRNAs) formed from double stranded RNA and PIWI-interacting RNAs (piRNAs) processed from retrotransposons by PIWI proteins (Chen and Heard 2013). These small ncRNAs are important regulators of gene expression and so play important functions in various biological and disease processes.

miRNAs are dysregulated in various pathologies including cancer. Both oncogenic and tumor suppressor roles have been identified for miRNAs in breast cancer. Oncogenic miRNAs block the expression of tumor suppressor genes while tumor suppressor miRNAs target genes which drive tumor growth and cancer progression(Wang and Luo 2015). Oncogenic miRNAs have been reported to drive cancer cell proliferation, invasion, metastasis and TIC function. miR-155 was shown to increase proliferation and suppress apoptosis in breast cancer cells (Zhang, Zhao et al. 2013). Furthermore, elevated levels of miR-10b were reported in metastatic breast cancer cells where it increased cell migration and invasion (Haque, Banerjee et al. 2011). miR-21 was found to be elevated in serum and tissue samples taken from patients with

metastatic breast cancer, suggesting a role for miR-21 in promoting metastasis (Li, Zhang et al. 2013). Tumor-suppressor miRNAs also play an integral role in negatively regulating various cancer promoting properties. The let-7 family of tumor-suppressor miRNAs was found to reduce TIC properties and induce a more differentiated phenotype in breast cancer (Yu, Yao et al. 2007). miR-205 is a key regulator of EMT (Gregory, Bracken et al. 2008) and reduces TIC capacity in breast cancer (Chao, Chang et al. 2014). Other miRNAs, such as the miR-30 family, were shown to inhibit the self-renewal capacity of and induce apoptosis in breast TICs, reduce metastasis (Yu, Deng et al. 2010), as well as inhibit breast cancer cell migration and invasion (Cheng, Wang et al. 2012). Several other miRNAs have been identified for their roles in regulating TIC properties including self-renewal, differentiation and migration, and cancer progression (Wang and Luo 2015, Chakraborty, Chin et al. 2016). Other miRNAs have been implicated in tamoxifen resistance (Zhang, Xu et al. 2015).

miRNAs are currently being explored for their clinical applicability as diagnostic and prognostic cancer biomarkers or as potential therapeutic targets (Kaboli, Rahmat et al. 2015). However, despite the immense potential for miRNAs in anticancer therapy, further studies are needed to better translate laboratory findings into patient care.

PIWI-interacting RNAs (piRNAs) are slightly longer than miRNAs. They are an approximate 24-32nt long sub-class of small ncRNAs that associate with the PIWI subfamily of Argonaute proteins to form a piRNA-induced silencing complex (piRISC) (Sato and Siomi 2013). piRNAs are important in maintaining genomic stability by suppressing transposons, facilitating the formation of telomere-protecting complexes, RNA silencing and chromatin regulation (Hashim, Rizzo et al. 2014). piRNAs are

generally associated with genetic and epigenetic regulation in germline cells and piRNA-mediated regulation of transposons is essential to normal gametogenesis and reproduction (Iwasaki, Siomi et al. 2015). However, there is evidence to support a role for piRNAs in pathological conditions such as breast cancer. Small RNA-sequencing data of breast cancer cell lines and patient biopsy samples identified differentially expressed piRNAs in breast carcinoma samples relative to normal samples; predicted piRNA-target mRNAs were associated with cancer-related networks including cell-to-cell signaling and interaction, cell death and survival, cell cycle, and DNA replication and repair (Hashim, Rizzo et al. 2014). Furthermore, PIWI-like protein 2 (PIWIL2) and piR-932 were found to be overexpressed in TICs relative to control cells and breast cancer patients with positive PIWIL2 expression demonstrated significantly higher occurrences of distant metastases compared to those without PIWIL2 expression (Zhang, Ren et al. 2013). In addition, a panel of piRNAs, expressed in breast cancer tissues, were found to be predictive of overall survival (OS) and Recurrence Free Survival (RFS) (Krishnan, Ghosh et al. 2016). Considering their role in maintaining genomic stability in gametogenesis and the fact that behaviors observed in gametogenesis, such as immortalization, implantation, and migration are analogous to transformation, invasion, and metastasis seen in cancer, ectopic expression of piRNAs and PIWI proteins in tumors may confer features of replicating germline stem cells to TICs thereby driving cancer progression (Moyano and Stefani 2015). Interestingly, overexpression of PIWIL1 was found to positively mediate chemotherapy resistance in cervical cancer cells, as well as increase *in vitro* tumorsphere formation and expression of stemness markers(Liu, Gao et al. 2014). Overall, piRNAs appear to play both prognostic and functional roles in cancer, with aberrant expression

being associated with poorer clinical outcomes. However, the extent of piRNA function and importance in cancer is still poorly understood thus warranting further exploration.

Overall, we demonstrated the ability of AF to modulate the expression profile of small ncRNAs, including miRNAs and piRNAs, in both a tamoxifen sensitive and resistant ER^+ TIC model.

Materials and Methods

Mammosphere Formation Assay and RNA Extraction

Cells were cultured in suspension as mammospheres using the MammoCult™ Human Medium Kit (Stem Cell Technologies, Vancouver, BC, Canada). Mammospheres were cultured for 5 days in Falcon 6-well non-treated polystyrene plates (cat# 351146) before being exposed to 1μM AF or 0.1% DMSO for an additional 2 days. After treatment, mammospheres were harvested, washed in PBS and total RNA extracted using the miRNeasy Mini Kit (cat # 217004, Qiagen, Germantown, MD, USA) in accordance with the manufacturers' instructions.

Preparation of miRNA NGS Libraries

RNA concentration and integrity were determined using the NanoDrop™ 2000 Spectrophotometer (ThermoScientific, Rockford, IL) and the Agilent 2200 TapeStation system (Santa Clara, CA, USA). Library preparation was carried out using the QIAseq miRNA Library Kit (cat # 331505, Qiagen), in accordance with the manufacturers' instructions. Briefly, adapters were ligated sequentially to the 3' and 5' ends of miRNAs. Subsequently, universal cDNA synthesis with Unique Molecular Indices (UMI) assignment, cDNA cleanup, library amplification and library cleanup were performed.

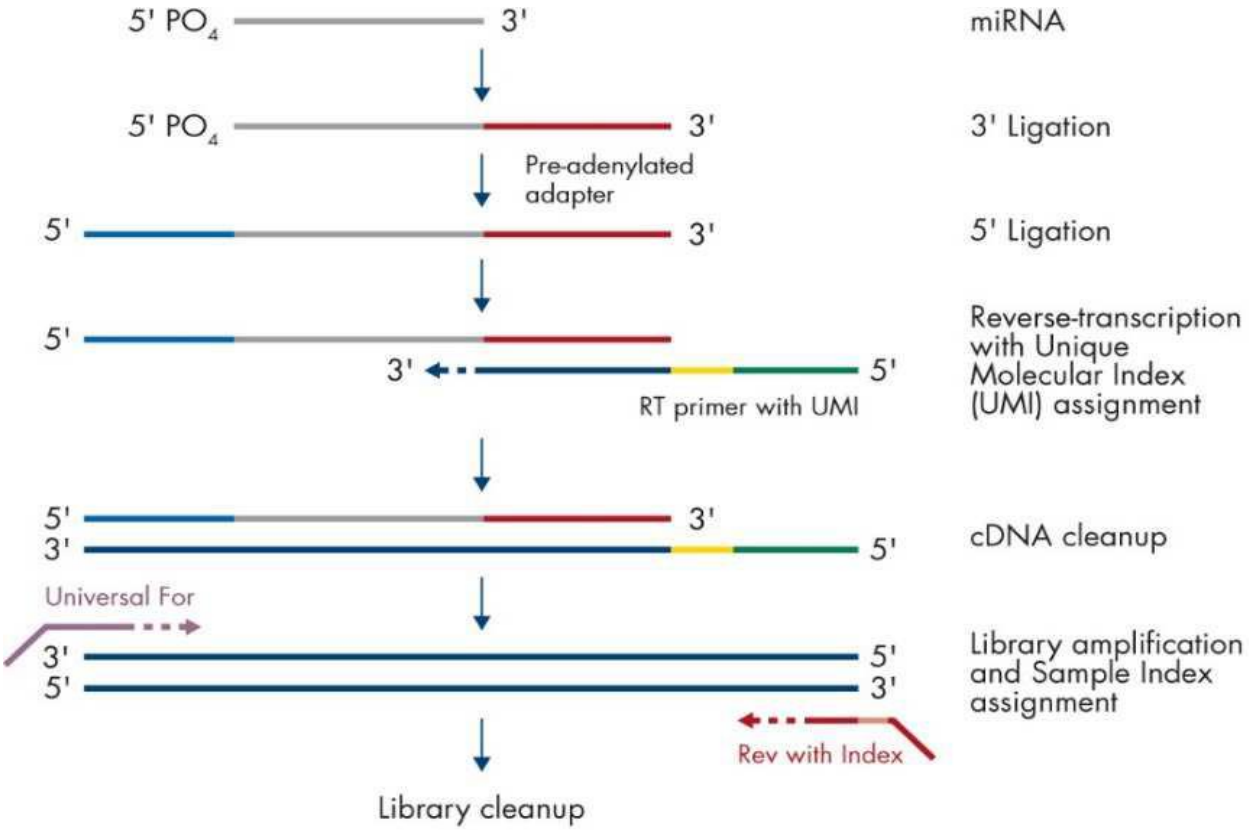

Figure 28. miRNA sequencing library preparation using the QIAseq miRNA Library Kit (QIAseq miRNA Library Kit Handbook 11/2016, page 13).

miRNA Library Pre-Sequencing Quality Control (QC) and NGS

After library cleanup, miRNA library QC was performed using the Agilent 2100 Bioanalyzer (cat # G2939BA, Agilent). Library concentration was then determined using a Qubit 4 Fluorometer (cat# Q33226, ThermoFisher Scientific). miRNA-seq libraries prepared with the QIAseq miRNA Library Kit were later sequenced using an Illumina NGS system, HiSeq 4000 (Illumina, San Diego, CA, USA).

Data Analysis

After sequencing differential miRNA expression analysis was carried out using the QIAseq miRNA Quantification software (http://ngsdataanalysis.sabiosciences.com/QIAseqmiRNA/). Further analysis of the miRNA expression data was performed using the IPA web-based software from Qiagen to gain insight into relevant molecular interactions.

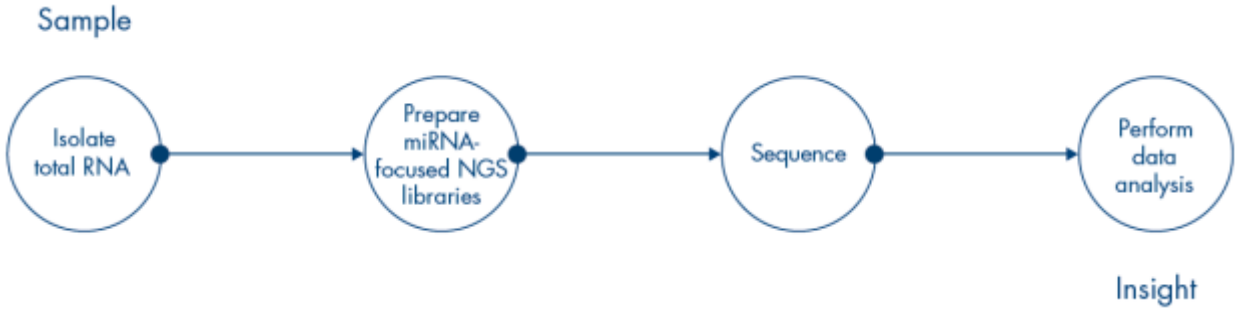

Figure 29. QIAGEN's Sample to Insight QIAseq miRNA workflow (QIAseq miRNA Library Kit Handbook 11/2016, page 11).

Results and Discussion

Aminoflavone Treatment Altered Small ncRNA Expression in both Tamoxifen Sensitive (Par MCF-7) and Resistant (TamR MCF-7) Mammospheres

In total, 366 small ncRNAs, including miRNAs and piRNAs, were differentially expressed in TamR MCF-7 mammospheres relative to Par MCF-7 controls. Furthermore, AF treatment altered the expression of several miRNAs and piRNAs in both tamoxifen sensitive and resistant mammospheres (**Table 3**). AF treatment modulated the expression of 315 and 341 small ncRNAs in TamR and Par MCF-7 mammospheres respectively.

Interestingly, AF showed a trend towards downregulating ncRNAs in Par MCF-7s while upregulating ncRNAs in TamR MCF-7s (data not shown). Moreover, it is noteworthy to mention that miR-135a-5p was the miRNA most significantly downregulated in TamR relative to Par mammospheres (-8.53 fold; **Table 4**) that AF was able upregulate to a relatively equivalent level (7.13 fold; **Table 6**).

Table 3. Differentially expressed ncRNAs including miRNAs and piRNAs in Tamoxifen sensitive (Par MCF-7) and resistant (TamR MCF-7) mammospheres with and without AF treatment.

DER	TOTAL DER	UPREGULATED	DOWNREGULATED
TAMR MCF-7 (DMSO) VS PAR MCF-7(DMSO)	366	152	214
PAR MCF-7 AF VS DMSO	315	79	236
TAMR MCF-7 AF VS DMSO	341	261	80

DER, differentially expressed RNAs; Par MCF-7, Parental MCF-7 mammospheres; TamR MCF-7, Tamoxifen resistant MCF-7 mammospheres; Fold Change ≥2

Table 4. Differentially Expressed miRNAs in DMSO exposed TAMR MCF-7 Mammospheres relative to Par MCF-7 Mammospheres (List is not exhaustive).

MiRNA	*Fold Change*	*P-value*
hsa-miR-10a-5p	65.27	0.005322072
hsa-miR-31-5p	31.59	0.004382023
hsa-miR-598-3p	13.62	0.002952352
hsa-miR-4304	11.94	0.008112487
hsa-miR-218-5p	11.78	0.00278191
hsa-miR-6744-5p	11.2	0.002107345
hsa-miR-125b-5p	10.27	0.006799104
hsa-miR-1292-5p	10.01	0.002188333
hsa-miR-496	9.29	0.001788734
hsa-miR-4286	8.85	0.003365543
hsa-miR-2277-5p	8.48	0.002148845
hsa-miR-10a-3p	8.12	0.007333525
hsa-miR-548a-3p	7.8	0.000107675
hsa-miR-125b-2-3p	7.52	0.000133411
hsa-miR-3616-3p	7.51	0.005229024
hsa-miR-1200	7.47	0.002946848
hsa-miR-338-5p	7.28	0.003277315
hsa-miR-23b-5p	6.29	0.001989827
hsa-miR-25-5p	5.9	0.008991789
hsa-miR-3611	-8.39	0.007787988
hsa-miR-450a-5p	-8.52	0.00732991
hsa-miR-135a-5p	**-8.53**	**0.001130404**
hsa-miR-520a-3p	-8.99	0.003812623
hsa-miR-545-3p	-10	0.004139045
hsa-miR-196a-3p	-10.05	0.000626069
hsa-miR-1537-5p	-10.09	0.000238783
hsa-miR-33b-5p	-10.09	0.001997735
hsa-miR-33a-5p	-10.44	0.003577703
hsa-miR-548d-3p	-11.19	0.006173072
hsa-miR-3665	-11.74	0.000127181
hsa-miR-30c-2-3p	**-13.51**	**0.015323421**
hsa-miR-4466	-15.65	0.001850961
hsa-miR-208b-5p	-19.41	0.007331074
hsa-miR-4760-5p	-33.07	0.000661868
hsa-miR-4787-5p	-47.54	0.008469147
hsa-miR-30a-3p	**-67.65**	**0.000830052**
hsa-miR-30a-5p	**-169.27**	**0.000688837**

miR-30a-3p and miR-30a-5p were the most significantly downregulated miRNAs in DMSO (control) treated TamR mammospheres relative to tamoxifen sensitive mammospheres (**Table 4**). They were downregulated -67.65 and -169.27 fold respectively. miR-30c-2-3p was also downregulated -13.51 fold. In a study of 246 ER^+ advanced breast cancers, higher expression of miR-30a-3p and miR-30c were associated with better response to tamoxifen treatment as measured by longer progression-free survival (PFS). However, only miR-30c was shown to be an independent predictor of patients with advanced breast cancer who are likely to benefit from tamoxifen therapy (Rodriguez-Gonzalez, Sieuwerts et al. 2011). Interestingly, miR-30 a/b/c/e were all predicted to target α6-integrin by web-based miRNA target prediction tools miRanda, TargetScan and Pictar. It is therefore plausible that TamR cells express elevated levels of α6-integin due to loss of miRNA-30 family. The loss of this family of miRNAs could also explain the lack of response to tamoxifen seen in resistant cancer cells and tumors.

Table 5. Differentially Expressed miRNAs in AF vs DMSO Exposed Par MCF-7 Mammospheres (List is not exhaustive).

miRNA	*Fold Change*	*P-value*
hsa-miR-191-5p	7.26	0.007117955
hsa-miR-381-5p	6.58	0.000103081
hsa-miR-1226-3p	5.86	0.000633311
hsa-miR-769-3p	4.59	0.004544094
hsa-miR-500a-3p	4.57	0.001678099
hsa-miR-125a-5p	4.12	0.000577201
hsa-miR-28-3p	3.62	0.010065743
hsa-miR-146b-5p	3.51	0.005650753
hsa-miR-103a-3p	3.44	0.001180643
hsa-let-7e-5p	3.06	0.001479374
hsa-miR-4784	3.06	0.006563964
hsa-miR-6792-3p	2.96	0.001295471
hsa-miR-532-5p	2.96	0.003823204
hsa-let-7a-5p	2.93	0.002694924
hsa-miR-559	-8.17	0.006887317
hsa-miR-32-3p	-8.2	0.000783909
hsa-miR-29a-5p	-8.45	0.031013798
hsa-miR-585-3p	-8.73	0.024825695
hsa-miR-548-3p	-8.89	0.00833722
hsa-miR-4502	-8.91	0.044523407
hsa-miR-3657	-8.93	0.038137809
hsa-miR-4493	-8.99	0.008403592
hsa-miR-190a-5p	-9.17	0.003884838
hsa-miR-1252-5p	-9.54	0.013949298
hsa-miR-106b-5p	-9.64	0.021061429
hsa-miR-450a-5p	-9.78	0.015770924
hsa-miR-552-5p	-10.14	0.000878949
hsa-miR-6844	-10.23	0.03382314
hsa-miR-548c-3p	-10.26	0.004492784
hsa-miR-302b-5p	-11.48	0.004998656
hsa-miR-4272	-15.68	0.000794873
hsa-miR-1277-5p	-16.66	0.023249426
hsa-miR-3613-5p	-17.74	0.030604574
hsa-miR-8067	-18.22	0.049065858
hsa-miR-33b-5p	-19.41	0.010057754
hsa-miR-1277-3p	-20.19	0.046818826
hsa-miR-33a-5p	-22.12	0.011339815
hsa-miR-545-3p	-24	0.003540819
hsa-miR-5692b	-25.41	0.009836664

Table 6. Differentially Expressed miRNAs in AF vs DMSO Exposed TamR MCF-7 Mammospheres (List is not exhaustive).

miRNA	***Fold Change***	***P-value***
hsa-miR-222-5p	88.61	0.019325345
hsa-miR-636	50.28	0.01933999
hsa-miR-802	27.95	0.006651284
hsa-miR-3653-5p	13.12	0.005837849
hsa-miR-7641	12.88	0.00998836
hsa-miR-1303	12.43	0.001228392
hsa-miR-487a-3p	12.06	0.002083502
hsa-miR-6837-3p	10.41	0.001199994
hsa-miR-5696	9.91	0.004563973
hsa-miR-6504-3p	9.62	0.000274539
hsa-miR-3667-3p	9.53	0.000735536
hsa-miR-6817-5p	8.29	0.000622734
hsa-miR-921	8.11	0.000225968
hsa-miR-5701	7.75	0.000263198
hsa-miR-4419b	7.4	0.004680569
hsa-miR-135a-5p	**7.13**	**0.006002407**
hsa-miR-4528	7.06	0.002468669
hsa-miR-129-1-3p	**2.28**	**0.013065301**
hsa-miR-133b	-3.11	0.024523604
hsa-miR-27a-5p	-3.13	0.003941545
hsa-miR-548ao-3p	-3.13	0.005437068
hsa-miR-6501-5p	-3.32	0.000431637
hsa-miR-200c-5p	-3.41	0.005512858
hsa-miR-4732-5p	-3.42	0.014154245
hsa-miR-3692-5p	-3.78	0.009437729
hsa-miR-6081	-4.04	0.033572412
hsa-miR-4729	-4.11	0.005792419
hsa-miR-548h-3p	-4.14	0.000324279
hsa-miR-26a-1-3p	-4.69	0.00020983
hsa-miR-25-5p	-4.78	0.002955803
hsa-miR-4304	-5.33	0.004004949
hsa-miR-1292-5p	-5.93	0.00318512

miR-129-1-3p was upregulated 2.28 fold in TamR mammospheres following AF treatment (**Table 6**). miR-129 was found to be down-regulated both in breast cancer tissues compared with paired adjacent normal breast tissues, and in breast cancer cell lines compared with normal breast epithelial MCF10A cells and suppressed cancer cell mobility and migration (Wang, Tang et al. 2012). α6-integrin is a predicted target of miR-129. AF may downregulate α6-integrin by increasing miR-129-3p expression.

miR-135a-5p was the most significantly downregulated miRNA in TamR mammospheres relative to Par mammospheres that AF treatment was able to upregulate to equivalent levels. miR-135a-5p was downregulated 8.5-fold in control TamR vs tamoxifen sensitive mammospheres (**Table 4**). However, AF treatment increased miR-135a-5p 7.3-fold in TamR mammospheres (**Table 6**). HIF-1α is a predicted target of miR-135a based on TargetScan and PicTar analyses. HIF-1α directly upregulates α6-integrin transcription to enhance invasion and TIC activity in models of metastatic breast cancer. The authors found that knockout models of HIF-1α showed depleted α6-integrin expression (Brooks, Schwab et al. 2016). Even more interesting is the fact that AF inhibits the expression of HIF-1α and HIF-1α target genes (Terzuoli, Puppo et al. 2010). Thus, one can speculate that AF suppresses α6-integrin expression, at least in part, following miR-135a-mediated down-regulation of HIF-1α. Furthermore, miR-135a was found to reduce invasion in breast and prostate cancer cells by targeting the Estrogen-Related Receptor α (ERRα) (Tribollet, Barenton et al. 2016). On the contrary, others have proposed a tumor promotor role for miR-135a in breast cancer where this miRNA was found to promote breast cancer cell migration and invasion by targeting HOXA10 (Chen, Zhang et al. 2012). Consequently, more studies are needed to clearly delineate the role of

miR-135a in breast cancer especially as it relates to its regulation of TIC function and tamoxifen resistance.

IPA Revealed an Association between Differentially Regulated miRNAs and Cancer-Related Networks in AF Treated TamR Mammospheres

Use of the web-based analysis software, IPA to examine differentially expressed miRNAs and their target genes in AF treated TamR mammospheres revealed five top miRNA- regulated networks. These networks were linked to various aspects of cancer regulation.

Cell Cycle and Cellular Movement was impacted (**Figure 30**). In this network, AF treatment upregulated several miRNAs including miR-135a-5p, miR-193-5p, and miR-148-3p. Others such as miR-125a-3p, miR-125b-5p, and miR-145-5p were downregulated.

Cellular Development, Cellular Growth and Proliferation was also affected in AF treated TamR mammospheres (**Figure 31**). AF downregulated four miRNAs in this network including miR-30c-5p, miR-133a-3p, miR-200-3p, and miR-185-3p.

Cell Death and Survival was also impacted (**Figure 32**). AF treatment upregulated miR-4651 which was predicted to interact with Bcl-2-like protein 1 (BCL2L1). BCL2L1 is a potent inhibitor of apoptosis (Kurita, Izumi et al. 2012).

Another network that was impacted was Cell-to-Cell Signaling and Interaction, Hematological System Development and Function and Immune Cell Trafficking (**Fig. 32**). miR-511-5p was predicted to target CD209, CD80 and Toll-like receptor 4 (TLR4).

TLR4 overexpression was found to be associated with breast cancer metastasis and lower overall survival (Chen, Zhao et al. 2015).

Finally, Cell-mediated Immune Response, Cellular Development, Cellular Function and Maintenance was another network impacted in AF treated TamR mammospheres (**Fig. 33**). miR-292b-5p was upregulated and predicted to target BCL11A, BCL11B, BTG1, and KPNA4.

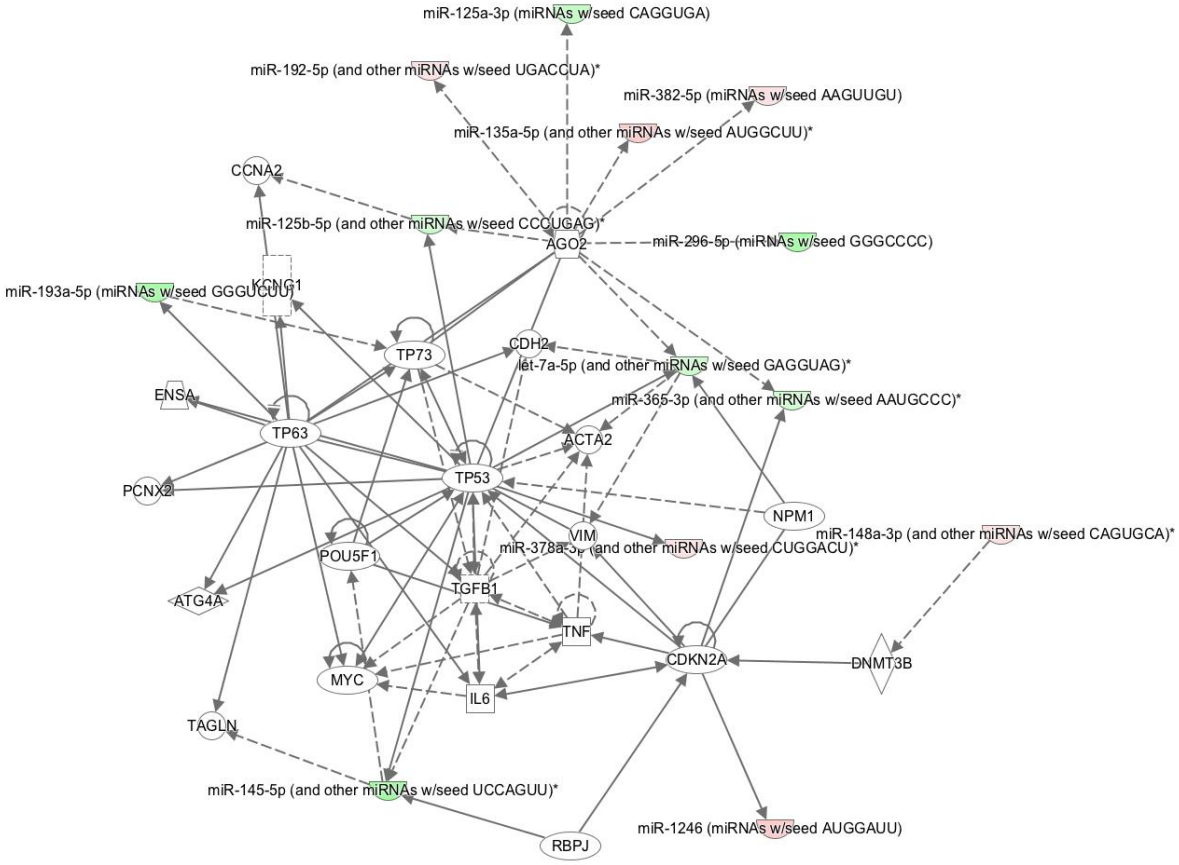

Figure 30. Differentially Expressed miRNAs with functions in Cell Cycle and Cellular Movement; Increased Measurement ; Decreased Measurement

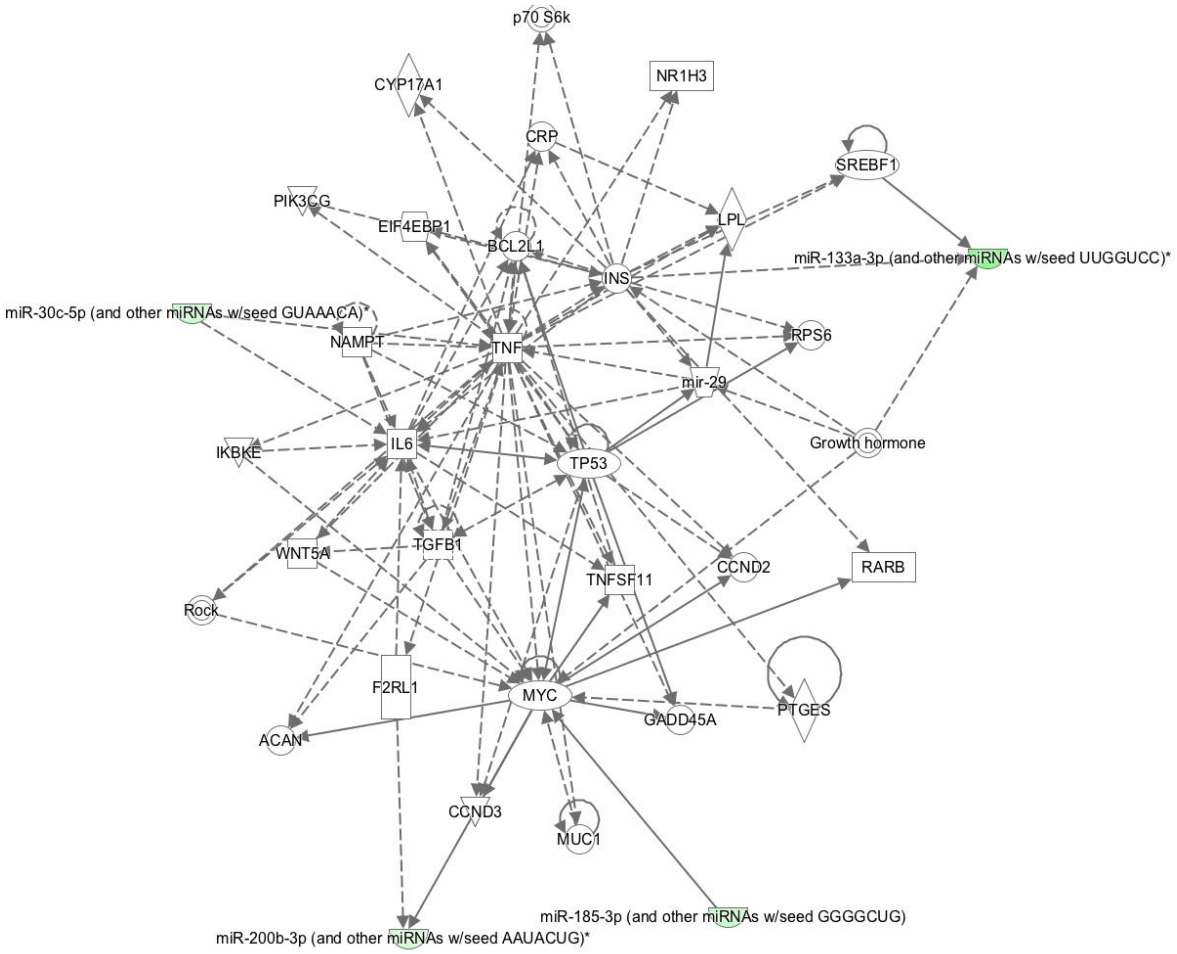

Figure 31. Differentially Expressed miRNAs with functions in Cellular Development, Cellular Growth and Proliferation; Decreased Measurement

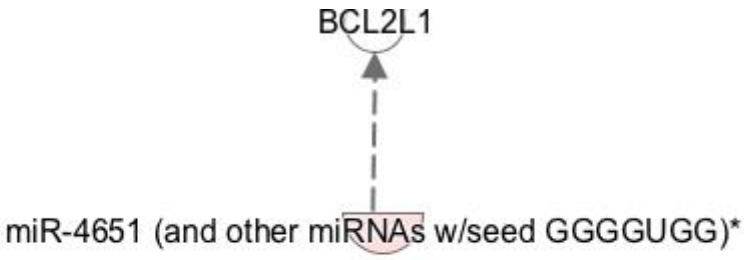

Figure 32. Differentially Expressed miRNA with functions in Cell Death and Survival; Increased Measurement

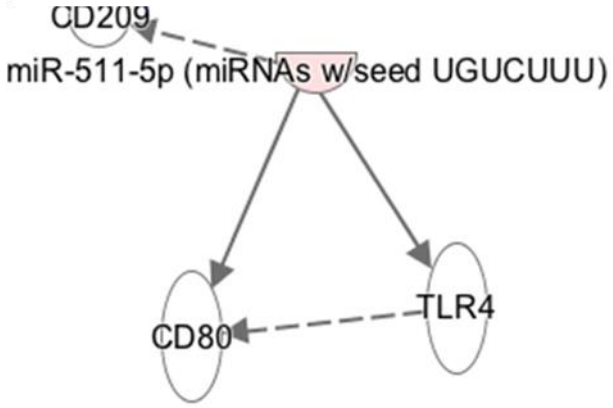

Figure 33. Differentially Expressed miRNA with functions in Cell-to-Cell Signaling and Interaction, Hematological System Development and Function, Immune Cell Trafficking; Increased Measurement

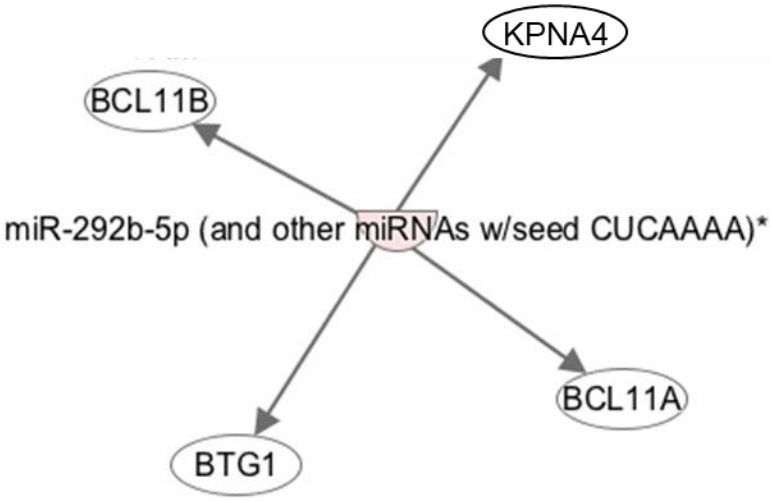

Figure 34. Differentially Expressed miRNA with functions in Cell-mediated Immune Response, Cellular Development, Cellular Function and Maintenance;
Increased Measurement

Disease and Function Analysis of the Top Differentially Expressed miRNAs Showed a Correlation with Invasive Ductal Breast Carcinoma

As mentioned previously, expression of miR-129 suppresses cell mobility and migration in breast cancer. Therefore, the ability of AF to increase the expression of this miRNA in TamR cells is likely indicative of AF treatment inducing a less invasive phenotype in these cells. Furthermore, AF upregulated miR-507 in TamR mammospheres. miR-507 was also shown to inhibit breast cancer cell migration and invasion (Jia, Liu et al. 2016).

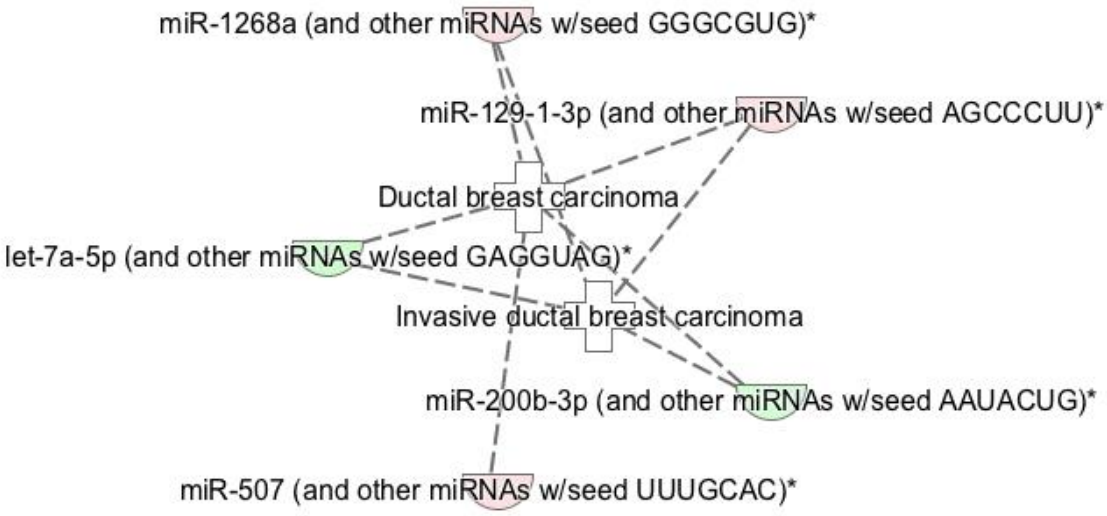

Figure 35. Differentially Expressed miRNA Correlated with Invasive Ductal Breast Carcinoma; Increased Measurement ; Decreased Measurement

Conclusion

To the best of our knowledge, we are the first to show that AF modulates the expression profile of small ncRNAs including miRNAs and piRNAs in a TamR mammosphere model. These findings are important as mammospheres represent an *in vitro* model system that may be more representative of breast tumors as compared to monolayers. Mammospheres are also enriched for TICs and so may give better insight into how miRNA expression and function are altered in this population of cells. We have identified several miRNAs with tumor suppressor functions that are downregulated in TamR mammospheres and others that are upregulated following AF treatment. Our preliminary data also include identification of tumor-suppressor miRNAs that may directly or indirectly target α6-integrin. miRNA-135-5p is a promising target as it has tumor suppressor functions and is significantly downregulated in TamR mammospheres compared to Tamoxifen sensitive controls, while AF treatment reestablishes its expression. Furthermore, AF-mediated regulation of miR-135a-5p may offer insight into how this agent represses α6-integrin expression to impede TIC properties. Future studies will continue to delineate the role of specific miRNAs in mediating TIC function in tamoxifen resistant cancer and the ability of AF to counter some of these pro-cancer effects.

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CHAPTER FIVE

GENERAL DISCUSSION

TICs are believed to be major contributors in the development of tamoxifen resistance, owing to their ability to evade the impact of therapy and self-renew. Resistance and disease recurrence account largely for the high mortality rate associated with breast cancer and remain major obstacles in effective disease management and establishment of a 'cure'. As such, it is imperative that we target the both the bulk tumor cells and the TIC population as a means of limiting disease recurrence and improving patient outcomes. We have shown that AF has significant activity against bulk tumor cells and impedes the TIC population in breast cancer by downregulating stemness gene α6-integrin (Brantley, Callero et al. 2016). Furthermore, we've shown that AF inhibits α6-integrin and its downstream signaling to overcome tamoxifen resistance (*in press*).

Notwithstanding this, successfully targeting the TIC population may not be as straightforward as one may think. Current approaches to identify TICs rely heavily on specific cell surface 'stemness' markers. It has been proposed that some of these markers also play functional roles in maintaining TICs and may therefore serve as viable therapeutic targets. Breast TICs are characterized as $CD44^{+}/CD24^{-/low}$. Additional markers of breast TICs include CD133, aldehyde dehydrogenase (ALDH), C-X-C chemokine receptor type 4 (CXCR4) and CD49F (α6-integrin) (Hwang-Verslues, Lee et al. 2012). In a human breast cancer xenograft model, a monoclonal antibody against CD44 reduced the frequency of recurrence to 31% when administered during remission following adriamycin/cyclophosphamide (AC) treatment (Marangoni, Lecomte et al. 2009). CD26 has also been proposed as a TIC marker and potential therapeutic target in

various cancer types including that of the breast (Davies, Beckenkamp et al. 2015). From this, one can infer that TICs not only differ in their expression of markers but that this difference in expression may bear functional relevance. Hence, developing effective therapy against TICs, based on marker expression, would require thorough and exhaustive knowledge of both inter-tumoral and intra-tumoral TIC heterogeneity. As such sequencing of tamoxifen resistant tumors may be necessary to identify the relevant patients with tumors that overexpress α6-integrin who would most likely benefit from AhR ligands like AF that suppress the expression of this gene.

Another way in which TICs avoid current treatment approaches is via their ability to harness specific growth or survival signaling pathways. Some of these key pathways include Notch, Hedgehog, Wnt/β-catenin, and PI3K/Akt signaling. Pharmacologic or genetic inhibition of Notch1 and Notch4 was shown to inhibit breast TIC growth *in vitro* and reduce tumor initiation *in vivo* (Harrison, Farnie et al. 2010). Patients expressing major components of the Hedgehog signaling cascade as well as $CD44^{+}/CD24^{-}$ TIC markers, were shown to have lymph node metastasis, higher grade tumors and poorer survival (Zhao, Tang et al. 2016). It was shown that Wnt/Beta-catenin signaling plays a role in maintaining the viability and self-renewal capacity of breast TICs *in vitro* and that prior incubation of primary tumor cells with a Wnt/β-catenin signaling inhibitor, PKF118-310, limited their ability to initiate tumor growth *in vivo* (Hallett, Kondratyev et al. 2012). Furthermore, TICs may be regulated by cross-activation or cross-communication between multiple pathways. As an example, it has been shown that activation of EP4 (prostaglandin E-2 receptor)/PI3K/AKT/NOTCH/WNT signaling enhances TIC activity (Majumder, Xin et al. 2016). One of the main concerns around

targeting TIC signaling pathways is the commonality in pathways such as Wnt, Notch and Hedgehog between normal stem cells and TICs (Abetov, Mustapova et al. 2015) which may account for the low selectivity of these targeted therapies. AF inhibits the TIC population by downregulating α6-integrin/PI3K/AKT signaling in tamoxifen resistant cells (*in press*). Considering its role in normal physiology, one might question the lack of specificity of agents aimed at eliminating the TIC population by targeting α6-integrin. However, it should be noted that cancer tissues often have elevated levels of α6-integrin compared to normal tissues (Friedrichs, Ruiz et al. 1995, Kwon, Lee et al. 2013). In addition, there appears to be a change in the spatial localization of α6-integrin in carcinoma compared to normal tissue. It was reported that α6-integrin is mainly distributed in the basement membrane of normal cells, while basal membrane staining was diminished or lost in all breast tumors examined (D'Ardenne, Richman et al. 1991). This change in the expression and localization of α6-integrin is also seen in other cancers such as pancreatic cancer (Cruz-Monserrate, Qiu et al. 2007). The knowledge of this change or redistribution of α6-integrin as cells progress from a normal to a cancer phenotype and the implications on immunoreactivity and function may also be manipulated to devise more specific therapeutic approaches.

Furthermore, normal and less aggressive, luminal breast cancer sub-types were shown to predominantly express α6A, while the more aggressive, triple negative cells expressed predominantly α6B, responsible for the more mesenchymal TIC sub-population (Goel, Gritsko et al. 2014). The authors of this study furthered identified Epithelial splicing regulatory protein 1 (ESRP1) as the transcriptional regulator which determines splicing of the α6-integrin gene in favor of the α6A variant which

demonstrates less association with stemness. Interestingly, ESRP1 is abrogated during EMT which may explain the increase in α6B and a switch to a more mesenchymal phenotype during this transition (Warzecha, Jiang et al. 2010). Considering this, we can increase the specificity of anti-α6 integrin agents by targeting the α6B variant predominantly expressed in the TIC population. Otherwise, we may be able to use genetic techniques to control the splicing activity of α6-integrin regulatory proteins such as ESRP1 in favor of α6A thereby priming cancer cells to switch to a more epithelial, less aggressive phenotype.

Another aspect of TIC regulation that may offer therapeutic avenues is the role of miRNAs. miRNAs are involved at various stages of TIC development and function and may be effective targets for combating TIC-induced cancer resistance and progression. miRNAs regulate TIC formation. p53 was shown to activate miR-200c in breast tumor samples which in turn suppressed genes which promoted EMT and stemness properties (Chang, Chao et al. 2011). Knockdown of other tumor suppressor miRNAs such miR-205 was shown to increase the TIC population and stemness properties in breast epithelial cells and miR-205 knockdown mice (Chao, Chang et al. 2014). miRNAs also regulate the self-renewal capacity of TICs. As an example, miR-10b was found to inhibit PTEN and upregulate Akt signaling to promote self-renewal of breast TICs (Bahena-Ocampo, Espinosa et al. 2016). Other miRNAs such as miR-200c and miR-100 stimulate differentiation of TIC cells into a less invasive, lower grade phenotype that can be more easily targeted by therapy (Shimono, Zabala et al. 2009, Petrelli, Carollo et al. 2015). miRNAs have also been directly linked to resistance. Loss of tumor suppressor miR-26a/b and associated upregulation of Erb-B2 receptor tyrosine kinase 2 (ERBB2)

expression was shown to confer resistance to tamoxifen in ER^+ breast cancer cells (Tan, Ding et al. 2017). Several other miRNAs have been linked to tamoxifen responsiveness including miR-30a-3p, miR-30c and miR-182, the expression of which correlated with better response to tamoxifen (Rodriguez-Gonzalez, Sieuwerts et al. 2011) and miRs-10a, 22, 29a, 125b, and 222 which were found to be elevated in TamR cells compared to sensitive cells (Manavalan, Teng et al. 2011). Considering the diverse roles of miRNAs in TIC regulation, TICs may be ideal targets for miRNA-based therapy as a therapeutic strategy to overcome cancer growth, resistance and metastasis. Notwithstanding, thorough delineation of both oncogenic and tumor suppressor miRNAs and their mRNA targets is essential in optimizing miRNA-based therapy. We currently have ongoing studies to investigate the role of miRNAs in facilitating AF-mediated anticancer actions in both tamoxifen sensitive and resistant mammospheres.

Targeting key factors in the tumor microenvironment that are essential for TIC survival has been explored as another weapon in the fight against tamoxifen-resistant breast cancer. Studies have shown that the ability of TICs to evade conventional therapies is not solely due to the intrinsic properties of these cells, but also influence of the TME. TICs enhance their survival and evade treatment by interacting with their microenvironment through various cell growth or self-renewal pathways. As examples, TICs are able to evade the immune system via the induction of regulatory T-cell production, changing the expression of antigens on cancer cells, changing the microenvironment to a more anti-inflammatory state, and by overcoming hypoxic conditions by inducing the release of factors such as HIF-1α or VEGF or by promoting tumor vascularization (Albini, Bruno et al. 2015). Interestingly, AF has been shown to

regulate both innate and adaptive immune responses to mimic a more pro-inflammatory environment (Callero, Rodriguez et al. 2017). Additionally, AF has been shown to inhibit the expression of HIF-1α and HIF-1α target genes (e.g. VEGF, CA9 and PDK-1) in MCF-7 xenografts (Terzuoli, Puppo et al. 2010). Additionally, α6-integrin itself is a proangiogenic gene and a direct transcriptional target of HIF-1α and HIF-2α. It is interesting to note that α6-integrin overexpressing cells are also enriched for HIF-1α, and demonstrate enhanced invasion and TIC properties in metastatic breast cancer models (Brooks, Schwab et al. 2016). Thus, one can postulate that AF may reduce α6-integrin levels, at least in part, my controlling the transcriptional activity of HIFs.

Acknowledging the contribution of the microenvironment to TIC maintenance and associated therapy resistance has led to the development of novel therapies targeting components of the TME. The efficacy of antiangiogenic drugs as therapeutic strategies for breast cancer has been called into question due to their limited effectiveness in clinical trials (Bergers and Hanahan 2008). Interestingly, it has been shown that antiangiogenic drugs such as sunitinib and bevacizumab enrich the TIC population *in vivo* (Conley, Gheordunescu et al. 2012) which may explain the limited benefit of such drugs in patients. Immune check-point inhibitors such as the PD-1 or PD-L1 antibodies with efficacy in malignancies such as melanoma (Naidoo, Page et al. 2014) and breast cancer(Ma, Chen et al. 2017) have recently been introduced into the clinics. A recent editorial entitled, 'Microenvironment and endocrine resistance in breast cancer: Friend or foe?' highlighted the role and importance of the microenvironment in endocrine therapy resistance and proposed the need for a more thorough understanding of the interaction

between cancer cells and other cellular components in different microenvironmental contexts (Recouvreux, Sampayo et al. 2015).

Other means of targeting the TIC population include re-sensitizing these cells to apoptotic signals using agents such as Zoledronic acid (ZA), a third-generation bisphosphonate, which upregulates pro-apoptotic genes in TICs (Rouhrazi, Turgan et al. 2018). Recently, we discovered that AF treatment was sufficient to upregulate the expression of pro-apoptotic BAX in both tamoxifen sensitive and resistant cells (*in press*).

Otherwise, methods of promoting TIC differentiation have been employed. The suppression of PKCζ, a cell polarity protein important in the asymmetric division of TICs, converted TICs into a more luminal-like state and subsequently re-sensitized these cells to tamoxifen (Wu, Kim et al. 2017). We have published data to show that AF treated tumors demonstrate a less invasive phenotype. AFP464 treated animals developed a mucinous phenotype with mucinous breast carcinomas being less prone to metastases and associated with better prognosis (Brantley, Callero et al. 2016).

As is evident, there are many ways in which TICs are regulated which offer a wide frame work of possibilities for targeting these sub-population of cells with the hope of overcoming cancer development, resistance and progression. However, one main consideration when targeting TICs are the similarities between the TIC niche and the normal stem cell niche. Consequently, TIC related therapies must be optimized not just for efficiency but specificity to offset toxicities in healthy tissues. For the first time, we show that AhR ligand AF targets the TIC population in tamoxifen resistant breast cancer by downregulating α6-integrin. This agent holds promise in overcoming the issues

associated with the lack of specificity of TIC targeting agents especially as it potently inhibits malignant cancer cells while being non-toxic to normal cells at therapeutically relevant concentrations.

General Conclusion and Future Directions

Our work is novel since, to the best of our knowledge, we are the first to link α6-integrin with AhR ligand-mediated suppression of TIC proliferation. We have also identified AF as a potential therapeutic agent capable of impeding α6-integrin expression and function in tamoxifen resistant breast cancer. Our contribution to the field therefore involves identification of α6-integrin as a functional mediator of tamoxifen resistance, the overexpression of which may be useful as a predictive biomarker for patients less likely to respond favorably to tamoxifen. We have also shown that inhibition of α6-integrin is sufficient to re-sensitize resistant cells to tamoxifen, thus highlighting the potential for anticancer AhR agonists such as AF to effectively counteract tamoxifen resistance by modulating stemness genes such as α6-integrin. This contribution is significant as the elucidation of new, dysregulated molecular targets that are regulators of pathways known to promote tamoxifen resistance and TIC function will form the basis for more effective breast cancer therapeutic approaches aimed at improving the long-term clinical efficacy of tamoxifen and possibly other endocrine therapies. Our findings provide greater insight into novel molecules and mechanisms which confer tamoxifen resistance, as well as greater understanding of AF-mediated anticancer mechanisms. Furthermore, the identification and use of agents that effectively target TICS have the potential to improve cancer treatment outcomes (Ricci-Vitiani, Pagliuca et al. 2008). Overall, our work forms the basis for developing specific AhR agonists as new therapeutic agents for use in

combination with tamoxifen or other endocrine therapies to ultimately circumvent therapy resistance and disease recurrence thereby improving disease outcome.

This study has several opportunities for expanding our current understanding of breast cancer treatment and resistance. Firstly, while *in vitro* studies and evaluation of patient tumor samples strongly support the potential of α6-integrin as a predictive biomarker of tamoxifen resistance, more robust studies are needed to confirm its clinical utility. As such, we intend to design retrospective studies using biobank/biorepository patient samples to gain insight into the clinical utility of α6-integrin as a predictive biomarker of tamoxifen responsiveness. Our proposed retrospective cohort study will allow for the generation of relevant data similar to a prospective study but with the added benefits of taking less time and being more cost-effective. These studies are relevant and timely especially as we are in the era of 'Precision Medicine', which hinges largely on the identification and validation of biomarkers which are potentially targetable by highly selective molecular targeted therapy. Even Former President Obama recognized the immense potential of targeted therapies as seen in the 2015 launch of the Precision Medicine Initiative, which remains in place under the current President Trump administration. Successful evaluation of α6-integrin as a biomarker of tamoxifen resistance will better enable clinicians to appropriately stratify luminal breast cancer patients that would ultimately benefit from tamoxifen alone or tamoxifen in combination with AhR ligands such as AF.

Furthermore, we have generated miRNA-sequencing data showing differential expression of miRNAs in mammospheres (enriched for TICs) derived from tamoxifen sensitive and resistant cells with or without AF treatment. Future studies will therefore be

dedicated to further evaluate these datasets. We hope to identify and clearly define the roles of tumor suppressor miRNAs relevant to the TIC population and cancer progression that are lost during the acquisition of resistance but are re-expressed upon exposure to AF. To date, there are no reported studies showing the ability of AF to regulate miRNA expression and, particularly, not using a TIC drug resistant model. Furthermore, we will increase our mechanistic understanding of tamoxifen resistance by examining the known or putative functions of differentially expressed miRNAs in tamoxifen sensitive and resistant mammospheres. We also plan to evaluate the potential of novel miRNAs identified to serve as therapeutic, prognostic or tamoxifen resistance biomarkers.

Finally, our data provides a rationale for the continued development of AF prodrug, AFP464 as an agent to enhance the therapeutic management of breast cancer. Despite its potent *in vitro and in vivo* efficacy in several preclinical solid tumor models, liver and pulmonary toxicities have limited the development of AFP464 (Brinkman, Chen et al. 2016). Drug-induced lung disease is often linked to immunological reactions that are T-cell mediated (Matsuno 2012). Interestingly, AF has been shown to regulate both innate and adaptive immune responses, and its pro-drug AFP 464 was found to increase the activity of cytotoxic T lymphocytes (CTL) in a murine M05 adenocarcinoma model (Callero, Rodriguez et al. 2017). The anticancer effects of AF's pro-inflammatory profile may be compromised by off-target toxic effects seen in normal tissues like the lung. This calls for development of novel analogs with enhanced efficacy but decreased cytotoxicity. It is quite plausible that the method of delivery of the drug may help lower toxicity. A recent publication has identified a novel method of delivering the drug which involves loading unimolecular micelle nanoparticle (NP) with AF to specifically target

the EGFR known to be overexpressed in TNBC. This approach was shown to improve the therapeutic index of the drug in TNBC xenograft models without causing toxicity in the lung, liver and kidney of the AF treated mice (Brinkman, Chen et al. 2016). Therapeutic index is a measure of drug safety and is a ratio of the toxic dose for 50% of subjects (TD50) relative to the therapeutic dose for 50% of subjects (ED50). Thus, a safer drug exhibits a higher therapeutic index. As such, this method of drug delivery provides new avenues for overcoming toxicities associated with AF found in previous clinical trials. Therefore, we believe that by altering the structure and changing the method of drug delivery AF, or an analog thereof, may offer great clinical benefit not only in endocrine resistant ER^+ breast cancer but in other types of breast cancers including aggressive TNBC for which therapeutic options are already limited. As such, we plan to use AF as a template to identify and/develop other agents for use alone/in combination with endocrine therapies. Otherwise, we will look at optimizing drug delivery. Our overall aim is to ultimately combine AhR ligands and established endocrine therapy agents to prevent the emergence of endocrine therapy resistance and combat therapy resistance once it has developed. Overall, our studies have the potential to impact current clinical guidelines for treating endocrine resistant breast cancer.

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