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Original Articles

AhR ligand Aminoflavone inhibits α6-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. Alpha6-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 [1,2]. 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 [3]. Cytoreduction of breast cancer frequently fails to eliminate the TIC population [4] which can self-renew and undergo multilineage differentiation [5], to drive disease recurrence and chemoresistance. Furthermore, TICs are enriched in mammospheres [6,7].

Integrins function as cell adhesion molecules that are vital to signaling pathways that regulate tumor development, migration, and angiogenesis [8,9]. α6-integrin promotes metastasis [10] and represents a putative stemness marker [5,9]. The overexpression of this gene is associated with aggressive breast cancer and poor prognosis [11], as well as, increased mammosphere formation and tumorigenesis [12]. Of significance, α6-integrin is overexpressed in malignant breast cancer cells compared to normal, non-malignant breast epithelial cells [13,14].

Recent studies indicate that aryl hydrocarbon receptor (AhR) signaling impedes mammosphere formation [15,16]. Dysregulation of this pathway is associated with tumor formation, growth and

Abbreviations: TICs, tumor-initiating cells; α6-integrin, alpha6-integrin; AhR, aryl hydrocarbon receptor; AF, Aminoflavone; AFP464, Aminoflavone pro-drug; ER+, estrogen receptor positive; ER−, estrogen receptor negative; 4OHTam, 4OH-Tamoxifen; DMSO, dimethyl sulfoxide; M05, spontaneous hormone-dependent mammary mouse model; AHR100, AhR ligand-unresponsive MCF-7 breast cancer cells; LM05-E, epithelial cells derived from tumors from the M05 mouse model; LM05-Mix, mixture of fibroblastic and epithelial cells derived from tumors from the M05 mouse model; Fra-1, Fos-related antigen 1; αNF, alpha-naphthoflavone; EMT, epithelial to mesenchymal transition.

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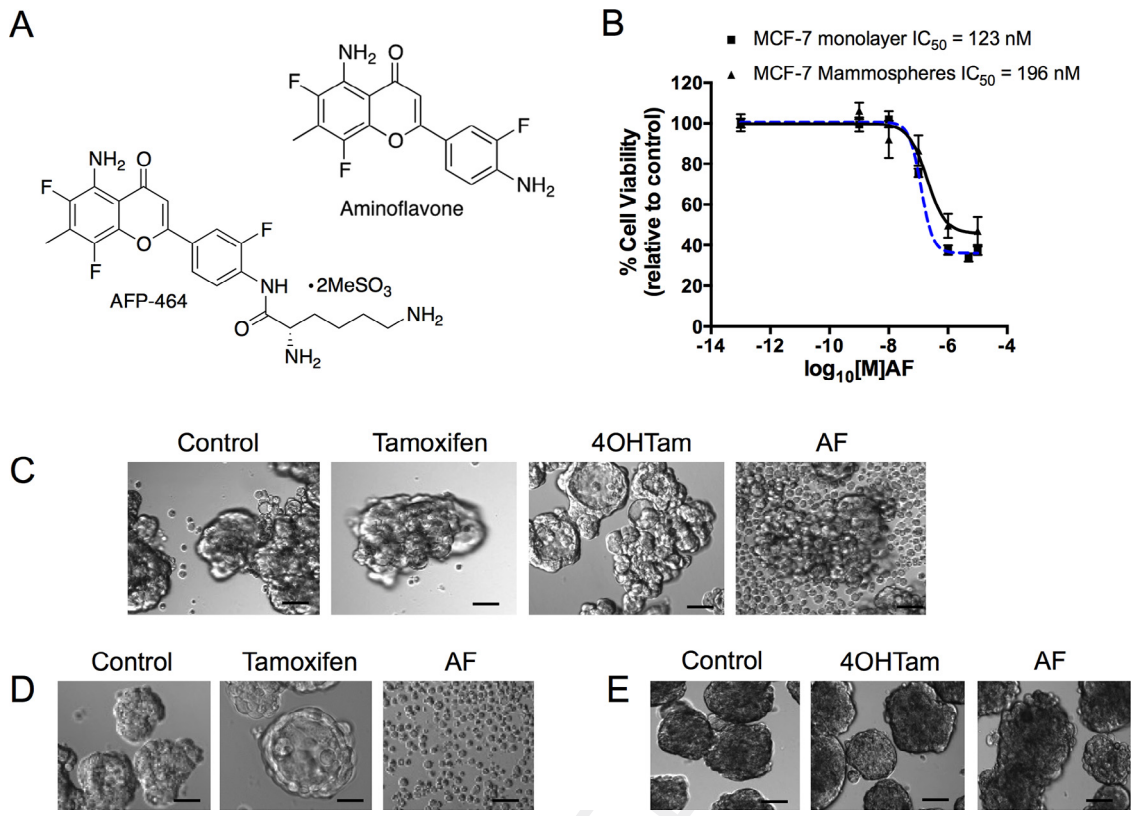

Fig. 1. AF, Tamoxifen and 4OHTam differentially impact mammosphere formation. (A) Structures of AF and AFP464, (B) MCF-7 cells and MCF-7 cell-derived mammospheres were treated with media containing 0.1% DMSO or AF (0.001–10 μM) for 48 h after which cytotoxicity was assessed using the Alamar Blue assay 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.

progression [17]. Aminoflavone (AF) is an investigational agent and AhR ligand (Fig. 1A) with potent activity against estrogen receptor positive (ER^+) and certain estrogen receptor negative (ER^-) breast cancer cells [18–20]. AF-mediated anticancer activity is linked to reactive oxygen species production, oxidative DNA damage and apoptosis [20], and AF pro-drug AFP464 (Fig. 1A) 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 [21]. Cell culture conditions for the MCF-7 and AHR100 cells have been described previously [20]. LM05-E and LM05-Mix cells were cultured in DMEM/F12 medium as previously described [22]. 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 °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 [22]. 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 [22]. 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 [23]. Alternatively, cytotoxicity of AF in MCF-7 cells was evaluated using the Alamar Blue assay as previously described [20]. MCF-7 derived mammospheres treated with AF were harvested and trypsinized as previously described before cytotoxicity was determined using the Alamar Blue assay [24].

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 were 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 analysis

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

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Research, Irvine, CA, USA) according to manufacturer instructions. Semi-quantitative RT-PCR analysis was performed as previously described [25] 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 Supplementary Table S1. 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).

Immunofluorescence assay

Cells were fixed in 4% formalin in PBS and permeabilized with 0.1% Triton X-100 in PBS as previously described [26]. 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 40 × 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 [27]. 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 × 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 [28]. 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 [15]. Additionally, while Tamoxifen facilitates the selection of cells which favor mammosphere formation [28], the non-toxic AhR agonist Tranilast readily disrupts mammospheres [16]. 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) (Fig. 1B). 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 (Fig. 1C). We found that another antitumor AhR agonist 2-(4-amino-3-methylphenyl)-5-fluorobenzothiazole [29] 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 (Fig. 1D).

Mammospheres contain a higher percentage of TICs compared to 2D cell monolayers [30]. 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 (Fig. 2A). A further increase was detected under hypoxia as determined by relief contrast mode microscopy and flow cytometry (Fig. 2A). Using fluorescence microscopy, we identified Aldefluor™ positive cells (which stain green) within mammospheres (Fig. 2B). MCF-7 mammospheres in the presence of hypoxia showed the greatest induction of the OCT-4 gene, a key stemness biomarker (Fig. 2C). Both Tamoxifen and 4OHTam increased the percentage of cells in mammospheres that stained for CD44$^+$/CD24$^{-/low}$, an effect substantially reduced by AF (Fig. 2D). This suggests AF has the capacity to suppress the growth of cells with TIC capacity.

AF lacks the ability to disrupt AHR100-derived mammospheres

Since activation of the AhR signaling pathway has been shown to impair mammosphere formation [15,16], 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 (Fig. 1E) 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 [31–34]. In particular, α6-integrin promotes mammosphere formation and tumorigenicity in MCF-7 cells [12]. Among the panel of integrins examined, AF only inhibited the expression of α6-integrin (Fig. S1A).

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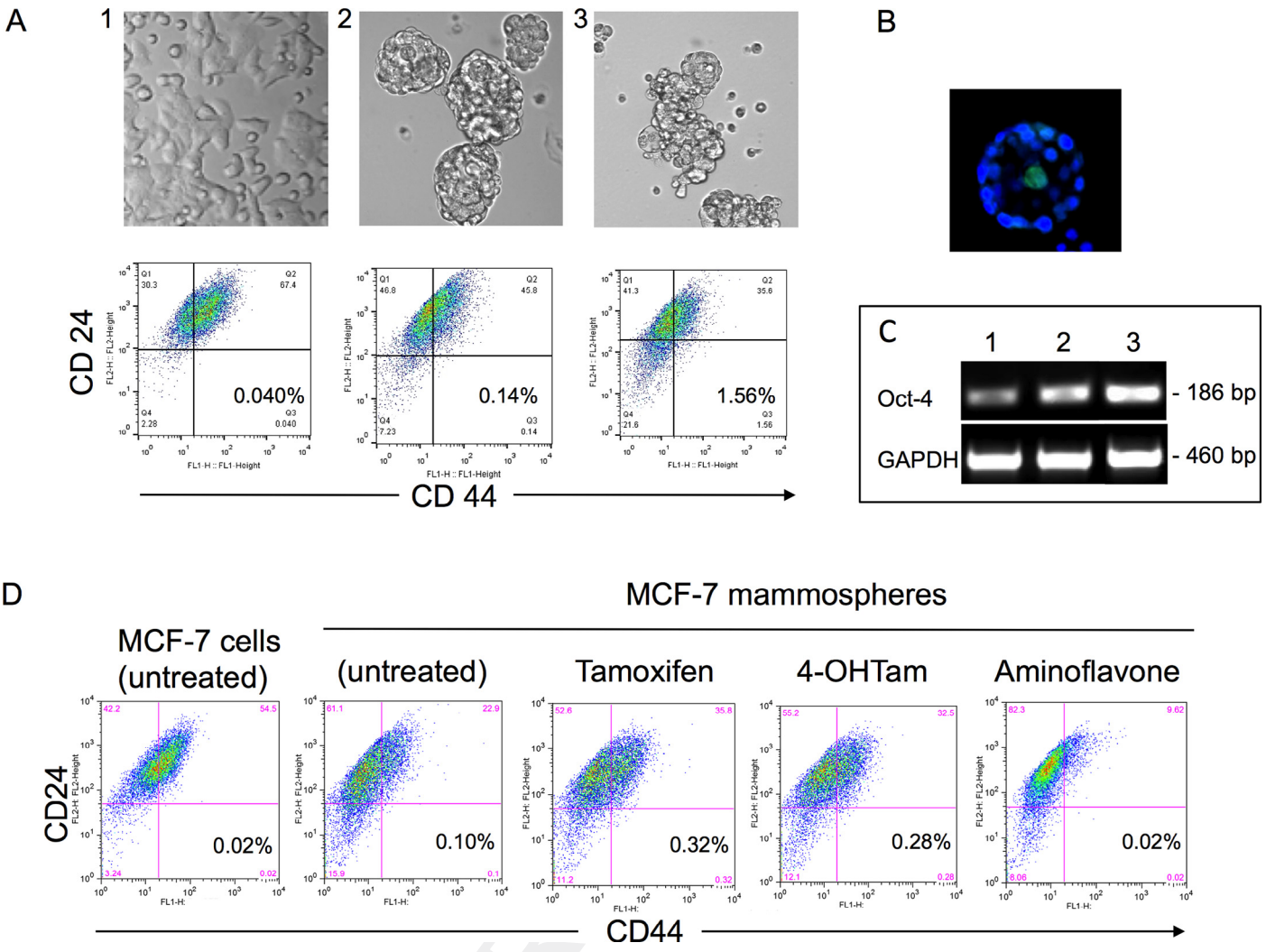

Fig. 2. MCF-7-derived mammospheres show an increase in $CD44^{+}/CD24^{-/low}$ levels in the presence of hypoxia. (A) 2D monolayer of MCF-7 cells cultured under normoxia (1), MCF-7derived mammospheres obtained from 7 d culture under normoxia (20.8% O_2) (2), or hypoxia (1% O_2) (3). Scale bar = 50 µm. 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 $CD44^{+}/CD24^{-/low}$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

AF-mediated suppression of α6-integrin was also demonstrated using flow cytometry (Fig. 3A). C-myc promotes breast tumorigenesis [35]. Since the α6-integrin promoter contains a c-myc binding site [36], 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 [37]. Moreover, the α6-integrin promoter contains an AP-1 binding site (Fig. S1B). Therefore, we evaluated α6-integrin, c-myc and Fra-1 gene expression in mammospheres following exposure to AF or Tamoxifen (Fig. 3B,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 (Fig. 3D). Taken together, Tamoxifen and AF differentially modulate genes to cause differences in cell fate, with Tamoxifen promoting mammosphere formation [28] and AF disrupting mammosphere formation.

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 CD44hi/CD24 low/- (Fig. S2A). 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 (Fig. S2B).

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 [18–20,38]. T47D ER+ breast cancer cells also exhibit sensitivity to AF [20,38]. We therefore sought to

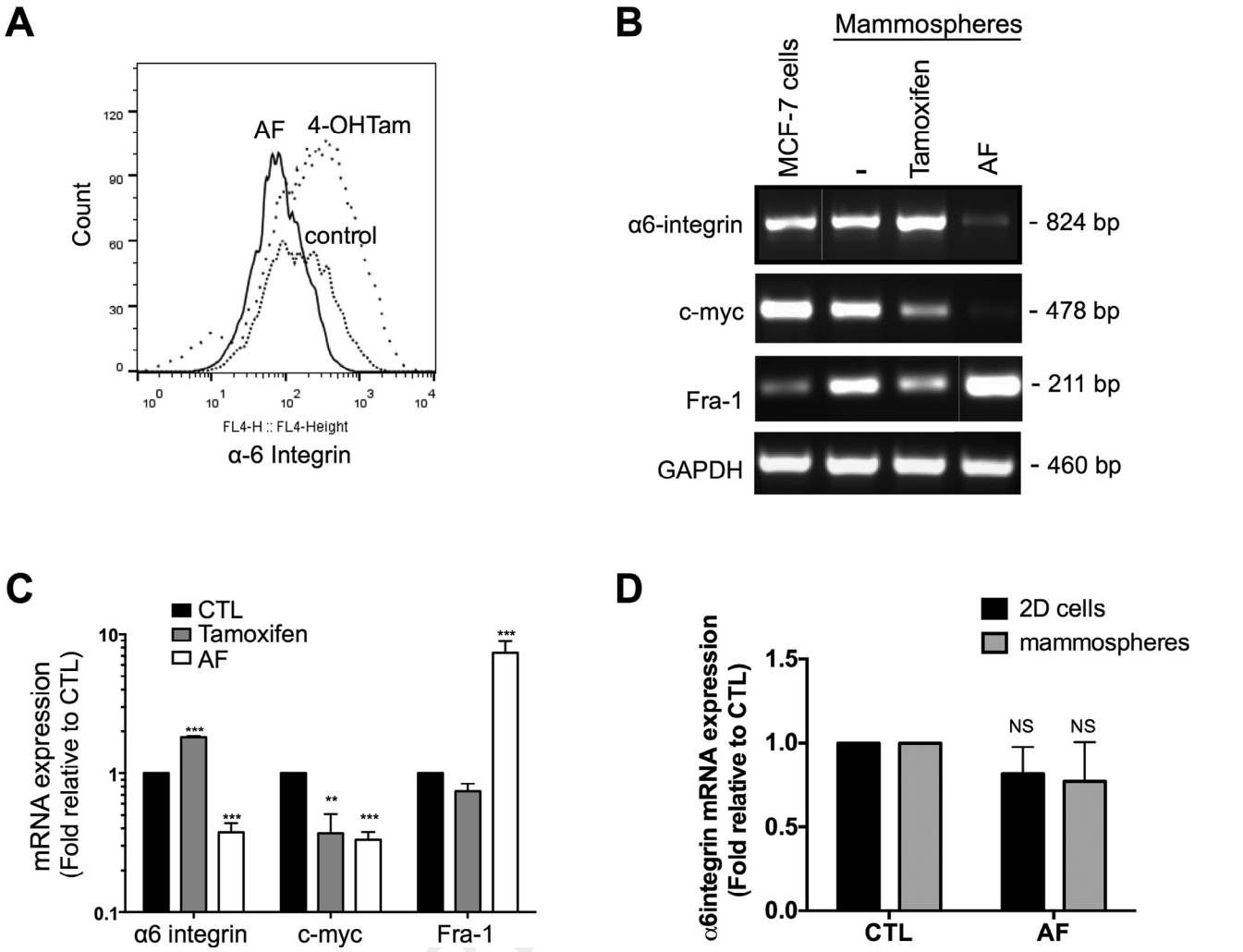

Fig. 3. AF and Tamoxifen differentially modulate gene expression in mammospheres. (A) Flow cytometry analysis was performed on cells from MCF-7 derived mammospheres exposed to control (0.1% DMSO), 1 μM 4OHTam or 1 μM AF for 48 h. (B) RNA was isolated from mammospheres exposed to 0.1% DMSO, 1 μM Tamoxifen 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 1 μM 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.

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 [22,26,39] 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 (Fig. 4A). 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 [22,26].

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 [18]. Similarly, we found that AF promoted AhR translocation in LM05-E breast cancer cells more so than LM05-Mix cells (Fig. 4B). 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.

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 (Fig. 5A,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.

AFP464 induces a less invasive phenotype in M05 tumors

Anticancer agents frequently alter breast tumor phenotype while suppressing tumor growth [40–42]. Histopathological analyses of M05 tumors revealed a semi-differentiated adenocarcinoma with papillary differentiation. Fig. 6 shows two different cell popula-

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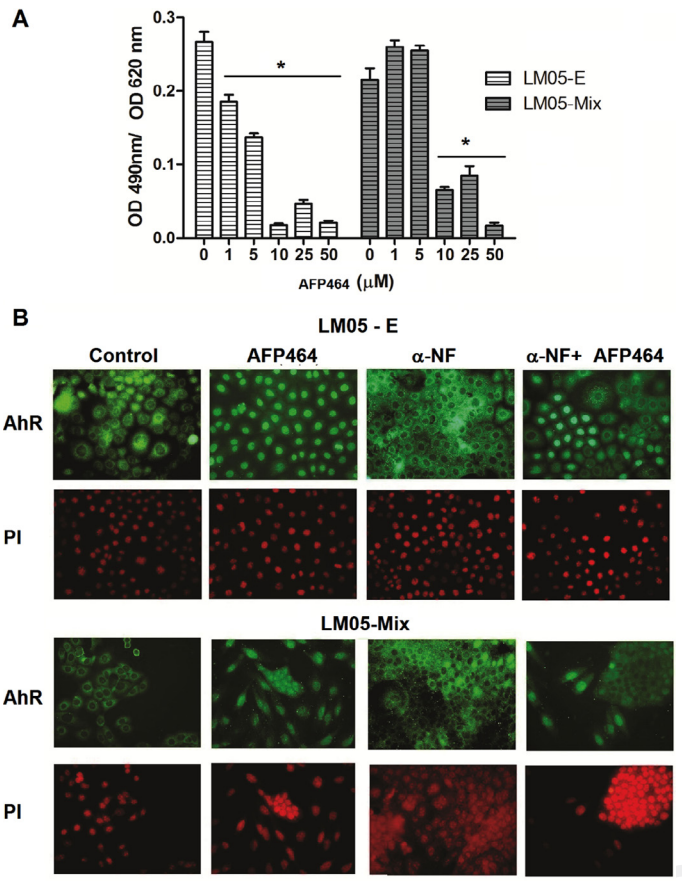

Fig. 4. AFP464 exhibits cytotoxicity in LM05 epithelial cells in an AhR-dependent fashion. (A) LM05-E and LM05-Mix cells were incubated with AFP464 for 5 d. Cellular viability was evaluated by the MTS assay. The values represent the average of three independent experiments ($n = 9$), *$p < 0.05$ compared 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). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

tions: 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 [43]. Our data suggest AFP464 promotes mucin lake formation resulting in a less invasive breast cancer phenotype.

AFP464 decreases mammosphere forming capacity of M05 mouse breast tumors

Chemotherapy and radiotherapy often increase the frequency of cells with stem cell properties [44]. 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 [27]. Tumors were then removed and prepared as single cell suspensions for mammosphere formation. AFP464 (12 mg/kg) effectively decreased M05-tumor derived mammosphere formation (Fig. 7A). This

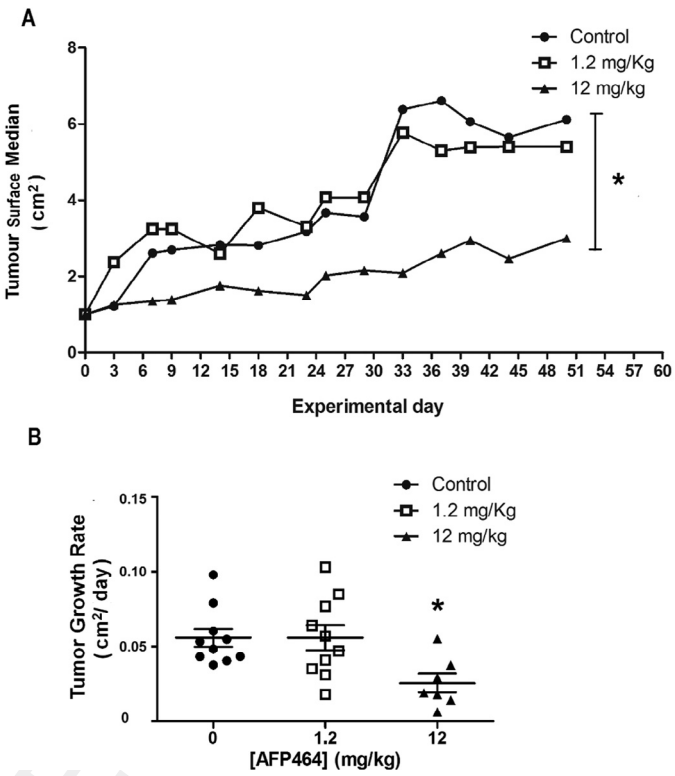

Fig. 5. AFP464 decreases M05 mouse breast tumor growth rate *in vivo*. Mice were inoculated with M05 tumor cells. When the tumors reached an average size of 1 cm^2, the mice were randomly divided into 3 groups and treated with vehicle or AFP464 (1.2 mg/kg, i.p., QD × 5 or 12 mg/kg, i.p., QD × 5) as outlined 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.

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

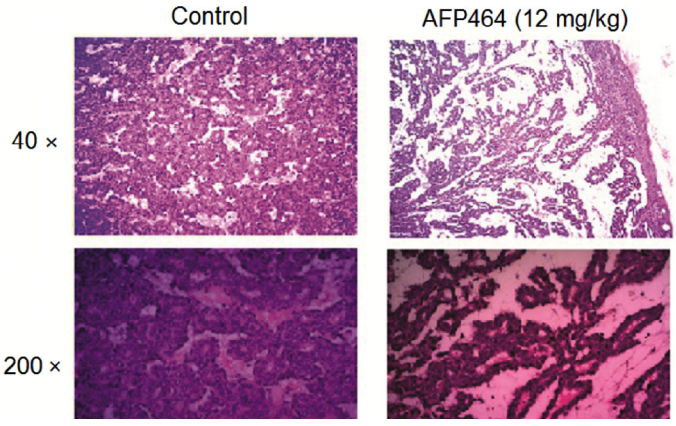

Fig. 6. AFP464 alters M05 breast tumor phenotype to resemble a less invasive subtype. Tumors excised from mice exposed to either vehicle or 12 mg/kg AFP464 were sectioned and stained with hematoxylin and eosin as described in materials and methods before being visualized microscopically (40× and 200×).

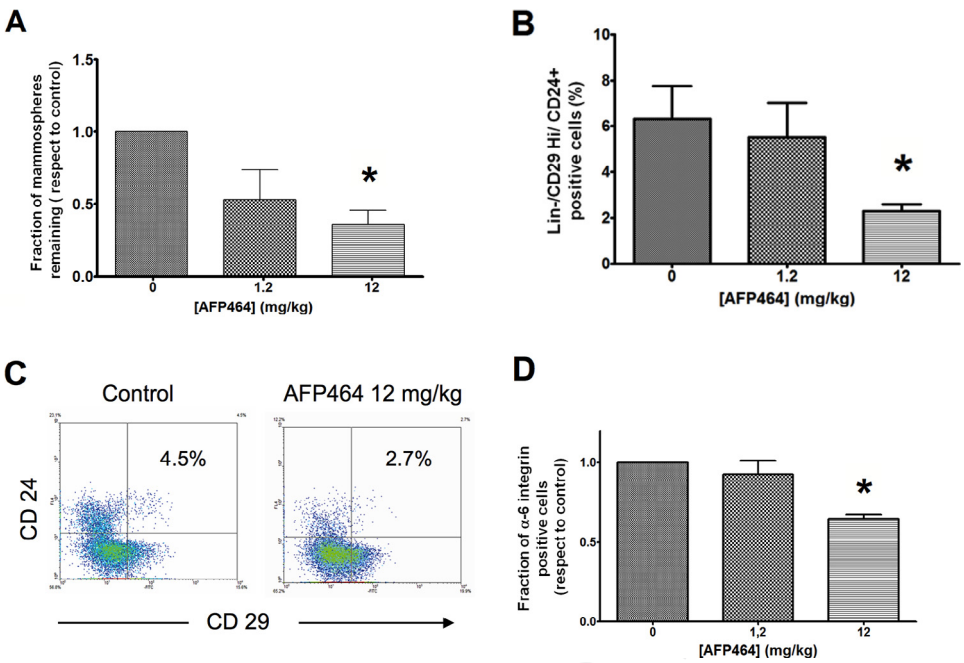

Fig. 7. AFP464 decreases the number of cells with mammosphere forming capacity and stem cell-like phenotype within the M05 tumor. Quantification of mammospheres derived from tumors grown in M05 mice treated with either vehicle (control) or AFP464 as detailed previously in Materials and Methods. Cells 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.

tumor readily stain for Lin (-)/CD29^h/CD24$^+$ [45,46]. AFP464 significantly decreased this population of cells at 12 mg/kg (Fig. 7B,C) AFP464 (12 mg/kg) also significantly decreased α6-integrin positive cells (Fig. 7D). These data suggest AFP464 suppresses breast cancer stem cell-like characteristics *in vivo*.

Discussion

Tumor initiating cells (TICs) resist the anticancer actions of chemotherapeutic and targeted anticancer agents [47,48]. This creates a rationale for combination therapy involving small molecules that target TICs and established anticancer agents [49–51]. AF and AFP464 elicit anticancer actions *in vitro* and *in vivo* respectively following AhR signaling activation [19,20,23,52,53]. 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 [54]. 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 [15,16]. 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 [55]. However, AhR agonist AF readily suppressed mammospheres similar to what was detected with another non-toxic AhR agonist [16].

Integrins regulate numerous cellular processes including proliferation and self-renewal [56–58]. In particular, α6-integrin is overexpressed in breast cancer to promote breast tumorigenesis and metastases [12]. α6-integrin overexpression is linked to reduced breast cancer patient survival [11].

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 (Fig. S2). MDA-MB-231 cells are completely unresponsive to the cytotoxic actions of AF [20,38]. 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 (Fig. 3B,C). This is consistent with findings that reveal Tamoxifen increases the propensity of cells to form mammospheres and the expression of stemness genes [28]. 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 [59]. Though TIC capacity occurs independently of EMT [60], 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 [61]. However, Fra-1 has also been found to drive stem cells out of dormancy to promote chemo-sensitization [37]. Fra-1 up-regulation may therefore constitute a facet of AF's

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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 [28] 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 [19]. AFP464 appears to alter tumor morphology to a mucinous phenotype (Fig. 6). Pure mucinous breast cancer carries a more favorable prognosis than mucinous breast cancer mixed with other invasive subtypes [62]. Mucins exhibit tumor suppressor activity in colorectal cancer [63] and a reduction in mucin lakes in breast tumors corresponds to a more aggressive state [64]. 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 [65], 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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Conflict of interest

The authors declare no conflict of interest.

Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.canlet.2016.03.025.

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