Targeting Treatment-Resistant Breast Cancer Stem Cells with FKBPL and Its Peptide Derivative, AD-01, via the CD44 Pathway

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Abstract

Purpose: FK506-binding protein like (FKBPL) and its peptide derivative, AD-01, have already shown tumor growth inhibition and CD44-dependent antiangiogenic activity. Here, we explore the ability of AD-01 to target CD44-positive breast cancer stem cells (BCSC).

Experimental Design: Mammosphere assays and flow cytometry assays were used to analyze the effect of FKBPL overexpression/knockdown and AD-01 treatment ± other anticancer agents on BCSCs using breast cancer cell lines (MCF-7/MDA-231/ZR-75), primary patient samples, and xenografts. Delays in tumor initiation were evaluated in vivo. The anti–stem cell mechanisms were determined using clonogenic assays, quantitative PCR (qPCR), and immunofluorescence.

Results: AD-01 treatment was highly effective at inhibiting the BCSC population by reducing mammosphere-forming efficiency and ESA⁺/CD44⁺/CD24⁻ or aldehyde dehydrogenase (ALDH)⁺ cell populations in vitro and tumor initiation in vivo. The ability of AD-01 to inhibit the self-renewal capacity of BCSCs was confirmed; mammospheres were completely eradicated by the third generation. The mechanism seems to be due to AD-01–mediated BCSC differentiation shown by a significant decrease in the number of holoclones and an associated increase in meroclones/paraclones; the stem cell markers, Nanog, Oct4, and Sox2, were also significantly reduced. Furthermore, we showed additive inhibitory effects when AD-01 was combined with the Notch inhibitor, DAPT. AD-01 was also able to abrogate a chemo- and radiotherapy-induced enrichment in BCSCs. Finally, FKBPL knockdown led to an increase in Nanog/Oct4/Sox2 and an increase in BCSCs, highlighting a role for endogenous FKBPL in stem cell signaling.

Conclusions: AD-01 has dual antiangiogenic and anti-BCSC activity, which will be advantageous as this agent enters clinical trial. Clin Cancer Res; 1–13. ©2013 AACR.

Introduction

Chemo- and radiotherapy are still the best treatment options for metastatic breast cancer. However, while these therapies are very effective in reducing tumor size, resistance invariably becomes a major issue. It is now well established that cancer stem cells (CSC) are considered one of the main causes of tumor recurrence and resistance to both chemo- and radiotherapy (1). In breast cancer, about 40% of patients experience tumor recurrence often as distant metastasis, with only a small percentage of tumors recurring locally (2).

Breast CSCs (BCSC), in particular, are enriched in the epithelial surface antigen (ESA)⁺/CD44⁺/CD24⁻/low subfraction of cells. These cells have the ability to self-renew, have enhanced tumorigenic potential in vivo, and are associated with poor outcome in patients with breast cancer (3). BCSCs are also able to generate progenitor cells that can differentiate into mature and differentiated cells. The CD44⁺/CD24⁻ cell subpopulation has been associated with an abundance of proinvasive genes (4) and increased tumor aggressiveness (5); higher proportions of CD44⁺/CD24⁻ cells were also associated with distant metastasis (6). Furthermore, there is evidence to suggest that CD44⁺/CD24⁻ cells are resistant to various chemo- and radiotherapy regimens, leading to an increase in CD44⁺/CD24⁻ cells, following standard treatment.
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Translational Relevance

FK506-binding protein like (FKBPL) is emerging as an important antitumor protein. FKBPL (i) inhibits tumor growth, (ii) is a prognostic and predictive breast cancer biomarker, and (iii) is a naturally secreted antiangiogenic protein that inhibits blood vessel development by targeting the cell surface receptor, CD44. Here, we have shown that FKBPL’s ability to target CD44 makes it useful for targeting cancer stem cells (CSC), which are enriched for CD44. CSCs can self-renew, are highly chemo- and radioresistant and highly metastatic, and are associated with poor outcome. Our data strongly suggest that FKBPL has a role in CSC signaling, and that its therapeutic peptide targets CD44 and can reduce this resistant cell population by differentiating these cells into a treatment-sensitive phenotype. This additional antitumor activity will add significant therapeutic benefit, as FKBPL-based peptides approach phase I/II clinical trials, with potential implications for scheduling, in combination with standard therapies, in late-stage trials.

FK506-binding protein like (FKBPL) is a divergent member of the FK506-binding protein family. FKBPL was identified as having a role in the response of cells to radiation (21, 22). In a complex with Hsp90, FKBPL stabilizes p21 (23) and regulates estrogen receptor (ER), androgen receptor, and glucocorticoid receptor signaling (2, 24, 25). Furthermore, in breast cancer, because of FKBPL’s association with ER, FKBPL has shown potential as both a prognostic and predictive biomarker of response to endocrine therapy (2, 26). However, the most recently identified role for FKBPL is as a secreted antiangiogenic protein, which is dependent on CD44 for its activity (27, 28), and more recently, we have generated data that strongly suggests that FKBPL/AD-01 bind and regulate CD44 (28). Peptides based on the active antiangiogenic domain of FKBPL, including a 24 amino acid peptide, AD-01, also showed potent anti-tumor activity in the subnanomolar range, in breast and prostate xenograft models; an FKBPL-based peptide will enter phase I clinical trials shortly (29). In light of FKBPL/AD-01’s dependency on CD44, it was hypothesized that they might also target CD44+ BCSCs. Here, we describe the role of FKBPL in stem cell signaling and provide evidence that AD-01 indeed has activity against BCSCs in addition to its well-characterized antiangiogenic action, suggesting enhanced clinical use.

Materials and Methods

Cell culture

All cells were obtained from the American Type Culture Collection and were authenticated by short-tandem repeat (STR) profiling carried out by the suppliers, and routine testing revealed that these cells were Mycoplasma-free. The MCF-7 and MDA-231 cell lines were grown in Dulbecco’s modified Eagle medium (DMEM; Life Technologies) and the ZR-75 cell line was maintained in RPMI-1640 medium (Life Technologies) both supplemented with 10% fetal calf serum (FCS; PAA). Cells stably overexpressing FKBPL (3.1D2 and A3) were additionally grown in the presence of 375 μg/mL (3.1D2) or 750 μg/mL (A3) G418 (Sigma). All experiments were carried out at 37°C in a humidified atmosphere of 95% O2/5% CO2.

Mammosphere assay

Briefly, 500 cells/cm2 were seeded in 6-well dishes in nonadherent culture as described previously (30). Second- and third-generation mammospheres were formed by disaggregating primary or secondary mammospheres and seeding the same number of cells in new wells ± fresh drug (31). The results were expressed as secondary/primary or tertiary/secondary ratio normalized to control. Mammospheres were counted using Nikon Eclipse TE300 (Japan) microscope under ×4 magnification.

protocols (7, 8). Although this seems to be somewhat controversial with some reporting on a reduction in the CD44+/CD24− cell population following therapy (9, 10) and others reporting no association found between chemoresistance and the percentage of CD44+/CD24− cells measured by pathologic complete response (11).

The functional relevance of CD44/CD24 markers in relation to breast cancer stemness is poorly understood. However, CD44 seems to play a major role in the homing of leukemic stem cells to bone marrow and initiation of acute myelogenous leukemia (AML; ref. 12). An activating anti-CD44 antibody prevented the tumorogenic potential of AML cells, and leukemia cells lacking CD44 could not home to bone marrow. Also, when CD44 was knocked down in CD44+/CD24− breast cancer cell subpopulation, the stemness of the cells was lost in relation to their tumorogenic potential, cell cycle, and gene expression leading to differentiation of stem cells to non-stem cells (13). This shows the importance of CD44 in maintaining the stemness of the BCSCs. CD44 is also involved in the feedback communication from the microenvironment to the CSCs (14). Therefore, CD44 seems to be associated with epithelial-to-mesenchymal transition, apoptosis, and drug resistance, all features of CSCs (15). Furthermore, the well-established stem cell markers, Nanog and Oct4, seem to be regulated by hyaluronic acid (HA)-induced CD44 signaling; HA binding to CD44 at the surface, mediates the intracellular domain of CD44 to form a complex with Nanog, followed by Nanog nuclear translocation and activation of the Nanog-Oct4 network (16). CD44, HA, and Nanog have been reported to be overexpressed in breast and ovarian tumors and heavily involved in tumor initiation and development (17, 18). Nevertheless, the role of CD44 in BCSCs needs further investigation as a potential therapeutic target.

Another important marker of stemness, Sox2, physically binds to the Oct4 and Nanog protein complex and regulates cell differentiation (19). Sox2 was found to be highly expressed in early-stage breast tumors and activated in BCSCs during early tumor initiation (20).

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Clonogenic assay

Methods were described previously (2). AD-01 treatment was added from day 2 for the duration of the experiment. Morphologically distinct colonies representing holoclones, meroclones, and paraclines were counted manually.

Combination drug treatments with AD-01 in vitro

The MDA-231 and MCF-7 monolayers were treated with IC_{50} doses of docetaxel (0.2 nmol/L, MDA-231) and cisplatin (7 nmol/L, MCF-7; 70 nmol/L, MDA-231) or radiation (2 Gy of radiation in a single dose) ± AD-01 (1 or 100 nmol/L) for 3 days. Subsequently, a single-cell suspension was prepared from trypsinized monolayers and 500 cells/cm² were seeded in triplicate in low adherent 6-well plates for mammosphere formation. For inhibition of the Notch pathway, gamma secretase inhibitors (GSI), compound E (0.025–1.25 μmol/L; Calbiochem) or DAPT (N-[N-((3, 5-difluorophenacetyl-L-alanyl)-S-phe-nylglycine t-butyl ester}; 10 μmol/L, Calbiochem) were added ± AD-01 (0.025, 1, and 100 nmol/L) directly into mammosphere culture.

Flow-cytometric analysis and sorting

Cell monolayers were treated with AD-01 for 72 hours. Flow cytometry was conducted as previously described (30). Fluorescence was measured using BD FACS Canto II and analyzed by WinMDI 2.9.

ALDEFLUOR assay

Following 72 hours AD-01 treatment of MDA-231 and MCF-7 monolayers, 1 × 10^6 cells were mixed with BAAA [BODIPY—aminoacetalddehyde diethyl acetate reagent; ALDEFLUOR kit (Stem Cell Technologies)] ± diethylami-nobenzaldehyde (DEAB; Stem Cell Technologies) inhibitor. Cells were incubated for 40 minutes at 37°C before being suspended in the ALDEFLUOR assay buffer (Stem Cell Technologies). Fluorescence was measured using FACSCalibur and analyzed using WinMDI 2.9. The aldehyde dehydrogenase (ALDH)^+ subpopulation was obtained in the absence of DEAB.

Ex vivo ZR-75 xenograft mammosphere assay

ZR-75 cells were implanted into severe combined immunodeficient (SCID) mice. Once tumors were established (~150 mm³), nanoparticles containing a delivery vehicle and FKBPL-targeted (n = 4) or nonto-targeted (n = 3) siRNA were delivered biweekly into intratumoral injection. Tumors were excised at quadrupling volume (~2–3 weeks after the start of the treatment), disaggregated, and assessed using the ex vivo mammosphere assay as described below for primary tumors.

Primary tumor cell isolation

Frozen single-cell suspensions from harvested pleural effusions collected from patients with metastatic breast cancer (n = 3) with fully informed consent (COREC# 05/ Q1403/25 and 05/Q1403/159; Paterson Cancer Institute, Manchester, United Kingdom) were used in the mammosphere assay and treated directly in mammosphere culture plates for 72 hours ± AD-01 (0.05 and 5 nmol/L). Patient-derived xenografts (PDX) were also used, where cells from primary metastatic breast samples were grown in mice, excised, and used in the mammosphere assay (n = 2). Six solid breast tumor mastectomy samples were collected from patients with fully informed consent (11/NI/0013/-.NIB11-0014; Northern Ireland Biobank), cut into small pieces (≤1 mm), and digested overnight in DMEM/HEPES (Gibco) containing 10% collagenase/hyaluronidase (Stem Cell Technologies). Digested tissue was then filtered through 100, 70, and 40 μm cell strainers (BD Technologies) and seeded for the mammosphere assay ± AD-01.

Gene expression analysis

The raw microarray gene expression data from GSE7390 (32) were downloaded from National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) database (33) into Partek Genomic Suite (Partek Inc.) and processed using robust multichip average (RMA; ref. 34) method to generate the normalized expression values. Expression values for the probe set 219187_at representing FKBPL were assessed for normal distribution using Kolmogorov–Smirnov test and used to stratify samples with high or low FKBPL based on median expression. Similarly, samples were stratified into high or low Nanog (probe set 220184_at) or Oct4 (probe set 208266_s_at representing POU5F1) or Sox2 (probe set 213721_at) expression based on median expression values. For further analysis, only samples with opposing expression pattern between Nanog and FKBPL were selected, that is, samples with high FKBPL-low Nanog or low FKBPL-high Nanog (n = 94). Similarly, for a separate analysis samples with high FKBPL-low Oct4 or low FKBPL-high Oct4 were only taken forward (n = 104). Another analysis was conducted with samples that are high FKBPL-low Sox2 or low FKBPL-high Sox2 (n = 94). Kaplan–Meier survival curves were generated on the basis of median-stratified data on Nanog, Oct4, or Sox2, respectively, with the associated survival data and compared using Mantel–Cox log-rank test. Multivariate Cox regression analysis was conducted with parameters including age, surgery type, histopathology type, angio-invasion, lymphocytic infiltration, histopathological grade, and ER status along with Nanog, Oct4, or Sox2 stratification.

In vivo tumor initiation assay

A total of 5 × 10^6 MCF-7 cells were implanted subcutaneous into SCID mice bearing estrogen pellets (0.25 mg) on day 1. PBS (vehicle control) or AD-01 (0.3 mg/kg/d) were administered daily, from day 1, by subcutaneous injection until tumors reached 100–150 mm³ size (n = 4/group). Tumor volume was calculated as 4/3πr³ [r is ½ GMD and GMD = (length × breadth × height)]. Tumor take and growth was monitored every 3 days.
Limiting dilution assay in vivo
MCF-7 cells were implanted subcutaneously as described previously and SCID mice bearing established MCF-7 xenografts were treated subcutaneously with PBS (vehicle control) and AD-01 (0.3 mg/kg/d) for 2 weeks and tumors were measured every 3 days. Following 2 weeks of treatment, tumors were excised, disaggregated, and reimplanted subcutaneously into SCID mice at varying concentrations (5 \times 10^6, 2.5 \times 10^6, and 1 \times 10^6 cells/mouse; n = 6/treatment group). Time taken for tumor initiation and growth were measured up to 80 days. All animal experiments were carried out in accordance with the Animal (Scientific Procedures) Act 1986 and conformed to the current United Kingdom Co-ordinating Committee on Cancer Research guidelines.

Quantitative real-time PCR
MCF-7 and MDA-231 cell monolayers were treated with AD-01 (100 nmol/L) for 48 hours \pm 10 minutes treatment of HA [0.1 mg/ml (234 kDa; Lifecore Biomedicals)] or transfected with a pool of FKBPL siRNA (50 nmol/L; Invitrogen) or nontargeted siRNA for 48 hours before being harvested for PCR. Cells were prepared for quantitative real-time PCR (qRT-PCR) using Power SYBR Green (Life Technologies). 18S, forward: AGTCCCTGCCCTTTGTACACA, reverse: GATGGCTCGAGAAGGATGTGGTCCG, and 100 nmol/L AD-01, although the effects were less dramatic (Supplementary Fig. S1B). Stable overexpression of FKBPL in MDA-231 (A3; Fig. 1E) and MCF-7 (3.1D2; Supplementary Fig. S1C) cells also led to a significant reduction in MFE normalized to control (MDA-231 vs. A3; MFE = 1 vs. MFE = 0.64; and MCF-7 vs. 3.1D2; MFE = 1 vs. MFE = 0.77). Nevertheless, there was no further reduction in the formation of secondary mammospheres in both cell lines.

To further validate these findings, flow cytometry was used to quantitate the AD-01–mediated reduction in BCSC using the well-characterized BCSC surface markers, ESA+/CD44+/CD24−, following treatment of MDA-231 and MCF-7 monolayers with AD-01 for 72 hours. Figure 2A shows the change in the ESA+/CD44+/CD24− subpopulation of MDA-231 cells following exposure to AD-01; a significant reduction in the ESA+/CD44+/CD24− cell subpopulation was observed following treatment with 1 and 100 nmol/L AD-01 in both MCF-7 (Fig. 2B and Supplementary Table S1A) and MDA-231 (Fig. 2C and Supplementary Table S1B) cell lines. Furthermore, the ALDEFLUOR assay was used to analyze the effect of AD-01 on the ALDH+ cell subpopulation, which is also representative of the BCSCs (33). In support of our previous observations, a significant more than 50% reduction in the ALDH+ subpopulation was achieved after AD-01 treatment in the MCF-7 (Fig. 2D and E and Supplementary Table S1C) and MDA-231 cell lines (Fig. 2F and Supplementary Table S1D). Statistical significance was obtained in all datasets.

Results
FKBPL and AD-01 reduce BCSC number and AD-01 affects the self-renewal capacity of cell lines in vitro
The mammosphere assay was used to assess the ability of AD-01 to affect BCSC numbers in vitro in ER+, MCF-7 and ZR-75 cells, and the ER− MDA-231 cell line. A significant reduction in mammosphere-forming efficiency (MFE) was obtained across all cell lines at 1 and 100 nmol/L (Fig. 1A–C). However, there was no clear linear dose response (Supplementary Fig. S1A), as seen previously with this peptide (27); the most effective dose in MDA-231 was 100 nmol/L showing approximately 40% reduction in MFE (Fig. 1A). In MCF-7 and ZR-75 cells, 1 and 100 nmol/L AD-01 reduced MFE by approximately 30% and 35%, respectively (Fig. 1B and C).

To establish whether the peptide was effective at inhibiting the self-renewal capacity of BCSCs over successive mammosphere generations, primary, secondary, and tertiary mammosphere generations were seeded. AD-01 (100 nmol/L) clearly and significantly reduced the MFE in the MDA-231 cell line, across 3 generations, until tertiary mammospheres were completely eradicated (Fig. 1D); mimicking the repeat dosing of drugs that patients would receive clinically. Similar results were achieved using 1 nmol/L AD-01, although the effects were less dramatic (Supplementary Fig. S1B).

Immunofluorescence
Following the same treatments as described earlier, MCF-7 and MDA-231 cells were plated onto coverslips, washed with PBS, and fixed using 4% formaldehyde (Thermo Scientific) for 10 minutes, permeabilized using 0.2% Triton X-100 (Sigma) for 10 minutes, blocked with 0.2% fish skin gelatin (FSG) for 1 hour at room temperature, and incubated overnight at 4°C with primary antibodies against Nanog (1:100; Abcam) and Oct-4 (1:100; Millipore). Subsequently, the coverslips were washed with PBS, incubated with Alexa Fluor–conjugated secondary antibodies (1:500; Invitrogen) in 0.2% FSG for 1 hour at room temperature, with Alexa Fluor–conjugated secondary antibodies (1:500; Invitrogen) in 0.2% FSG for 1 hour at room temperature, washed again with PBS, dehydrated, air-dried, and embedded in 4’,6-diamidino-2-phenylindole (DAPI) containing Prolong Gold antifade reagent (Invitrogen). Fluorescence was detected using a Nikon Eclipse 90i microscope. Images were processed using NIS-Elements software.

Statistical analysis
Data presented are a mean of at least 3 independent experiments \pm SE. One-way ANOVA or two-tailed t tests were used to assess differences between various treatments. Statistical significance was determined by the P values less or equal to 0.05; *, P < 0.05; **, P < 0.01; ***, P < 0.001.
such as quiescence with extended G2 cell cycle, improved ability to repair DNA double-strand breaks (36), lower levels of reactive oxygen species (37), and drug efflux pumps, BCSCs are able to resist the fatal effects of the conventional therapies. Therefore, it was necessary to show that AD-01 could reduce chemo- and radioresistance. We show that treatment of MDA-231 cells with a single radiation dose of 2 Gy, increased MFE significantly (Fig. 3A; MFE = 2.05); however, this was abrogated when AD-01 was added to the treatment regimen (1 nmol/L: MFE = 1.07; 100 nmol/L: MFE = 0.98). Similarly, when MDA-231 monolayers were treated with docetaxel, there was an enrichment in MFE [Fig. 3B; MFE = 1.57 (docetaxel) vs. MFE = 1 (control)]. Nevertheless, this was abrogated when AD-01 was added to the treatment regimen [Fig. 3B; MFE = 1.15 (docetaxel/AD-01) vs. MFE = 1.57 (docetaxel alone)]. AD-01 was also able to abrogate the cisplatin-induced increase in MFE in both MDA-231 (Fig. 3C) and MCF-7 (Fig. 3D) cell lines, suggesting that AD-01 would be a useful therapy to sensitize this treatment-resistant tumor cell population.

AD-01 and the Notch inhibitor, DAPT, have an additive inhibitory effect on BCSCs

To assess whether AD-01 could potentiate the effects of notch inhibitors, strongly implicated for their anti-BCSC effect (38), we evaluated the Notch pathway GSI, DAPT, in combination with AD-01. Following a 72-hour combinational treatment with 100 nmol/L AD-01 and 10 μmol/L DAPT, a 65% reduction in MFE (Fig. 3E) was observed, compared with just more than 40% reduction in MFE when DAPT was used on its own (Fig. 3E). Similar results were shown with another GSI, compound E (Supplementary Fig. S2). The additive effects of AD-01 in combination with DAPT or compound E suggest that the anti–stem cell effect mediated by AD-01 could possibly target pathways additional to the CD44 pathway.

AD-01 reduces the number of BCSCs by differentiating them into more "typical" cancer cells

In an attempt to investigate the fate of the BCSCs following treatment with AD-01, we assessed colony morphology using a clonogenic assay. Tan and colleagues (39) and

![Figure 1. FKBPL and its peptide derivative, AD-01, reduce mammosphere formation in breast cancer cell lines and AD-01 affects self-renewal capacity. The effect of AD-01 treatment on primary MFE in the (A) MDA-231 (representative image of MDA-231 mammospheres ± AD-01 in inset), and (B) MCF-7 (a representative image of MCF-7 mammospheres ± AD-01 treatment in inset), and (C) ZR-75 cell lines. D, primary (1st), secondary (2nd), and tertiary (3rd) generations of mammospheres in the MDA-231 cell line after treatment with 100 nmol/L AD-01. E, first and second generations of mammospheres in the MDA-231 cell line versus MDA-231 cells stably overexpressing FKBPL (A3). MFE was calculated for each generation. Data points are mean ± SEM. n ≥ 3. *, P < 0.05; **, P < 0.01; *** , P < 0.001 (one-way ANOVA). GAPDH, glyceraldehyde-3-phosphate dehydrogenase.](www.aacrjournals.org)
Harrison and colleagues (40) showed that holoclones were morphologically distinct colonies resembling CSCs with regard to CD44<sup>high</sup>/CD24<sup>low</sup> levels, and were more tumorigenic/undifferentiated and chemoresistant in comparison with meroclones and paraclones. A holoclone will change its colony morphology to meroclones or paraclones as it becomes more differentiated represented by MCF-7 cells (Fig. 4A). AD-01 did not affect overall cell survival compared with control indicating its lack of toxicity in both MCF-7 and MDA-231 cells (Supplementary Fig. S3), as previously reported (27). In support of our previous MFE data, the number of holoclones was reduced by approximately 25% to 30% after AD-01 treatment in both the MCF-7 and MDA-231 cell lines (Fig. 4A and B). Furthermore, AD-01’s ability to differentiate BCSCs was reinforced by the same (~30%) increase in the number of meroclones and paraclones in both MCF-7 and MDA-231 cell lines (Fig. 4A and B).

AD-01 and endogenous FKBPL affect BCSC signaling associated with regulation of Nanog, Oct4, and Sox2 downstream from CD44 pathway

Three markers associated with self-renewal and maintenance of stem cell fate, Nanog, Oct4, and Sox2, which act downstream of CD44, were examined following treatment with AD-01 (41, 42, 20). When AD-01 was added exogenously to MCF-7 (Fig. 4C and D) or MDA-231 (Fig. 4E and Supplementary Fig. S4A) cell monolayers, there was a significant reduction in Nanog and Oct4 protein levels and mRNA levels of Nanog and Oct4; and Sox2. In MCF-7 cells, the CD44 ligand, HA, mediated an increase in Oct4 protein (Fig. 4C) and mRNA levels (Fig. 4D) and a slight upregulation of Nanog mRNA levels as previously reported (14); this was abrogated when AD-01 was added alone or in combination with HA (Fig. 4C and D), supporting an AD-01–mediated attenuation of CD44 signaling. In MDA-231 cells, AD-01 treatment showed a statistically significant reduction in all three stem-cell markers, Oct4, Nanog, and Sox2 at the mRNA level (Fig. 4E). Furthermore, AD-01 seems to abrogate nuclear accumulation of Nanog and Oct4, which is opposite to the HA-CD44 signaling effect.

To establish the role of endogenous FKBPL in CSC signaling, mice harboring ZR-75 xenografts were treated via direct intratumoral injection with FKBPL-targeted or -nontargeted siRNA. FKBPL knockdown induced a 56% increase in the number of mammospheres formed ex vivo (FKBPL siRNA: MFE 1.76 vs. nontargeted siRNA: MFE 1.00).
MFE = 0.78; Fig. 5A). This result was also confirmed in vitro (Fig. 5B). FKBPL knockdown was confirmed in vivo (Fig. 5A, inset) and in vitro (Fig. 5B, inset).

Furthermore, the role of endogenous FKBPL in CSC differentiation was investigated. FKBPL knockdown in MCF-7 cells led to a significant increase in Nanog and Oct4 protein levels measured by immunofluorescence (Fig. 5C) and Nanog, Oct4, and Sox2 mRNA levels (Fig. 5D). Also, there seems to be an increased nuclear translocation of Nanog and Oct4 when FKBPL is silenced. Similar results were obtained in the MDA-231 cell line (Fig. 5E and Supplementary Fig. S4B).

**AD-01 treatment reduces the BCSC population in clinically derived primary and metastatic breast tumors and high FKBPL levels and low Nanog levels are associated with improved patient survival**

AD-01’s anti-stem cell effect was further evaluated on clinically relevant primary solid breast tumor samples and highly metastatic breast cancer samples from both pleural effusions and PDX. Treatment with 1 and 100 nmol/L AD-01 was able to significantly reduce the number of BCSCs in primary breast tumors by more than 50% in the mammosphere assay (Fig. 6A); there seemed to be no difference in response between tumor stage and grade (Supplementary Table S2A). Importantly, AD-01 was also able to reduce the number of CSCs in metastatic samples, indicated by a statistically significant reduction of mammospheres even at low AD-01 concentrations (0.05 and 5 nmol/L), resulting in approximately 20% MFE inhibition, when normalized to control (Fig. 6B). However, there was a wide variation in the %MFE in the metastatic samples, lowering the significance of the raw data. Nevertheless, the modest AD-01-mediated effect observed in these metastatic samples, is important given that they are derived from end-stage highly treatment-resistant tumors, as indicated by the increased MFE compared with primary tumor isolates (1.2 vs. 0.4). To evaluate whether endogenous FKBPL could predict survival
outcomes in patients with breast cancer, we assessed levels of FKBPL and the stem cell markers, Nanog, Oct4, and Sox2, in a publicly available breast cancer microarray dataset (GSE7390). We show that tumor FKBPL and Nanog inversely correlate with survival outcomes in patients with breast cancer (n = 94); high FKBPL and low Nanog, correlates with improved overall survival (OS; Fig. 6C), distant metastasis-free survival (DMFS; Supplementary Fig. S5) and recurrence-free survival (RFS; Supplementary Fig. S5). In a multivariate Cox regression analysis, to test the independent prognostic relevance of these two combined markers, high FKBPL/low Nanog expression were independent markers of improved OS (log-rank test, P = 0.03; HR, 3.01) and DMFS (log-rank test, P = 0.023; HR, 3.18), whereas a trend toward better RFS was observed (log-rank test, P = 0.06; HR, 2.00). In a similar analysis, high FKBPL/low Sox2 expression (Supplementary Fig. S6) showed borderline significance toward better OS (log-rank test, P = 0.06; multivariate Cox analysis HR, 2.6), DMFS (log-rank test, P = 0.07; multivariate Cox analysis HR, 2.44) and a trend toward better RFS (log-rank test, P = 0.18; multivariate Cox analysis HR, 1.89). Furthermore, high FKBPL/low Oct4 showed a trend toward improved OS, DMFS, and RFS (Supplementary Fig. S7).

AD-01 modulates tumor initiation and the BCSC population in vivo

To validate the anti–stem cell activity of AD-01 in vivo, a tumor initiation experiment and limiting dilution assays were used for the MCF-7 xenograft model. For the tumor initiation assay, mice were treated with AD-01 (0.3 mg/kg/d) from day 1 of tumor implantation and observed for tumor initiation over a period of 29 days. The AD-01 treated group showed a 7-day delay in tumor initiation and a significantly lower tumor growth rate (Fig. 6D and Supplementary Table S2B). In the limiting dilution assay, mice with established xenografts were treated with PBS or AD-01 for 15 days. Tumors were then excised and disaggregated;
tumor cells were used in the mammosphere assay, for quantitative PCR (qPCR) analysis or reimplanted into secondary mice at three different concentrations. Following reimplantation, a statistically significant delay in tumor initiation was shown in secondary (untreated) mice injected with three different concentrations of AD-01–treated xenograft cells (Fig. 6E).

Ex vivo analysis of mammospheres showed a 50% reduction in the MFE in the AD-01 treatment group (Fig. 6F). Furthermore, qPCR analysis of MCF-7 xenografts showed statistically significant reduction in all three stem cell markers, Nanog, Oct4, and Sox2 in the AD-01 treatment group (Fig. 6G).

Discussion
We have provided evidence that FKBPL and its peptide derivative, AD-01, are effective at targeting BCSCs in vitro, using cell line models and primary breast tumor tissues, and in vivo using breast tumor xenografts. This was established using three independent assays, as there is evidence in the literature that there are no "perfect" markers of stemness (43). Although we do not see identical effects across all 3 assays, we consistently observe a significant AD-01–mediated reduction in the BCSC population across all endpoints used. Furthermore, the functional mammosphere assays showed that AD-01 was able to reduce BCSCs by up to 40% in the first generation and completely eradicate BCSCs by the third generation. Repeat dosing of AD-01 in a clinical setting should therefore prevent tumor recurrence by the AD-01–mediated targeting of BCSCs. This newly identified mode of action for AD-01, in addition to its already well-established antiangiogenic activity (27) will be advantageous clinically, in light of the hypothesis that current
High antiangiogenic agents could increase the pool of CSCs by creating hypoxia (44). We also provide evidence that endogenous FKBPL plays a role in CSC signaling. FKBPL overexpression reduced BSCSs in first-generation mammosphere assay only; there was no enhancement of this effect in second-generation mammospheres, probably because there was no further modulation of FKBPL levels overexpression reduced BSCSs in first-generation mammospheres, probably because there was no further modulation of FKBPL levels resulting in decreased FKBPL expression (high FKBPL) and tumors grown to 150 mm³. We also provide evidence that endogenous FKBPL plays a role in CSC signaling. 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We have previously shown using publicly available microarray datasets (GSE7390) and a breast cancer tissue microarray (n = 498) that high levels of FKBPL were associated with better OS, DMFS, and prolonged RFS (2, 26). Here, we have also shown that high FKBPL levels were associated with improved patient survival.

Figure 6. AD-01 treatment reduces the BCSC population in clinically derived primary and metastatic breast tumors and in vivo in a xenograft model. High FKBPL and low Nanog levels are associated with improved patient survival. Mammosphere formation of (A) cancer cells derived from primary breast tumors (n = 6) and (B) metastatic tumor cells derived from pleural effusions of end-stage patients (n = 3) and PDX (n = 2). C, Kaplan–Meier survival curves of patients with breast cancer (n = 94) representing samples with opposing FKBPL and Nanog expression (high FKBPL/low Nanog and low FKBPL/high Nanog) and OS. Analysis was conducted on microarray data from the publicly available dataset GSE7390. D, tumor-initiation experiment following implantation of MCF-7 cells and AD-01 (0.3 mg/kg/d s.c.) or PBS treatment from day 1 (n = 4/group). E, tumor cells from the AD-01/PBS–treated xenografts were reimplanted into secondary mice; tumor occurrence was monitored twice a week and time to tumor initiation calculated. Each dot represents a mouse. F, mRNA levels of Nanog, Oct4, and Sox2; thus suggesting that FKBPL is a key protein involved in regulating cancer stemness. This was confirmed in an independent study, where FKBPL was pulled out of a siRNA screen to identify BCSC-enriching genes in MCF-7 cells (Rene Bernard; personal communication). This is highly supportive of the endogenous role of FKBPL in BCSC signaling, which may also help explain the prognostic value of FKBPL in patients with breast cancer. We have previously shown using publicly available microarray datasets (GSE7390) and a breast cancer tissue microarray (n = 498) that high levels of FKBPL were associated with better OS, DMFS, and prolonged RFS (2, 26). Here, we have also shown that high FKBPL/low Nanog or high FKBPL/low Sox2 expression was associated with improved patient survival.
This result further confirms the role of FKBPL in CSCs signaling and its association with Nanog expression.

The mechanism of action of AD-01 is most likely dependent on CD44, FKBPL and AD-01 bind to CD44 and mediate deregulation of the CD44 pathway, extensively shown by Yakkundi and colleagues (27, 28). Although protein and mRNA levels of CD44 were increased following FKBPL overexpression or AD-01 treatment, downstream targets of the CD44 pathway, such as Rho-Rac, cytoskeletal dynamics were disrupted (28), although the precise mechanism is yet to be determined. The functional relevance of the CD44 pathway in BCSCs is still in its infancy; CD44 is mainly associated with the CSC niche (12) and it may represent a novel target for improving breast cancer therapy outcomes. CD44 as a therapeutic target has been validated using a monoclonal antibody and siRNA approach. For example, Marangoni and colleagues (45) showed, using a CD44 targeting monoclonal antibody (P245), a significant inhibition of the tumor growth in breast cancer xenografts ± chemotherapy and delayed tumor recurrence following cessation of chemotherapy. Similarly, we have prevented a tumor regrowth with AD-01 following cessation of chemotherapy (27). Here, we showed that AD-01’s dependence on the CD44 pathway, in relation to its anti–stem cell effect, is further supported by the reduction in stem cell differentiation markers, Nanog, Oct4, and Sox2. Bourguignon and colleagues (16) showed that the CD44 ligand, HA, initiates the interaction between CD44 and Nanog and also leads to activation of Nanog–Oct4 network. This process has also been associated with activation of multidrug-resistant genes, tumor progression (16), and chemoresistance in various carcinoma cells (46). Therefore, it is possible that AD-01 and FKBPL are exerting their anti–stem cell effects by deregulating CD44 signaling with downstream effects on the stem cell markers, Nanog, Oct4, and Sox2, leading to differentiation of stem cells to more mature, “typical” cancer cells. The AD-01–mediated differentiation of BCSCs to more mature cancer cells was further supported using a clonogenic assay where the number of holoclones was reduced, whereas the number of paraclines and meroclines increased following AD-01 treatment. Therefore, AD-01 represents a very unique anticancer agent which targets the CD44 pathway and differentiates stem cells to more mature cancer cells. Moreover, extensive toxicologic evaluation of FKBPL-based peptides, in preparation for clinical trial, has shown no indication of toxic effects (Almac Discovery; personal communication).

Furthermore, the AD-01–mediated decrease in the ESA+/CD44+/CD24– cell subpopulation was more effective than the 30% reduction reported using the Notch inhibitor, DAPT (30). In addition, we have shown that a combination of AD-01 and the GSIs is additive. The significance of the Notch pathway in BCSC maintenance, development, and cell fate is already well established, and as a result most of the agents under clinical development to eliminate BCSCs target the Notch pathway (47).

Finally, we have shown here that AD-01 is also able to abrogate the increase in BCSC numbers initiated by chemo- and radiotherapy. We suggest that AD-01 might be differentiating CSCs to a more chemo- and radiosensitive cancer cell population. It is also possible that cancer cells become more responsive to chemo- and radiotherapy once the CSC pool is reduced. AD-01 may therefore be a useful adjuvant therapy that could be used in conjunction with, or in advance of, standard chemo- or radiotherapy to sensitize these treatment resistant CSCs; this would complement its well-established antiangiogenic activity.

In summary, it is encouraging that the FKBPL-derived therapeutic peptide, AD-01, might have other anticancer roles in addition to its well-established role in inhibiting tumor growth and angiogenesis, which would make it a very desirable therapeutic candidate.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions

Acknowledgments
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