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

Lana McClements1, Anita Yakkundi1, Angelos Papaspyropoulos2, Hannah Harrison4, Matthew P. Ablett4, Puthen V. Jithesh6, Hayley D. McKeen1, Rachel Bennett1, Christopher Donley1, Adrien Kissenpfennig2, Stuart McIntosh3, Helen O. McCarthy1, Eric O’Neill5, Robert B. Clarke4, and Tracy Robson1

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 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 subpopulations 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 chemotherapy and radiotherapy (1). In breast cancer, about 40% of patients experience tumor recurrence often as distant metastasis, with only a small percentage of tumors recurrences locally (2).

Breast CSCs (BCSC), in particular, are enriched in the epithelial surface antigen (ESA)+/CD44+/CD24− or aldehyde dehydrogenase (ALDH)+ 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

Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

Acknowledgments: L. McClements and A. Yakkundi contributed equally to this work.

Corresponding Author: Tracy Robson, School of Pharmacy, Queen’s University Belfast, 97 Lisburn Road, Belfast BT9 7BL, United Kingdom. Phone: 44-2890247794; E-mail: t.robson@qub.ac.uk

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 x4 magnification.
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 paraclones 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 doxetaxel (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-difluorophenacyl-L-alanyl)]-S-penicillamine 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 described previously (30). Fluorescence was measured using BD FACSCanto II and analyzed by WinMDI 2.9.

ALDEFLUOR assay

Following 72 hours AD-01 treatment of MDA-231 and MCF-7 monolayers, 1 × 10⁶ cells were mixed with BAAA (BODIPY—aminocetaldehyde diethyl acetate reagent; ALDEFLUOR kit [Stem Cell Technologies]) ± diethylaminonobenzaldehyde (DEAB; Stem Cell Technologies) inhibitors. Cells were incubated for 40 minutes at 37°C before being suspended in the ALDEFLUOR assay buffer (Stem Cell Technologies). Fluorescence was measured using FACS-Calibur 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 nontargeted (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/
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 (5×10^5, 2.5×10^6, and 1×10^6 cells/mice; 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 ±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 Cells-to-CT Kit (Life Technologies). 18S was used as an internal control to normalize all data. The following primers were used: Nanog, forward: GGTGTGACGCAGAAGGCCG; reverse: CCCATAGCCTGGGGT; Sox2, forward: GGGGAAAGTAGTTCA; reverse: CCCAGTCGGGTTCACCAGGCA; Oct4, forward: GGTGTGACGCAGAAGGCCG; reverse: CCCATAGCCTGGGGT; AD-01 (100 nmol/L) for 48 hours 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).

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 in vivo using the well-characterized BCSC surface marker, 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.

AD-01 abrogates enrichment in BCSCs after chemo- and radiotherapy

One of the aberrant properties of BCSCs is chemo- and radiotherapy resistance. Through various mechanisms,
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 combinatorial 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

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**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.
Harrison and colleagues (40) showed that holoclones were morphologically distinct colonies resembling CSCs with regard to CD44high/CD24low 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, 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; *P* < 0.01; one-way ANOVA). SSC, side scatter.

**Figure 2.** AD-01 reduces the ESA+/CD44−/CD24−low and the ALDH+ cell subpopulation. A change in ESA+/CD44−/CD24−low was analyzed by flow cytometry following 72 hours AD-01 treatment of the MDA-231 (A and C) and MCF-7 (B) monolayers. MCF-7 cells were stained for high ALDH activity (+) DEAB, and the shift in this population was observed after AD-01 (1 and 100 nmol/L) treatment (D). Percentage reduction in the ALDH+ cell population was quantified in both MCF-7 (E) and MDA-231 (F) cell lines. Data points are mean ± SEM. n ≥ 3. *, *P < 0.05; **, *P < 0.01; ***, *P < 0.001 (one-way ANOVA). SSC, side scatter.
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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...
FKBPL 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 (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 GSI 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 chemotherapeutic and radiotherapy. We suggest that AD-01 might be differentiating CSCs to a more chemoresistant cancer cell population. It is also possible that cancer cells become more responsive to chemotherapeutic 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 chemotherapeutic 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
Conception and design: A. Yakkundi, R.B. Clarke, T. Robson
 Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): L. McClements, A. Yakkundi, A. Papaspyropoulos, M.P. Ablett, H.D. McKeen, R. Bennett, C. Donley, A. Kissenpfennig, S. McIntosh, H.O. McCarthy, E. O’Neill, R.B. Clarke, T. Robson
Writing, review, and/or preparation of the manuscript: L. McClements, A. Yakkundi, A. Papaspyropoulos, H. Harrison, M.P. Ablett, P.V. Jithesh, H.D. McKeen, S. McIntosh, R.B. Clarke, T. Robson
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): L. McClements, A. Yakkundi, H.O. McCarthy
Study supervision: A. Yakkundi, T. Robson

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