Targeting treatment resistant breast cancer stem cells with FKBPL and its peptide derivative, AD-01, via the CD44 pathway

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Translational relevance

FK506 binding protein like - FKBPL, is emerging as an important anti-tumour protein. FKBPL (i) inhibits tumour growth, (ii) is a prognostic and predictive breast cancer biomarker and, (iii) is a naturally secreted anti-angiogenic protein that inhibits blood vessel development by targeting the cell surface receptor, CD44. Here we have demonstrated that FKBPL’s ability to target CD44 makes it useful for targeting cancer stem cells (CSCs), which are enriched for CD44. CSCs can self-renew, are highly chemo and radioresistant, highly metastatic and are associated with poor outcome. Our data strongly suggest that FKBPL has a role in CSC signalling, 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 anti-tumour 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 later stage trials.
Abstract

Purpose: FKBPL and its peptide derivative, AD-01, have already demonstrated tumour growth inhibition and CD44 dependent anti-angiogenic activity. Here we explore the ability of AD-01 to target CD44 positive breast cancer stem cells (BCSCs).

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

Results: AD-01 treatment was highly effective at inhibiting the BCSC population by reducing mammosphere forming efficiency (MFE) and ESA+/CD44+/CD24− or ALDH+ cell subpopulations in vitro and tumour 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 appears to be due to AD-01-mediated BCSC differentiation demonstrated 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 demonstrated 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 signalling.

Conclusions: AD-01 has dual anti-angiogenic and anti-BCSC activity which will be advantageous as this agent enters clinical trial.
Introduction

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

Breast CSCs (BCSCs), in particular, are enriched in the ESA (epithelial surface antigen)⁺/CD44⁺/CD24⁻ subfraction of cells. These cells have the ability to self-renew, have enhanced tumourigenic potential in vivo and are associated with poor outcome in breast cancer patients (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 pro-invasive genes (4) and increased tumour 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 chemotherapy and radiotherapy regimens, leading to an increase in CD44⁺/CD24⁻ cells, following standard treatment protocols (7, 8). Although this appears 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 pathological complete response (11).

The functional relevance of CD44/CD24 markers in relation to breast cancer stemness is poorly understood. However, CD44 appears to play a major role in the homing of leukaemic stem cells to bone marrow and initiation of acute myeloid leukaemia (AML) (12). An activating anti-CD44 antibody prevented the tumourigenic potential of AML cells, and
leukaemia 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 tumourigenic potential, cell cycle and gene expression leading to differentiation of stem cells to non-stem cells (13). This demonstrates 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 appears 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 signalling; 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 tumours and heavily involved in tumour 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 tumours and activated in BCSCs during early tumour initiation (20).

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 stabilises p21 (23) and regulates oestrogen receptor (ER), androgen receptor and glucocorticoid receptor signalling (2, 24, 25). Furthermore, in breast cancer, because of FKBPL’s association with ER, FKBPL has demonstrated 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 anti-angiogenic 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 anti-angiogenic domain of FKBPL, including a 24 amino acid peptide, AD-01, also demonstrated potent anti-tumour activity in the sub-nanomolar range, in breast and prostate xenograft models; an FKBPL-based peptide will enter phase 1 clinical trials shortly (29). In light of FKBPL/AD-01’s dependency on CD44; it was hypothesised that they might also target CD44+ BCSCs. Here we describe the role of FKBPL in stem cell signalling and provide evidence that AD-01 indeed has activity against BCSCs in addition to its well-characterised anti-angiogenic action, suggesting enhanced clinical utility.

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 DMEM medium (Life Technologies) and the ZR-75 cell line was maintained in RPMI-1640 medium (Life Technologies) both supplemented with 10% foetal 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 non-adherent culture as previously described (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 normalised to control. Mammospheres were counted using Nikon Eclipse TE300 (Japan) microscope under 4x 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.

**Combinational drug treatments with AD-01 in vitro.** The MDA-231 and MCF-7 monolayers were treated with IC50 doses of docetaxel (0.2 nM-MDA-231) and cisplatin (7 nM-MCF-7; 70 nM-MDA-231) or radiation (2 Gy of radiation in a single dose) ± AD-01 (1 nM or 100 nM) for 3 days. Subsequently, a single cell suspension was prepared from trypsinized monolayers and 500 cells/cm² were seeded in triplicate in low adherent six-well plates for mammosphere formation. For inhibition of the Notch pathway, gamma secretase inhibitors (GSI), compound E (0.025-1.25 µM; CalbioChem) or DAPT (\{N-[N-(3, 5-difluorophenacetyl-L-alanyl)]-S-phenylglycine-\}butyl ester\}; 10 µM; Calbiochem) were added ± AD-01 (0.025 nM, 1 nM and 100 nM) directly into mammosphere culture.

**Flow cytometric analysis and sorting.** Cell monolayers were treated with AD-01 for 72 h. Flow cytometry was carried out as described previously (30). Fluorescence was measured using BD FACS Canto II and analysed by WinMDI 2.9.

**ALDEFLUOR© assay.** Following 72 h AD-01 treatment of MDA-231 and MCF-7 monolayers, 1x10⁶ cells were mixed with BAAA (BODIPY® – aminoacetaldehyde diethyl acetate reagent; Aldefluor kit (Stem Cell Technologies)) ± DEAB (Diethylaminobenzaldehyde; Stem Cell Technologies) inhibitor. Cells were incubated for 40 min at 37°C before being suspended in the Aldefluor® assay buffer (Stem Cell Technologies). Fluorescence was measured using FACSCalibur and analyzed using WinMIDI 2.9. The aldehyde dehydrogenase (ALDH)⁺ subpopulation was obtained in the absence of DEAB.
**Ex vivo ZR75 xenograft mammosphere assay.** ZR75 cells were implanted into SCID mice. Once tumours were established (~150 mm³), nanoparticles containing a delivery vehicle and FKBPL targeted (n=4), or non-targeted (NT, n=3) siRNA were delivered biweekly via intratumoural injection. Tumours were excised at quadrupling volume (approximately 2-3 weeks after the start of the treatment) disaggregated and assessed using the *ex-vivo* mammosphere assay as described above for primary tumours.

**Primary tumour 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) were used in the mammosphere assay and treated directly in mammosphere culture plates for 72 h ± AD-01 (0.05 nM and 5 nM). 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 tumour 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 Technology). Digested tissue was then filtered through 100 µm, 70 µm 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 NCBI GEO database (33) into Partek Genomic Suite (Partek Inc., St.Louis, MO, USA) and processed using Robust Multichip Average (RMA; 34) method to generate the normalised 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...
208286_x_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, i.e., 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 based on 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 performed 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 tumour initiation assay.** 5x10⁶ MCF-7 cells were implanted subcutaneously into SCID mice bearing oestrogen pellets (0.25 mg) on day 1. PBS (vehicle control) or AD-01 (0.3mg/kg/day) were administered daily, from day 1, by s.c. injection until tumours reached 150 mm³ size (n=4 per group). Tumour volume was calculated as $\frac{4}{3}\pi r^3$ (r is $\frac{1}{2}$ GMD and $GMD = \sqrt[3]{(\text{length} \times \text{breadth} \times \text{height})}$). Tumour-take and growth was monitored every 3 days.

**Limiting dilution assay in vivo.** MCF-7 cells were implanted subcutaneously as described above and SCID mice bearing established MCF-7 xenografts were treated subcutaneously with PBS (vehicle control) and AD-01 (0.3mg/kg/day) for 2 weeks and tumours were measured every 3 days. Following 2 weeks of treatment, tumours were excised, disaggregated, and re-implanted subcutaneously into SCID mice at varying concentrations (5x10⁶, 2.5x10⁶ and 1x10⁶ cells per mice; n=6 per treatment group). Time taken to tumour 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 UKCCCR guidelines.

**Quantitative real-time PCR.** MCF-7 and MDA-231 cell monolayers were treated with AD-01 (100 nM) for 48 h ±10 min treatment of hyaluronic acid (HA; 0.1 mg/ml (234 kDa, Life core Biomedicals, USA) or transfected with a pool of FKBPL siRNA (50 nM; Invitrogen) or NT siRNA for 48 h before being harvested for PCR. Cells were prepared for quantitative RT-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, FW: GGTGTCAGCAGAAGGCCTCA; R: CCCAGTCTGGTTCACCAGGCA, Oct4, FW: GGCTCGAGAAGGATGTGGTCCG; R: GGGCTCCCATAGCCTGGGGGT, Sox2, FW: GGGGAAGTAATGTGTGCTGCC; R: CGCCGCGATGATTGTATT, and 18S, FW: AGTCCCTGCCCCCTTTGTACACA; R: GATCCGAGGGCCCTCACTAAAC. The experiments were carried out in triplicate for each data point.

**Immunofluorescence.** Following the same treatments as described above, MCF-7 and MDA-231 cells were plated onto coverslips, washed with PBS and fixed using 4% formaldehyde (Thermo Scientific) for 15 min, permeabilized using 0.2% Triton X-100 (Sigma) for 10 min, blocked with 0.2% Fish Skin Gelatin (FSG) for 1 h at RT 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 h at RT, washed again with PBS, dehydrated, air-dried and embedded in DAPI containing Prolong Gold antifade reagent (Invitrogen). Fluorescence was detected using a Nikon Eclipse 90i microscope. Images were processed using NIS-Elements software.
**Statistical methods.** Data presented is a mean of at least three independent experiments ± standard error (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.

**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 utilized in order to assess the ability of AD-01 to affect BCSC numbers *in vitro* in ER⁺, MCF-7 and ZR75 cells, and the ER⁻ MDA-231 cell line. A significant reduction in MFE was obtained across all cell lines at 1 and 100 nM (Fig. 1A-C). However, there was no clear linear dose response (Fig. S1A), as seen previously with this peptide (27); the most effective dose in MDA-231 was 100 nM demonstrating ~ 40% reduction in MFE (Fig. 1A). In MCF-7 and ZR75 cells, 1 nM and 100 nM AD-01 reduced MFE by ~ 30% and ~35%, respectively (Fig. 1B and C).

In order 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 nM) clearly and significantly reduced the MFE in the MDA-231 cell line, across three 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 nM AD-01, although the effects were less dramatic (Fig. S1B).

Stable overexpression of FKBPL in MDA-231 (A3;Fig. 1E) and MCF-7 (3.1D2;Fig. S1C) cells also led to a significant reduction in MFE (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.

In order to further validate these findings, flow cytometry was used to quantitate the AD-01 mediated reduction in BCSC using the well-characterised BCSC surface markers, ESA+/CD44+/CD24−, following treatment of MDA-231 and MCF-7 monolayers with AD-01 for 72 h. Fig. 2A demonstrates 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 nM and 100 nM AD-01 in both MCF-7 (Fig. 2B and Table S1A) and MDA-231 (Fig. 2C and Table S1B) cell lines. Furthermore, the ALDEFLUOR® assay was used to analyse the effect of AD-01 on the ALDH+ cell subpopulation which is also representative of the BCSCs (32). In support of our previous observations, a significant over 50% reduction in the ALDH+ subpopulation was achieved after AD-01 treatment in the MCF-7 (Fig. 2D, E and Table S1C) and MDA-231 (Fig. 2F and Table S1D) cell lines. Statistical significance was obtained in all data sets.

**AD-01 abrogates enrichment in BCSCs after chemotherapy 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 demonstrate that AD-01 could reduce chemo and radioresistance. We demonstrate 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 nM: MFE=1.07; 100 nM: 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 tumour cell population.

**AD-01 and the Notch inhibitor, DAPT, have an additive inhibitory effect on BCSCs.** In order 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 h combinational treatment with 100 nM AD-01 and 10 µM DAPT, a 65% reduction in MFE (Fig. 3E) was observed, compared to just over 40% reduction in MFE when DAPT was used on its own (Fig. 3E). Similar results were demonstrated with another GSI, compound E (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 et al (39) and Harrison et al (40) demonstrated that holoclones were morphologically distinct colonies resembling CSCs with regard to CD44^high^/CD24^low^ levels; and were more tumourigenic/undifferentiated and chemoresistant in comparison to 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 to control indicating its lack of toxicity in both MCF-7 and MDA-231 cells (Fig. S3), as previously reported (27). In support of our previous MFE data the number of holoclones was reduced by approximately 25-30% after AD-01 treatment in both the MCF-7 and MDA-231 cell lines (Fig. 4A & 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 & B).

**AD-01 and endogenous FKBPL effect BCSC signalling 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 & D), or MDA-231 (Fig. 4E & 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-D), supporting an AD-01-mediated attenuation of CD44 signalling. In MDA-231 cells, AD-01 treatment demonstrated 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 signalling effect.

In order to establish the role of endogenous FKBPL in CSC signalling, mice harbouring ZR75 xenografts were treated via direct intra-tumoural injection with FKBPL targeted siRNA or NT siRNA. FKBPL knockdown induced a 56% increase in the number of mammospheres formed *ex vivo* (FKBPL siRNA: MFE=1.76 vs. NT siRNA: 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 appears 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 & Fig. S4B).

**AD-01 treatment reduces the BCSC population in clinically derived primary and metastatic breast tumours 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 tumour samples and highly metastatic breast cancer samples from both pleural effusions and PDX. Treatment with 1 nM and 100 nM AD-01 was able to significantly reduce the number of BCSCs, in primary breast tumours by over 50% in the mammosphere assay (Fig. 6A); there appeared to be no difference in response between tumour stage and grade (Table S2). 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 nM and 5 nM), resulting in ~20% MFE inhibition, when normalised 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 tumours, as indicated by the increased MFE compared to primary tumour isolates (1.2 vs. 0.4). To evaluate whether endogenous FKBPL could predict survival outcomes in breast cancer patients, we assessed levels of FKBPL and the stem cell markers, Nanog, Oct4 and Sox2, in a publically available breast cancer microarray data set (GSE7390). We demonstrate that tumour FKBPL and Nanog inversely correlate with survival outcomes in breast cancer patients (n=94); high FKBPL and low Nanog, correlates with improved overall survival (OS; Fig. 6C), distant metastasis-free survival (DMFS; Fig. S5) and recurrence free survival (RFS;
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; hazard ratio=3.01) and DMFS (log rank test p=0.023; hazard ratio=3.18), whereas a trend towards better RFS was observed (log rank test p=0.06; hazard ratio=2.00). In a similar analysis high FKBPL/low Sox2 expression (Fig. S6) showed borderline significance towards better OS (log rank test p=0.06; multivariate Cox analysis hazard ratio=2.6), DMFS (log rank p=0.07; multivariate Cox analysis hazard ratio=2.44) and a trend towards better RFS (log rank p=0.18; multivariate Cox analysis hazard ratio=1.89). Furthermore, high FKBPL/low Oct4 showed a trend towards improved OS, DMFS and RFS (Fig. S7).

**AD-01 modulates tumour initiation and the BCSC population in vivo.** To validate the anti-stem cell activity of AD-01 in vivo, a tumour initiation experiment and limiting dilution assays were employed for the MCF-7 xenograft model. For the tumour initiation assay, mice were treated with AD-01 (0.3 mg/kg/day) from day 1 of tumour implantation and observed for tumour initiation over a period of 29 days. The AD-01 treated group demonstrated a 7 day delay in tumour initiation and a significantly lower tumour growth rate (Fig. 6D; Table S2B). In the limiting dilution assay, mice with established xenografts were treated with PBS or AD-01 for 15 days. Tumours were then excised and disaggregated; tumour cells were used in the mammosphere assay, for qPCR analysis or re-implanted into secondary mice at three different concentrations. Following re-implantation; a statistically significant delay in tumour initiation was demonstrated 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 demonstrated 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 tumour tissues, and *in vivo* using breast tumour xenografts. This was established using three independent assays, as there is evidence in the literature that there are no ‘perfect’ markers of stemness (43). Whilst we do not see identical effects across all three assays, we consistently observe a significant AD-01-mediated reduction in the BCSC population across all endpoints used. Furthermore, the functional mammosphere assays demonstrated 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 tumour 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 anti-angiogenic activity (27) will be advantageous clinically, in light of the hypothesis that current anti-angiogenic agents could increase the pool of CSCs by creating hypoxia (44). We also provide evidence that endogenous FKBPL plays a role in CSC signalling. 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 across the generations. However, FKBPL knockdown *in vivo* increased the mammosphere forming potential of ZR75 cells *ex vivo* and *in vitro*. Furthermore, FKBPL knockdown in the MDA-231 and MCF-7 cells lines *in vitro* resulted in an increase in the stem cell associated markers, 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
signalling, which may also help explain the prognostic value of FKBPL in breast cancer patients. We have previously demonstrated using publically available microarray data sets (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 demonstrated 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 signalling 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 demonstrated by Yakkundi et al. (27, 28). Whilst 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 et al (45) demonstrated, using a CD44 targeting monoclonal antibody (P245), a significant inhibition of the tumour growth in breast cancer xenografts ± chemotherapy and delayed tumour recurrence following cessation of chemotherapy. Similarly, we have prevented a tumour re-growth with AD-01 following cessation of chemotherapy (27). Here, we demonstrated 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 et al (16) demonstrated 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 multi-drug resistant genes, tumour
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 signalling 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 while the number of paraclones and meroclones increased following AD-01 treatment. Therefore, AD-01 represents a very unique anti-cancer agent which targets the CD44 pathway and differentiates stem cells to more mature cancer cells. Moreover, extensive toxicological 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). Additionally, 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 demonstrated 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 anti-angiogenic activity.
In summary, it is encouraging that the FKBPL derived therapeutic peptide, AD-01, might have other anti-cancer roles in addition to its well-established role in inhibiting tumour growth and angiogenesis, which would make it a very desirable therapeutic candidate.

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Figure Legends

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 nM AD-01. (E) 1st and 2nd 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).

Figure 2. AD-01 reduces the ESA+/CD44+/CD24−/low and the ALDH+ cell subpopulation. A change in ESA+/CD44+/CD24−/low was analysed by flow cytometry following 72 h 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 nM and 100 nM) 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).

Figure 3. AD-01 is effective at sensitizing breast cancer cells to radiation, chemotherapy and a Notch inhibitor. (A) MDA-231 cells were pre-treated with AD-01 1 nM for 72 h, irradiated (2 Gy) and incubated for a further 48 h, and the single cell suspensions were used in the mammosphere assay. Cells were treated with (B) docetaxel (0.2 nM) for 24 h (MDA-231) or cisplatin (C) 70 nM; MDA-231 and (D) 7 nM; MCF-7 for 6 h ± AD-01 (1 nM) for a further 72 h. (E) Single cell suspension MDA-231 cells were treated with 10 µM DAPT ± 1 nM or 100 nM AD-01 directly in mammosphere culture. MFE was calculated for each point. Data points are mean ± SEM. n≥3. * p<0.05, *** p<0.001 (one-way ANOVA).

Figure 4. AD-01 reduces the number of BCSCs by differentiating BCSCs into more ‘typical’ cancer cells. Representative images of MCF-7 colonies; holoclones, meroclones and paraclones (A); reduction in the number of holoclones formed and a concomitant increase in the number of more differentiated, meroclone and paraclone colonies, following AD-01 treatment was observed in MCF-7 (A) and MDA-231 (B) cells. Different colonies were manually counted and expressed per 100 cells seeded. Protein (C) and mRNA (D) levels
of Oct4 and Nanog in MCF-7 cells following treatment with HA ± AD-01 (100 nM). (E) mRNA levels of Oct4, Nanog and Sox2 in MDA-231 cells following treatment with AD-01 (100 nM); protein levels of Oct4 (inset). Data points are mean ± SEM. n≥3. * p<0.05, ** p<0.01 (one-way ANOVA).

Figure 5. The role of endogenous FKBPL on BCSCs and their markers, Nanog, Oct4 and Sox2. MFE measured (A) ex vivo in ZR75 xenografts and (B) in vitro in ZR75 cells following transfection with FKBPL targeted or NT siRNA (inset: FKBPL immunohistochemistry of tumour xenograft sections (A) and western blot of cell lysates from ZR75 probed for FKBPL (B)). (C) Protein and (D) mRNA levels of Nanog, Oct4 and Sox2 in MCF-7 cells transfected with either NT siRNA or FKBPL siRNA. (E) mRNA levels of Nanog and Oct4 in MDA-231 cells following FKBPL knockdown. Data points are mean ± SEM. n≥3. * p<0.05, ** p<0.01, *** p<0.001 (two-tailed T-test or one-way ANOVA).

Figure 6. AD-01 treatment reduces the BCSC population in clinically derived primary and metastatic breast tumours 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 tumours (n=6) and (B) metastatic tumour cells derived from pleural effusions of end stage patients (n=3) and PDX (n=2). (C) Kaplan-Meier survival curves of breast cancer patients (n=94) representing samples with opposing FKBPL and Nanog expression (high FKBPL/low Nanog and low FKBPL/high Nanog) and overall survival. Analysis was performed on microarray data from the publicly available data set GSE7390. (D) Tumour initiation experiment following implantation of MCF-7 cells and AD-01 (0.3 mg/kg/day s.c) or PBS treatment from day 1 (n=4 per group). (E) Tumour cells from the AD-01/PBS treated xenografts were re-implanted into secondary mice; tumour occurrence was monitored twice a week and time to tumour initiation calculated. Each dot represents a mouse. (F) Mammosphere formation following excision and disaggregation of established MCF-7 xenografts treated with AD-01 or PBS in vivo (n=2 per group). (G) mRNA levels of Nanog, Oct4 and Sox2 in disaggregated MCF-7 xenografts treated with AD-01 or PBS in vivo (n=2). Data points are mean ± SEM. ** p<0.01, *** p<0.001 (one-way ANOVA).
Figure 4
Figure 5
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Targeting treatment resistant breast cancer stem cells with FKBPL and its peptide derivative, AD-01, via the CD44 pathway

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