Abstract

Purpose: Antiangiogenic therapies can be an important adjunct to the management of many malignancies. Here we investigated a novel protein, FKBPL, and peptide derivative for their antiangiogenic activity and mechanism of action.

Experimental Design: Recombinant FKBPL (rFKBPL) and its peptide derivative were assessed in a range of human microvascular endothelial cell (HMEC-1) assays in vitro. Their ability to inhibit proliferation, migration, and Matrigel-dependent tubule formation was determined. They were further evaluated in an ex vivo rat model of neovascularization and in two in vivo mouse models of angiogenesis, that is, the sponge implantation and the intravital microscopy models. Antitumor efficacy was determined in two human tumor xenograft models grown in severe compromised immunodeficient (SCID) mice. Finally, the dependence of peptide on CD44 was determined using a CD44-targeted siRNA approach or in cell lines of differing CD44 status.

Results: rFKBPL inhibited endothelial cell migration, tubule formation, and microvessel formation in vitro and in vivo. The region responsible for FKBPL’s antiangiogenic activity was identified, and a 24-amino acid peptide (AD-01) spanning this sequence was synthesized. It was potently antiangiogenic and inhibited growth in two human tumor xenograft models (DU145 and MDA-231) when administered systemically, either on its own or in combination with docetaxel. The antiangiogenic activity of FKBPL and AD-01 was dependent on the cell-surface receptor CD44, and signaling downstream of this receptor promoted an antimigratory phenotype.

Conclusion: FKBPL and its peptide derivative AD-01 have potent antiangiogenic activity. Thus, these agents offer the potential of an attractive new approach to antiangiogenic therapy.

Introduction

Neovascularization is critical to tumor growth and metastasis (1), and this has led to the development of antiangiogenic agents (2). The best validated include bevacizumab (3), sunitinib (4), and sorafenib (5), which target VEGF. While they have been effective in preclinical models, only modest responses were seen in the clinic (2). Nevertheless, these agents have been approved for metastatic colorectal cancer (mCRC), non–small-cell lung cancer (NSCLC), advanced breast cancer, advanced renal cell carcinoma and hepatocellular carcinoma, and gastrointestinal stromal tumors. Resistance and toxicity associated with these agents have also been reported (6), including evidence that these agents can drive tumor invasion (7, 8). Drugs targeting other pathways, such as the RGD-mimetic αvβ3/αvβ5 inhibitors, have also failed to produce significant clinical responses (9), with recent evidence suggesting that low (nanomolar) concentrations of these inhibitors can paradoxically stimulate tumor growth and angiogenesis (10). Thus, development of new antiangiogenic agents targeting alternative pathways may help to improve the therapeutic responses. In this respect, inhibitors of placental growth factor (PIGF; ref. 11) and Delta-like 4 (Dll4; ref. 12) are showing promise in preclinical models. However, numerous other angiogenic signaling pathways exist...
that could also be targeted within tumors (13). For example, alternative receptors such as CD44, a cell-surface receptor for hyaluronan (HA), has been implicated in endothelial cell functions, angiogenesis, and metastasis because of its ability to regulate migration of both tumor and endothelial cells (14, 15). Targeting multiple angiogenic pathways with a range of drugs may therefore provide an attractive way forward.

FKBPL belongs to the family of FK506 binding proteins (FKBP; ref. 16, 17). However, it is a divergent member of this group with shared homology mostly in the C-terminal tetratricopeptide repeat (tpr) domain, important for interactions with Hsp90. FKBPL shows low homology over the peptideyl-prolyl cis-trans isomerase (PPI) domain and lacks critical residues that are required for enzymatic activity (18). We have previously shown that FKBPL affects tumor radiosensitivity (16, 17), and Jascur’s group identified its role in stabilizing p21 in association with Hsp90 (19). We have also shown that its association with Hsp90 is also critical for regulating steroid receptor signaling, in particular the glucocorticoid receptor (GR; ref. 20) and more recently the androgen (21) and estrogen receptors (ER; ref. 22). The latter study suggests that FKBPL affects ER signaling and may have prognostic and predictive power in breast cancer patients (22). Overexpression of Hsp90 can also induce neovascularization in vivo (23). In addition, Hsp90 is a major regulator of the stability and activation of angiogenesis-associated molecules while Hsp90 inhibitors have antiangiogenic properties (24). Therefore, the interaction of FKBPL with Hsp90 underpinned an investigation into the antiangiogenic properties of this protein.

Here we present evidence that FKBPL is an endogenously secreted antiangiogenic protein and show that peptides based on the active region (AD-01) are potent inhibitors of angiogenesis. This region is quite distinct from that required for its intracellular role in Hsp90 complexes, suggesting that extracellular FKBPL acts independently of Hsp90, mediating its effects through CD44, with known roles in the regulation of endothelial and tumor cell migration (14, 15).

Materials and Methods

Cell culture

The immortalized human microvascular endothelial cell line (HMEC-1) was obtained from the Center for Disease Control and Prevention and was maintained in MCDB-131 medium (Gibco BRL) supplemented with 10% fetal calf serum (FCS, PAA) epidermal growth factor (EGF, 10 ng/mL; Roche) and l-glutamine (10 mM/ L; Gibco BRL). All other 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. DU145 and PC3 cells were cultured in RPMI 1640 medium (Life Technologies) supplemented with 10% FCS. MDA-231 and MCF-7 cells were maintained in DMEM (Gibco BRL) supplemented with 10% FCS. All experiments were carried out at 37°C in a humidified atmosphere of 5% CO2/95% O2.

Generation of the pcDNA3.1/endostatin construct

The pBLAST hENDO XV plasmid (InvivoGen) was digested with HpaI (Promega) and EcoV (Invitrogen) to release the hEndo XV insert. The hEndo XV insert was then ligated directionally into the ECoRV restriction site of pcDNA3.1 (Invitrogen).

Generation and transfection of full-length or truncated FKBPL constructs

The FKBPL/pDNA mammalian expression construct was described previously (18). To construct the 7 FKBPL truncated mutant plasmid constructs stop codons were introduced at amino acid positions 34, 40, 48, 58, 86, 151, or 200 by site-directed mutagenesis according to the manufacturer’s instructions (QuickChange kit; Stratagene) and confirmed by DNA sequencing. One microgram of each plasmid was used for transfection of HMEC-1 cells.

Preparation of recombinant FKBPL

Recombinant FKBPL (rFKBPL) was prepared commercially by Fusion Antibodies. Standard IMAC purification was followed by desalting to remove any contaminating Escherichia coli proteins.

Peptide synthesis

The 24mer peptide sequence (AD-01) NH2-QIQPRDPPTELELVSPDPAS-OH and FKBPL 1–57mer (AL-57) sequence NH2-MEITPPVNTIG EKDTSQPQEQ WEKNLRENLD SVIQIRQQPR DPPTETLELVSPDPAS-OH were assembled on an ABI 433 peptide synthesizer using modified Fmoc protocols for solid-phase peptide synthesis with commercially available protected amino acids (Novabiochem) and confirmed to be more than 95% pure by analytical reverse-phase high-performance liquid chromatography (RP-HPLC).
Cell migration assay
The in vitro migration assay is a modified version of an established method (25) and is described in our previous article (26). In brief, 90% confluent HMEC-1 cells were wounded and supplemented with fresh medium containing rFKBPL, AD-01, or AL-57. Monolayers were incubated for 7 hours and then fixed, and the extent of “wound” closure blindly assessed microscopically. Data are expressed as percentage of the initial wound size at time 0 (T0) or as percentage inhibition compared with a time-matched control. Significance was determined by one-way ANOVA.

All numerical data are expressed as means ± SEM (n = 3 unless otherwise stated in the figure legend).

Tubule formation assay
The in vitro tubule formation assay is a modified version of a method described by others (25) and was described previously by us (26). In brief, HMEC-1 cells were seeded at a density of 1 × 10^5 onto rehydrated Matrigel. After 1 hour, increasing concentrations of rFKBPL, AD-01, or AL-57 were added to each well, and the plate was incubated for a further 18 hours. Angiogenesis was determined from the degree of endothelial cell tubule formation (polygonal structures). An independent investigator assessed each well blindly. Each data point was expressed as an average of 5 readings per well, 3 wells per treatment group, in 3 independent experiments.

Rat aortic ring assay
This method is described in our previous article (26). In brief, male Wistar rats were euthanized and the thoracic aorta was aseptically removed and sectioned into 1-mm-thick rings, then embedded into Matrigel on 24-well plates. Rings were incubated for 8 days following exposure to each condition and then fixed in 4% PBS-buffered paraformaldehyde. Vessel development was assessed microscopically. In 4 fields of view (at the upper, lower, left, and right positions of the ring), the number of outgrowths from the ring edge into the Matrigel was counted and their lengths recorded to the furthest growth point. Only major branching points were included. The mean and maximum vessel lengths or numbers of vessels for each treatment group were compared with time-matched sham controls and the percentage inhibition calculated.

Viability/proliferation assay
An MTT assay was used to measure cell viability/proliferation as described previously (26). Briefly, HMEC-1 cells were seeded (2.5 × 10^5) into 96-well plates and allowed to attach for 5 hours. The cells were treated for 24 and 48 hours. Postincubation, the cells were exposed to a 5 mg/mL solution of MTT for 4 hours and the optical density measured spectrophotometrically. Effect on proliferation was determined by comparing treated cells with vehicle-treated controls.

Sponge assay model
Polyether sponges (Calligen Foam Ltd.) were subcutaneously implanted into C57 black mice and injected on alternate days with 10 ng fibroblast growth factor (β-FGF) alone (n = 5) or β-FGF in combination with 5 μg full-length rFKBPL (n = 5), 0.35 μg AD-01 (molar equivalent of 5 μg full-length rFKBPL; n = 3) or 0.11 ng AD-01 (equivalent to 10^{-9} mol/L in vitro; n = 3). Sponges were removed, fixed, paraffin embedded, sectioned, and stained and the vessels were counted. Vessels were blindly counted by 3 independent assessors using 40× magnification in 10 fields per section. The average number of vessels per field of view in each sponge was plotted. Significance was determined by one-way ANOVA.

In vivo tumor growth delay assays
About 5 × 10^5 DU145/MDA-231 tumor cells were intra-dermally injected into the rear dorsum of Balb-c severe compromised immunodeficient (SCID) mice (Harlan). For the gene delivery experiments, tumors were allowed to grow to a volume of 100 to 125 mm^3. The mice were randomly divided into the various treatment groups, and each mouse received intratumoral injections of Lipofectamine 2000 (Invitrogen) plasmid complexes (1 μg/μL), twice weekly, for the duration of the experiment. For the two AD-01 studies, mice with established tumors (150–175 mm^3) received daily intraperitoneal (i.p.) injections of AD-01 or PBS as a control. In the combination study, docetaxel (20 mg/kg i.p.) was administered once every 15 days in 3 cycles. Tumors were measured every 3 days and tumor volume was calculated as 4/3πr^3 (where r = 1/2GMD and GMD = √{Length × Breadth × Height}). All animal experiments were carried out in accordance with the Animal (Scientific Procedures) Act 1986 and conformed to the current UKCCCR guidelines. Significance was determined by the log-rank test. Kaplan–Meier analysis of the data sets was applied to determine time differences to specific events, for example, time-to-tumor doubling/tripling.

Histologic staining of mouse organs and immunohistochemistry
Formalin-fixed, paraffin-embedded tissues were sectioned and stained with hematoxylin and eosin or anti-FKBPL antibody (Proteintech), followed by detection by horseradish peroxidase (HRP)-conjugated secondary antibody.

Intravital microscopy of tumor blood vessels
Viewing chambers were inserted, under general anesthesia, under the dorsal skin of male Balb-c SCID mice (27). Fragments of DU145 tumors were placed on the microvascular bed within the surgical area and covered with a glass microslide. When tumors reached 2 ± 0.2 mm in diameter, AD-01 was delivered i.p. at 0.3 mg/kg/d for 14 days, while control mice received PBS. Imaging using fluorescein isothiocyanate (FITC)-labeled dextran (150 kDa) was carried out on days 0, 7, and 14 after initiating treatment, using epifluorescence microscopy. Intravital images (compressed Z-stacks) were analyzed by ImageJ software (NIH). The number of vessel branch points or the average vessel diameter (μm) at 7 and 14 days was
counted from 3-dimensional images; \( n = 5 \) mice per treatment group, 4 fields of view per tumor, and 30 vessels per field. Significance was determined by the 2-tailed \( t \) test.

**Immunoprecipitations**

To detect FKBP1 in conditioned medium, agarose G-FKBPL antibody conjugate or prewashed beads (negative control) were incubated with cell lysates/prefiltered medium according to our previously described methods (20, 22). The beads were then reconstituted in Læmmli buffer, and Western blot analysis was carried out to detect interacting proteins by probing for FKBP1.

**Western blot analysis**

Cell lysates were heated to 90°C for 10 minutes. Samples were subjected to SDS-PAGE electrophoresis using the Xcell SureLock Mini-cell system (Invitrogen), transferred to nitrocellulose membranes, blocked for 1 hour at room temperature in 1% milk solution, probed with monoclonal anti-CD44H antibody (R&D Systems) at dilution 1:1,000, anti-FKBPL antibody (Proteintech) at dilution 1:1,000, GAPDH at dilution 1:1,000, and actin at dilution 1:5,000 (Sigma), Rac-1 at dilution 1:1000 (Millipore), and then reprobed with either mouse or rabbit Ig HRP-linked secondary antibody (GE Healthcare) at dilution 1:10,000. Antibody binding was detected using the SuperSignal West Pico Chemiluminescent Substrate (Pierce).

**Rac assay**

HMEC-1 cells were pretreated with 1 nmol/L AD-01 prior to serum/HA stimulation (HA; molecular weight 220 kDa, medical grade purity; Lifecore Biomedical Inc.). Cell lysates were collected, and the Rac-GTPase assay was done on 1 mg protein using a Rac-1 activation assay kit (Upstate) according to the manufacturer’s instructions.

**siRNA transfections**

HMEC-1 cells were grown to 80% confluence and then transfected for 72 hours with 1.6 nmol/L smart pool CD44-siRNA transfections (Upstate) according to the manufacturer’s instructions. Cells were then replated on chamber slides and Western blot analysis was carried out to detect interacting proteins by probing for FKBP1.

**Statistical analyses**

All numerical data are expressed as means ± SEM. Data sets were analyzed for significance with either Student’s \( t \) test or ANOVA unless stated otherwise.

Further details on all methods can be found in Supplementary Methods.

**Results**

**Overexpression of FKBP1 or exposure to exogenous recombinant FKBP1 inhibits angiogenesis in vitro**

FKBP1 is an Hsp90 cochaperone. On the basis of the previous reports highlighting that Hsp90 inhibition can induce an antiangiogenic phenotype, we studied the role of FKBP1 in this process. Transient overexpression of FKBP1, by transfection of HMEC-1 cells with a FKBP1 cDNA mammalian expression construct, significantly inhibited cell migration in vitro (Fig. 1A). This effect was not restricted to the intracellular action of FKBP1 since exogenous administration of purified rFKBP1 protein also inhibited HMEC-1 migration in a dose-dependent manner (Fig. 1B), delaying full closure of a wounded cell monolayer by 18 hours compared with controls (Supplementary Fig. S1). Furthermore, administration of rFKBP1 resulted in dose-dependent inhibition of HMEC-1 tubule formation on Matrigel (Fig. 1C). rFKBP1 was also a potent, dose-dependent inhibitor of blood vessel development (Fig. 1D) in an ex vivo rat aortic ring angiogenesis assay (26) and was 500-fold more potent than another protein-based anti-angiogenic, endostatin (28), in the same assay. Proliferation was unaffected even after exposure to the highest concentrations of rFKBP1 (Supplementary Fig. S2A).

**FKBP1 inhibits angiogenesis in vivo in a model of neovascularization and inhibits the growth of DU145 human tumor xenografts**

The antiangiogenic activity of rFKBP1 was further measured in subcutaneously implanted sponges in vitro. Treatment with 10 ng β-FGF stimulated extensive cellular ingrowth into the sponges (Fig. 2A). In contrast, β-FGF–induced cellular ingrowth was inhibited by coculture with rFKBP1 (5 µg), forming significantly fewer microvessels than those treated with β-FGF alone \( (P = 0.032, \text{Fig. 2A}) \).

Inhibition of tumor angiogenesis and tumor growth by FKBP1 was compared with that of endostatin, by over-expressing these genes by direct intratumoral injection of the respective expression constructs into DU145 xenografts. Twice weekly injection of FKBP1 cDNA led to an increase in tumor FKBP1 levels (Fig. 2B) and caused dramatic inhibition of tumor growth, which persisted for over 3 months (Fig. 2C); injection of the endostatin construct gave similar growth inhibition. Kaplan–Meier curves were used to measure the animals’ tumor response and employed time-to-tumor tripling as the time for that specific event. The data show significant increases in time to tripling following delivery of FKBP1 or endostatin (Fig. 2D). A frequent consequence of FKBP1 treatment was the development of extensive central necrosis within the tumor, which healed to reveal an empty, clean core surrounded by a viable tumor rim (Supplementary Fig. S3). This is reminiscent of the cavitation reported in clinical trials of angiogenesis inhibitors (29).

A 24mer peptide (AD-01) spanning the N-terminal domain of FKBP1 is a potent inhibitor of angiogenesis in vitro and in vivo

To elucidate FKBP1’s active antiangiogenic domain truncated FKBP1 mutants were generated by site-directed mutagenesis, inserting a stop codon immediately downstream of the codons encoding aa34, aa40, aa48, aa58, aa66, aa151, and aa200 of the FKBP1 cDNA (see Supplementary Fig. S4).
for sequences). In the simple wound scrape assay, antimigratory activity was retained following transient transfection of the Δ58 mutant but lost using the Δ34 and Δ40 mutants (Fig. 3A), suggesting that the antimigratory activity of FKBPL must reside between aa34 and aa58. Two synthetic peptides were made, one consisting of amino acids 1–57 of the FKBPL protein (i.e., the peptide encoded by the Δ58 mutant) and a 24-amino acid peptide spanning the active domain of FKBPL (i.e., amino acids 34–57; Supplementary Fig. S4). Their activity was evaluated in the HMEC-1 migration and tubule formation assays. The FKBPL 24mer (AD-01) and 57mer (AL-57) were highly potent, with activity in the subnanomolar range in both of these assays (Fig. 3B; Table 1 Supplementary Data). However, AD-01 was superior to AL-57 in the ex vivo rat aortic ring assay (Fig. 3C; Table 1 Supplementary Data). A biphasic dose response similar to the rFKBPL dose response was observed for both of these peptides (Fig. 1B). This is consistent with studies of other drugs that target the endothelium; no specific mechanism has been clarified (30). Like rFKBPL, AD-01 did not affect endothelial cell proliferation, even at the highest doses when compared with a time-matched untreated control at time points up to 48 hours (Supplementary Fig. S2B).

Having shown that AD-01 had superior potency in terms of inhibiting vessel outgrowth from rat aortic rings ex vivo, we assessed the persistence of AD-01’s effect on endothelial cell sprouting to help establish the mechanism of the antiangiogenic effect. Vessels were allowed to develop for 7 days, and the medium was removed and resupplemented with medium that contained 10⁻⁹ mol/L AD-01. The addition of AD-01 caused complete inhibition of vessel development when compared with time-matched controls (Supplementary Fig. S5A). In contrast, aortic rings

Figure 1. rFKBPL protein inhibits angiogenesis in vitro and ex vivo. A, transient transfection of an FKBPL cDNA construct inhibits migration of wounded HMEC-1 monolayers compared with empty vector controls. Representative images of wounded monolayers and overexpression of FKBPL following transfection. The histogram shows the wound size relative to wound size at time = 0 h ± SEM; n = 3. Significance was determined by ANOVA. B, inhibition of HMEC-1 wound closure (compared with time-matched control) after exposure to a range of concentrations of rFKBPL. Data points show means ± SEM; n = 3. C, inhibition of HMEC-1 tubule formation in Matrigel following exposure to increasing concentrations of rFKBPL; data are corrected to a sham-treated control. Data points are means ± SEM; n = 3. D, microvessel sprouting from rat aortic rings incubated with increasing concentrations of rFKBPL (left). Quantitative determination of vessel length and number of vessels after 7 days compared with time-matched controls (right). Data points are means ± SEM; n = 3.
exposed to AD-01 for 7 days showed minimal sprouting, and removal of drug-supplemented medium and replenishment with complete medium resulted in the resumption of vessel outgrowth (Supplementary Fig. S5B). These experiments suggest that AD-01 is angiostatic and can control both immature and mature vessel outgrowth.

The antiangiogenic activity of AD-01 was also evaluated in vivo using the subcutaneous murine sponge assay as previously described. AD-01 inhibited blood vessel development at both a low dose \( (P = 0.0380) \) and a high dose \( (P = 0.0303) \), similar to rFKBPL (Fig. 3D).

Systemic delivery of AD-01 suppresses DU145 human tumor xenograft growth and inhibits blood vessel development in DU145 tumors

To further validate our novel peptide, AD-01 was administered daily by intraperitoneal injection to SCID mice bearing intradermal DU145 prostate tumors. While the volume of control tumors quadrupled within 40 days, the volume of those receiving AD-01 \( (\geq 0.003 \text{ mg/kg/d}) \) was significantly lower after 2 months’ treatment than at the start of treatment (Fig. 4A), suggestive of partial remission. Kaplan–Meier plots revealed significantly prolonged...
Figure 3. The active domain within FKBPL resides between aa-34–58 and a 24mer-based peptide, AD-01, spanning this domain inhibits angiogenesis and is more potent than rFKBPL. A, wound size of HMEC-1 monolayers 7 hours after transfection with truncated DNA constructs; n = 3. B, inhibition of migration (left; compared with time-matched controls) and tubule formation (right; compared with time-matched controls) of HMEC-1 cells after treatment with AD-01/AL-57 peptides across a range of concentrations. Data points are means ± SEM; n = 3. C, inhibition of microvessel sprouting from rat aortic rings (compared with time-matched controls ± SEM) incubated for 7 days with a range of concentrations of AD-01/AL-57; n = 3. D, AD-01 inhibited β-FGF–induced angiogenesis in the sponge assay in vivo. Microvessel densities in implanted sponges treated with β-FGF alone (10 ng) or β-FGF + AD-01 (0.35 μg or 0.11 ng). Each symbol represents the average number of vessels per 40× field, with 10 fields counted blindly in each sponge; n = 5 mice/sponges for β-FGF alone; n = 3 mice/sponges for AD-01 treatment.
tumor response with doses of 0.3 mg/kg (P = 0.0367) and 0.003 mg/kg (P = 0.0042), respectively (Fig. 4A). To determine the efficacy of the peptide in a second tumor model, female mice were implanted intradermally with the aggressive MDA-231 breast xenograft model. Again, our peptide exhibited significantly prolonged time to tripling in the experiment; as determined by Kaplan–Meier analysis, in animals treated with 0.003 mg/kg/d (P = 0.0415) and 0.3 mg/kg/d (P = 0.0093), respectively (Fig. 4B). No adverse toxicity was observed during the course of the experiment, and the weights of animals were stable over the treatment period (Supplementary Fig. S6). Central necrosis and cavitation of the majority of treated tumors were again observed at 60 days.

Intravital microscopy was used to assess intratumoral blood vessel development in DU145 tumors, implanted in the dorsal skin flap of mice (27). Blood vessels were imaged following intravenous injection of FITC–dextran on days 0, 7, and 14 after treatment with AD-01 (0.3 mg/kg/d i.p.), using epifluorescence microscopy (Fig. 4C). Tumor blood vessels of AD-01–treated animals showed a lack of perfusion and the number of vessels was clearly reduced compared with controls; this was more obvious at the lower magnification where the whole tumor mass was imaged. Several parameters were used to quantify these differences including vessel diameter and the number of branch points (indicative of vessel number). A decrease in vessel number was observed in AD-01–treated mice compared with controls within 7 days (Fig. 4C). There was also an increase in the diameter of vessels in AD-01–treated animals at 7 and 14 days, indicating vessel normalization (Fig. 4C). Importantly, in contrast to the marked effect of AD-01 on tumor

Figure 4. Systemic delivery of AD-01 inhibits blood vessel development and tumor growth. A, growth curves for DU145 xenografts or MDA-231. B, with or without daily intraperitoneal injection of a range of doses of AD-01. Data points are means ± SEM; n = 5–8 mice per treatment group and Kaplan–Meier survival curves with time-to-tumor tripling volume as the end point for survival; *, significance was determined by the log-rank test. C, intravital images (compressed Z-stacks) of DU145 xenografts showing blood vessels at 7 and 14 days after the start of treatment with AD-01 (0.3 mg/kg/d) or PBS control intraperitoneally (left). Number of vessel branch points or average vessel diameter (μm) at 7 and 14 days after initiating treatment with AD-01 (0.3 mg/kg/d i.p.) or PBS ± SEM; n = 5 mice per treatment group, 4 fields of view per tumor and 30 vessels per field. Significance was determined by the 2-tailed t test.
blood vessel number and integrity, there was no effect on the normal vasculature; retinal vasculature of animals treated with 0.3 mg/kg daily i.p. for 14 days was identical to controls (Supplementary Fig. S7).

Systemic delivery of AD-01 in combination with docetaxel

The combination of bevacizumab with cytotoxic chemotherapy is a promising approach to increasing cure rates (2). AD-01 was therefore combined with an established clinical regime of docetaxel, as there is evidence of improved efficacy when bevacizumab is combined with docetaxel (31). The combination of docetaxel with either high or low doses of AD-01 maintained the tumors in a state of stable disease for a period of 80 days. This was most apparent in the early stages compared with single agent (Fig. 5A). Tumors in the AD-01–alone group grew more slowly than controls but reached a size comparable with the combination group at around 60 days, due to the “cavitation” phenomenon observed previously. Animals receiving docetaxel as a single agent were controlled until treatment ended and then regrew rapidly with a tumor growth delay of 30 days compared with controls. The combined modality significantly increased time to doubling in these animals (Fig. 5B) compared with either agent alone, probably due to the enhanced control of tumor growth in the early stages. At the end of the experiment, organs were removed and processed, with no difference in histology detected between liver, spleen, and lung tissues excised from control or AD-01 (0.3 mg/kg/d)-alone treated animals; tumors from AD-01–treated animals showed signs of significant necrosis (Fig. 5C).

FKBPL is a naturally secreted antiangiogenic and is dependent on CD44 for its antiangiogenic/antimigratory activity

Although an extracellular role for FKBPL has not yet been described, we do see similar antiangiogenic activity irrespective of whether FKBPL is endogenously overexpressed or cells are treated exogenously with rFKBPL or AD-01. It is unlikely that exogenous rFKBPL or AD-01 is passively internalized, so we sought to determine whether or not endogenously expressed FKBPL is naturally secreted. FKBPL
protein was detected in the tissue culture medium of 2 normal cell lines, HMEC-1 and lung epithelial cells (L132), following immunoprecipitation with an anti-FKBPL antibody (Fig. 6A); however, we were unable to detect FKBPL in the medium of MDA-231 breast tumor cells unless FKBPL was stably overexpressed (Fig. 6A). This was despite similar endogenous levels of FKBPL in normal and tumor cell lines (Supplementary Fig. S8). This is consistent with antiangiogenic responses/secretion being downregulated in tumor cells to allow full growth potential. In order to identify a possible binding partner, we carried out bioinformatic analysis of the 24-amino acid sequence that conferred antiangiogenic activity. This region lies outside the domain that is necessary for FKBPL’s interaction with Hsp90 (i.e., FKBPL’s tpr domain), so an alternative target was sought. BLAST analysis identified homology between AD-01 and a region within CD74 (Supplementary Fig. S9), known to be important for its dimerization with CD44 (32). CD44’s role in metastasis and angiogenesis has already been clearly described (14, 15, 33, 34). We therefore investigated the role played by the cell-surface HA receptor CD44 in the FKBPL-mediated inhibition of angiogenesis.

Transfection of CD44 siRNA alone did not affect HMEC-1 migration compared with controls; however, knockdown of CD44 abrogated the AD-01-mediated inhibition of HMEC-1 migration, suggesting a dependency on CD44 (Fig. 6B). In addition, both AD-01 and rFKBPL inhibited the migration of a panel of tumor cell lines in a CD44-dependent manner (Fig. 6C; Supplementary Fig. S10). CD44 is a cell-surface receptor that mediates migratory processes through activation of the small GTPase Rac (34).

Figure 6. FKBPL is secreted and is dependent on CD44 for its antiangiogenic activity. A, immunoprecipitation (IP) Western blots using conditioned medium from HMEC-1, L132, and MDA-231 cells 24 hours after plating. FKBPL was immunoprecipitated using an anti-FKBPL antibody and then run on a Western blot and probed for FKBPL. Control medium (no cell exposure) or immunoprecipitation with an IgG control were used as negative controls. Whole cell lysates were used as positive controls. B, inhibition of migration of HMEC-1 compared with time-matched control 72 hours after transfection with nontargeted siRNA compared with CD44-targeted siRNA in untreated cells (top) and treated (10⁻⁹ mol/L AD-01; bottom) ± SEM; n = 5. C, migration of tumor cells after treatment with AD-01 (left) or rFKBPL (right) across a range of concentrations (± SEM; n = 3). D, representative gel showing inhibition of Rac activity in HMEC-1 cells after treatment with AD-01 (10⁻⁹ mol/L) for 10 min/60 min prior to serum/HA activation. CTRL, control.
after serum stimulation or 10 minutes after stimulation with CD44’s natural ligand HA, and this was inhibited by a 10- or 60-minute preexposure to AD-01 (Fig. 6D), suggesting that the observed inhibition of cell migration is a consequence of AD-01–mediated inhibition of CD44/Rac activation.

Discussion

VEGF is an established antiangiogenic target; however, an array of side effects is now emerging in clinical practice, emphasizing that VEGF and its receptors are not specific for endothelial cells (35, 36). Furthermore, VEGF inhibitors can promote metastasis (7, 8) and activate EPO receptors on tumor cells, which may counteract the benefits of VEGF inhibition (37). Thus, there is a need for new antiangiogenesis-targeting pathways not regulated by VEGF.

We have identified a potent, naturally secreted antiangiogenic protein, FKBPL, which mediates its activity through CD44. Although there are some data to suggest that FKBPL is an intracellular protein associated with Hsp90 chaperone complexes (16, 17, 19–22), an extracellular role has not previously been described. We now show that FKBPL is secreted. FKBPL does not contain a terminal signal peptide, but many cytoplasmic proteins are exported in response to specific stimuli by nonclassical, endoplasmic reticulum/Golgi-independent protein release, regulating cell adhesion/motility upon export (38–40). These include FGF1, Ku, RHAMM, epimorphin, and GAL3, among others (41). We have identified a new antiangiogenic protein that is secreted to control angiogenesis. This is supported by our data showing that FKBPL can be detected in the medium of “normal” cells but cannot be detected in the medium of tumor cells except when artificially overexpressed. This hypothesis may explain our recent demonstration that high FKBPL mRNA levels are prognostic for improved overall survival and distant metastasis-free survival in a cohort of untreated breast cancer patients (22), consistent with an antiangiogenic role for this protein. However, this would need to be further clarified.

The antiangiogenic activity of FKBPL resides within a 24-amino acid domain; this is outside the domain required for an interaction with Hsp90 (i.e., the tpr domain), suggesting that Hsp90 is not responsible for either the chaperoning of FKBPL to the extracellular milieu or for subsequent antiangiogenic signaling. We have now shown that FKBPL mediates its activity through the CD44 pathway. Although the exact mechanisms are yet to be fully elucidated, AD-01 inhibits endothelial cell migration (and this is dependent on high levels of CD44; Fig. 6B and C), tubule formation, and angiogenesis in several ex vivo and in vivo models while having no effect on the proliferative capacity of endothelial cells. This differentiates AD-01 from the other approved VEGF-targeted agents and is important, given that major adverse effects of antiangiogenic drugs are associated with their broad spectrum of antiproliferative activity (6).

The complexity of CD44 signaling has made its role in angiogenesis and metastasis difficult to elucidate. The presence of splice-variant forms of CD44 has been associated with tumor progression, while other studies on the standard form (CD44s) suggest an association with decreased metastasis and enhanced survival (42, 43). CD44 knockdown has been shown to inhibit angiogenesis (15), though treatment of mice with KM81, a bioactive antimurine CD44 antibody (which blocks HA binding), failed to inhibit the neovascularization of Matrigel implants (44). Furthermore, CD44-null mice are viable with no obvious morphologic defects, which suggests possible compensation by other receptors such as RHAMM (45). Our own data suggest that AD-01 signals through CD44, inhibiting the Rho GTPase Rac, a well-known regulator of actin assembly and activator of migration (Fig. 6D). Further studies will be required to appreciate the full role of CD44 in the AD-01–mediated inhibition of cell migration.

We have shown that AD-01 is a highly potent, antiangiogenic agent across a range of models in vitro. It was more potent than the larger peptide AL-57 in the rat aortic ring assay, which may be due to a lack of penetration of the larger peptide into this ex vivo tissue. Furthermore, AD-01 had no effect on proliferation, nor is it cytotoxic. In vivo, it was effective against 2 tumor xenograft models, prostate and breast. Its combination with docetaxel achieved complete control, particularly in the early stages, compared with AD-01 alone, which would be very favorable clinically. Nevertheless, tumors treated with AD-01 alone were equally well controlled at 60 days, suggesting that it could also be useful as a single agent. It would be useful in future combination studies to assess regrowth of these tumors after ceasing treatment; although we would expect regrowth to occur (supported by data in Supplementary Fig. S3B), we might also be able to show more effective cell killing in the combination group than in AD-01 alone. The in vivo tumor growth delay data (Figs. 4 and 5) also show that our targeting approach must be dependent on CD44 expression within the tumor vasculature and not in the tumor parenchyma, since DU145 cells do not express CD44 at detectable levels. This is supported by the window chamber data (Fig. 4C), which shows that the vasculature in the DU145 xenograft model is severely compromised within 7 days of AD-01 treatment. Thus, AD-01 has broad applicability across a wide range of solid tumors whether or not they express CD44.

In summary, this is the first description of a role for FKBPL as an antiangiogenic protein. Its peptide derivative, AD-01, holds promise as an antiangiogenic for cancer therapy and could have potential when resistance to and/or escape from existing VEGF therapies has developed.

Disclosure of Potential Conflicts of Interest

I. James, T. Harrison, G. Cotton, J. Roberts, and J. McGregor are all employees of Almac Discovery. T. Robson received research support over the last 3 years totalling around £500,000 from Almac Discovery.
FKBPL and Peptide Derivatives Inhibit Angiogenesis

T. Robson, D.G. Hirst, A. Valentine, and M. O’Rourke are all inventors on (WO 2007/141533) FKBPL AND USES THEREOF. This patent has been licensed to Almac Discovery. D.J.J. Waugh is on the Almac Discovery Advisory Board and R. Bicknell has acted as a consultant for Almac Discovery.

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