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Title

FKBPL based peptide, ALM201, targets angiogenesis and cancer stem cells in ovarian cancer

Running Title

The anti-tumour activity of ALM201 in ovarian cancer

Key words

FKBPL, ALM201, ovarian cancer, angiogenesis, cancer stem cells

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Abstract

Background

A novel therapeutic peptide derived from FKBPL, ALM201, has completed a Phase I clinical trial and obtained FDA orphan drug status in ovarian cancer.

Methods

In vitro cancer stem cell (CSC) assays were conducted in a range of HGSOC cell lines and fresh patient samples. In vivo tumor initiation, growth delay and limiting dilution assays were utilized. Signalling mechanisms determined using immunohistochemistry, ELISA, qRT-PCR, RNAseq and Western blotting. Endogenous FKBPL protein levels evaluated using tissue microarrays (TMA).

Results

ALM201 reduced CSCs in cell lines and primary patient samples in vitro by inducing differentiation. ALM201 treatment of the highly vascularized Kuramochi xenografts, resulted in a delay in tumor growth by disruption of angiogenesis and a 10-fold decrease in the CSC population. In contrast, ALM201 failed to elicit a strong anti-tumor response in non-vascularized OVCAR3 xenografts, due to high levels of IL-6 and vasculogenic mimicry. High endogenous tumor expression of FKBPL was associated with an increased progression free interval, supporting the protective role of FKBPL in HGSOC.

Conclusion

We show that FKBPL-based therapy can (i) dual target angiogenesis and CSCs (ii) target the

CD44/STAT3 pathway in tumours and (iii) is effective in highly vascularised HGSOC tumours with low levels of IL-6.

Background

Ovarian cancer affects one in 70 women in developed countries and high grade serous ovarian cancer (HGSOC) is the most common and aggressive subtype accounting for the majority of advanced cases (1,2). The ten year survival is lower than 30% and this has not improved substantially in 30 years, despite improved diagnostic and therapeutic intervention (3). The standard management consists of operative tumor debulking and administration of six cycles of paclitaxel and carboplatin chemotherapy (4). Approximately 80% of patients respond to first line treatment, however tumor recurrence and chemotherapy resistance eventually occurs in almost all patients within a median progression free interval of 15 months post diagnosis (4).

Angiogenesis has a pivotal role in the pathogenesis of ovarian cancer by promoting tumor growth and progression through ascites formation and metastatic spread (5). Targeting angiogenesis in ovarian cancer has been an active area of research and bevacizumab, a monoclonal antibody against VEGF-A, has been approved by the EMA and recently the FDA as a first line therapy in combination with chemotherapy (6,7). This is based on the pivotal Phase III GOG-0218 trial in which those women who received bevacizumab in combination with chemotherapy had a median progression-free survival (PFS) of 18.2 months compared to 12.0 months in women who received chemotherapy alone (HR=0.64; 95% CI 0.54 - 0.77, $p < 0.0001$) (8). However, concerns regarding toxicity and resistance remain major hurdles for the clinical use of anti-angiogenic therapy. Across all tumor types, bevacizumab is discontinued in 8.4% - 22% of all patients due to adverse reactions (9). Furthermore, anti-angiogenic resistance, at least in part, is attributed to hypoxia-driven cancer stem cell (CSC) enrichment (10). It is now recognised that CSCs have major roles in the etiopathogenesis, metastasis and chemo-resistance of ovarian cancer and their targeting is an

important therapeutic strategy (11). The successful elimination of CSCs could have unprecedented implications in the clinical management of patients (12).

FK506-binding protein like (FKBPL) is a divergent member of the FK506-binding protein family first identified as having a role in the response of cells to radiation (13,14). At the intracellular level, and in a complex with Hsp90, FKBPL stabilizes p21 and regulates oestrogen receptor (ER), androgen receptor, and glucocorticoid receptor signalling (15–18). Furthermore, FKBPL demonstrated prognostic potential in a meta-analysis of five independent breast cancer TMA cohorts (19). FKBPL is also a secreted anti-angiogenic protein and the cell surface receptor, CD44, is a potential target for its activity (20,21). In support of a role for FKBPL in angiogenesis, FKBPL knockout mice are embryonically lethal and FKBPL heterozygous embryos display vascular irregularities; suggesting a critical role for FKBPL in developmental angiogenesis (22). *In vitro* and *in vivo* knockdown of FKBPL in breast cancer cell lines increases mammosphere formation accompanied by an increase in the pluripotency transcription factors (*Nanog*, *Sox2* and *Oct4*) (23,24). Furthermore, FKBPL was identified using an shRNA genetic screen library as a regulator of breast cancer tumor initiation (25), and high tumor *Fkbpl* and low *Nanog* are associated with improved survival outcomes in breast cancer patients (n = 94) (23).

The highly potent anti-angiogenic and anti-CSC activity of FKBPL is due to a unique sequence within the N-terminal region. A 24 residue peptide comprising amino acids 34-58 of FKBPL was developed and termed, AD-01. AD-01 has demonstrated potent anti-angiogenic and anti-CSC activity potentially through binding to CD44 (23,26). Furthermore, FKBPL and its peptide derivatives inhibit breast cancer metastasis through Notch signalling (27). Analysis of the

structure, activity and stability of AD-01 led to the selection of ALM201, a 23 residue peptide as the clinical drug candidate. ALM201 lacks cytotoxicity and displayed a very good safety profile in a Phase I, first in man, clinical trial (EudraCT number: 2014-001175-31) (28,29). Given that anti-angiogenic agents are demonstrating efficacy in the HGSOc setting, a disease of unmet clinical need, we assessed whether ALM201 could elicit dual anti-angiogenic and anti-stemness activity in this disease. Indeed, this would differentiate this drug from other agents targeting angiogenesis only.

To begin addressing this, we investigated if ALM201 could target CSCs in a range HGSOc cell lines and patient samples. OVCAR3 cells were sensitive to ALM201 *in vitro*, however xenograft studies indicated no anti-tumor or anti-CSC efficacy *in vivo*. On the other hand, Kuramochi xenografts demonstrated significantly reduced tumor growth and CSC frequency following ALM201 treatment. Further studies indicated differences in tumor vascularization and cytokine levels between these two xenografts. OVCAR3 xenografts displayed extensive vasculogenic mimicry and limited CD31⁺ blood vessels whilst Kuramochi xenografts had an extensive blood vessel network. In addition, OVCAR3 cells dramatically increased the expression of IL-6 *in vivo* and we demonstrated that IL-6 could inhibit the ability of ALM201 to target CSCs.

Materials and Methods

Tumorsphere assay

Briefly, 250 cells/cm² were seeded in 6-well dishes in non-adherent culture as described previously and treated once with ALM201 upon seeding (30). Tumorspheres > 50 µm were counted using a Nikon Eclipse TE300 (Japan) microscope under ×4 magnification after 3-4 days for cells lines and 7 days for primary samples.

Tumor Initiation Experiment

A total of 1 x 10⁶ OVCAR3 or 5 x 10⁶ cells Kuramochi cells were resuspended in PBS and diluted 1:1 in Matrigel (BD Bioscience, UK) and immediately implanted into female, 6 week old, female SCID mice (Harlan Laboratories, UK). PBS (vehicle control) or ALM201 (0.3 mg/kg/day) were administered daily (d1-d5), from day 1, by subcutaneous injection (n=5 mice/group). The mice were randomly allocated to experimental groups, with a weight range of 18g -22g. Tumor volume was calculated as described previously (23). For all in vivo experiments, mice were housed in individually ventilated cages according to EU Directive 2010/63 at constant temperature and humidity with 12 h light/dark cycle and fed standard chow. The welfare of the all mice were monitored daily and health screening carried out regularly as per policy of licensed establishment. No adverse events were noted for in vivo experiments. The experimental protocols were compliant with the UK Scientific Act of 1986 and ARRIVE guidelines (Supplementary Table 3) and Personal License Number 1598 under the Project License Number 2794.

In vivo limiting dilution assay

SCID mice bearing Kuramochi xenografts from the above tumor initiation experiment were treated with PBS or ALM201 until tumors reached geometric mean diameter (GMD) of 12 mm³. Tumors were excised, disaggregated using a scalpel and added to a MACs C tube (Miltenyi Biotec, UK) containing collagenase type II (Invitrogen, UK), DNAase type 1 (Sigma-Aldrich, UK) in RPMI/1% penicillin/streptomycin (Invitrogen, UK). Tumors were minced using a gentleMACS dissociator (Miltenyi Biotec, UK) and incubated at 37°C in an orbital incubator for 45 min. The cell suspension was resuspended in red blood cell lysis buffer (Roche, UK) for 2-3 minutes. The cells were resuspended in ice cold PBS and counted using a haemocytometer. Cells were implanted intradermally, as described above, into secondary SCID mice at 2.5 x 10⁶, 1 x 10⁶, 5 x 10⁵, 1 x 10⁵, 1 x 10⁴ cells per mouse. Mice did not receive treatment and were observed for tumor initiation for six months. The tumor initiating cell frequency was calculated using ELDA software (31).

In vivo tumor growth delay

OVCAR3 and Kuramochi cells were implanted intradermally into SCID mice, as described previously. Established tumors (100 mm³) were then treated with PBS (vehicle control) or ALM201 (0.3 mg/kg/day) as described previously for 30 days or 56 days in the OVCAR3 or Kuramochi xenografts, respectively (n=5/group). Tumors were excised and used for downstream experiments.

Tissue Microarray

Individual patient data from four HGSOC tissue microarray (TMA) cohorts were obtained and summarised in Supplementary Table 1. TMAs were constructed at the various centres using formalin-fixed, paraffin embedded tissue from primary HGSOC with a 0.6 mm diameter core (Cohort 1, 2, 3) or 1 mm (Cohort 4) diameter core taken from tumor areas. Tissue staining was carried out at the Northern Ireland Molecular Pathology Laboratory of Queen's University Belfast as described (19). TMAs were scored fully by one 'trained' scorer (SA/GM), with a second, independent scorer (SA/GM) evaluating a minimum of 20% of the cohort. Two cohorts were further independently scored by a clinical gynaecological pathologist (GMcC). Each scorer was blinded to all pathological information, and slides were scored according to staining intensity; only cores which consisted of > 20% tumor were scored. A histoscore was calculated from the sum of (1 x % weakly positive tumor cells) + (2 x % moderately positive tumor cells) + (3 x % strongly positive tumor cells) with a maximum histoscore of 300 as described in (19) and sent to the independent statistics team at the University of Warwick for analysis.

Results

The FKBPL derived therapeutic peptide, ALM201, targets CSCs in HGSOC cell lines and patient samples

The tumorsphere assay was used to assess the ability of ALM201 to reduce ovarian CSCs *in vitro* and *ex vivo*. A significant reduction in tumorsphere forming efficiency (TFE) of 20 - 30% was obtained across all cells lines, PE01, PE04, OVCAR3 and OVCAR4 cells lines (Fig. 1A-D); similar to what we had observed in breast cancer cell lines with the preclinical peptide, AD-01 (23). FKBPL levels were assessed in all cell lines, with highest expression observed in OVCAR4 cells and lowest expression in PE01 cells (Fig. 1E). There was no difference between endogenous FKBPL levels and the response of the cell lines to ALM201 in the tumoursphere assay (Fig. 1 E, Supplementary Fig 1). RBCK1 is an FKBPL-interacting protein, which regulates FKBPL stability at the post-translational level via ubiquitination (32). RBCK1 was also measured in the ovarian cancer cell lines, Again, there was no correlation between RBCK1, USP19 and FKBPL in the ovarian cancer cell lines (Fig. 1 E, Supplementary Fig 1). The Kuramochi cell line, reported to closely resemble HGSOC (33), did not form tumorspheres (Fig. 1F). However, polypoid giant cancer cells (PGCCs) were routinely observed in the Kuramochi monolayer. PGCCs are induced by hypoxia or chemotherapy and they generate daughter cells with CSC-like properties through an evolutionary conserved, asymmetric budding mechanism. Zhang *et al*, reported that spheroids derived from PGCCs are positive for CSC markers and a single PGCCs spheroid from the ovarian HEY cell line was able to form tumors *in vivo* (34). Encouragingly, ALM201 (100 nmol/L) significantly reduced the number of spheroids formed, suggesting a reduction in the tumor initiating population in the Kuramochi cell line (Fig. 1G). The anti-CSC activity was further

evaluated using clinically relevant fresh primary HGSOC tissue directly from patients. Treatment with ALM201 (1 and 100 nmol/L) was able to reduce the number of tumorspheres representative of CSCs in three chemo-naive samples by approximately 40% (Fig. 1H). Neo-adjuvant chemotherapy is reserved for patients with aggressive tumors for whom optimal tumor debulking is not possible (35). Patients who received neoadjuvant chemotherapy demonstrated an approximately 10-fold increase in the TFE compared to chemo-naive patients (Fig. 1H and II). However, ALM201 also reduced CSCs in the neoadjuvant patients, albeit with a lower average reduction of approximately 20% TFE (Fig. 1I). Upon grouping the patient samples, treatment with ALM201 significantly inhibited tumoursphere formation in chemo-naive patients that did not receive chemotherapy, but not in the neoadjuvant patients (Supplementary Fig. 2). On the whole, ALM201 appears to effectively reduce tumoursphere formation in chemo-naive HGSOC indicating it may be more effective as a first line agent. ALM201 demonstrates a mixed anti-CSC response in other subtypes of ovarian cancer, with clear activity in the A2780 cell line, an endometrioid patient, adenocarcinoma patient, clear cell patient and a serous borderline patient (Supplementary Fig. 3).

To validate the tumorsphere assays, we used flow cytometry to quantitate the ALM201-mediated reduction in ovarian CSCs using well-characterised ovarian CSC surface markers, CD44⁺/CD117⁺ (36). There was a significant decrease in the CD44⁺/CD117⁺ subpopulation in OVCAR3 cells following ALM201 (100 nmol/L) treatment (Fig. 2 A-B). The Kuramochi cell line had no detectable CD44⁺/CD117⁺ subpopulation. The ALDEFLUOR assay was also used to analyse the effect of ALM201 on the ALDH⁺ subpopulation, which is also representative of ovarian CSCs (37). There was a significant decrease in OVCAR3 ALDH⁺ cells following ALM201 (1 nmol/L

and 100 nmol/L) treatment ($p < 0.05$; $n = 5$) and a decrease of Kuramochi ALDH⁺ cells following ALM201 treatment but this was not significant ($n = 4$) (Fig. 2 C - E). There was an average of 15.1% ALDH⁺ cells in the OVCAR3 cell line compared to 3.46% in the Kurmaochi cell line (Fig. 2 D, E). Together, this indicates that the stem cell like population is small in the Kuramochi *in vitro* population given the lack of ability to form tumorspheres, no detectable CD44⁺CD117⁺ subpopulation and a small ALDH⁺ subpopulation.

In order to investigate the fate of CSCs following treatment with ALM201, we assessed colony morphology using a clonogenic assay. Using this assay, we have previously reported that the preclinical peptide, AD-01, was not cytotoxic but rather differentiated breast CSCs into a more mature phenotype (23). Similar to what was observed with AD-01, ALM201 was not cytotoxic (Fig. 2F) and it significantly, reduced holoclone formation and increased meroclone and paraclone formation (Fig. 2 G - H). These results further support the hypothesis that ALM201 differentiates CSCs into more ‘mature’ cancer cells.

ALM201 does not target CSCs or angiogenesis in OVCAR3 xenografts

To validate the anti-tumor activity of ALM201 *in vivo*, a tumor initiation experiment was performed using the OVCAR3 xenograft model. Mice were treated with ALM201 (0.3 mg/kg/day) from day 1 of implantation. Surprisingly, ALM201 did not delay tumor initiation of the OVCAR3 xenografts (Fig 3A). We then used a tumor growth delay model to investigate the ability of ALM201 to inhibit angiogenesis. Established (100 mm³) OVCAR3 xenografts were treated with ALM201 (0.3 mg/kg/day; d1- d5). No significant delay in tumor growth was observed, suggesting that ALM201 does not inhibit angiogenesis in this model (Fig 3B). Following 30 days of treatment,

tumors were excised and dissociated. The dissociated OVCAR3 xenograft cells were assessed in an *ex vivo* tumorsphere assay, and no decrease in TFE was observed in the ALM201 treatment group (Fig. 3C). In addition, flow cytometry was conducted and ALM201 treated xenografts demonstrated no significant decrease in the CD44⁺CD117⁺ CSC-like subpopulation (Fig. 3D). Overall, these results indicate that ALM201 does not target CSCs or angiogenesis in OVCAR3 xenografts. We had previously shown that the preclinical peptide, AD-01, significantly decreased the mRNA expression of pluripotency markers *Oct4*, *Nanog* and *Sox2* in breast cancer xenografts, consistent with the differentiation of the CSCs (23). Likewise, *Sox2* mRNA levels were significantly reduced in OVCAR3 monolayer cells after 24 h *in vitro* treatment with ALM201 (1 nmol/L and 100 nmol/l) (Fig 3E). However, *Oct4*, *Nanog* and *Sox2* mRNA levels were significantly increased in the ALM201 treated xenografts; consistent with the lack of anti-CSC activity in the tumor xenograft setting (Fig 3F). The OVCAR3 xenografts were sectioned and stained for CD31⁺ blood vessels. Not surprisingly, given the lack of any significant anti-tumor efficacy in this xenograft model, there were very few CD31⁺ blood vessels in the OVCAR3 xenografts (Fig. 3G &H) and so we considered that other methods of vascularisation were driving growth. The xenografts were dual stained with CD31⁺/PAS⁺, a marker for vasculogenic mimicry (VM). An extensive network of PAS⁺ vessels were observed in the xenografts; suggesting a non-angiogenic tumor phenotype (Fig 3F). An *in vitro* model of VM was the evaluated, by inducing tubule formation in OVCAR3 cells. There was no difference in tubule formation after ALM201 (100 nmol/L) treatment in OVCAR3 cells (Fig. 3I). Together this data indicates that OVCAR3 xenografts induce VM channels for tumor growth and this cannot be inhibited by ALM201 (Fig. 3). The Kuramochi cells do not form tubules *in vitro* (Supplementary Fig. 4).

ALM201 targets CSCs and angiogenesis in Kuramochi xenografts

A tumor initiation experiment was performed using Kuramochi cells and a significant 28 day delay in tumor initiation and subsequent delay in tumor growth was observed in the ALM201 (0.3 mg/kg/day) treatment group (n>5) (Fig. 4 A & B). This was also reflected in the Kaplan Meier survival curves (Fig 4 C). Kuramochi xenografts from mice treated with PBS or ALM201 (0.3 mg/kg/d) were then stained for CD31⁺ blood vessels. Unlike the OVCAR3 xenografts, Kuramochi xenografts demonstrated a robust vascular network and there was a significant decrease in CD31⁺ vessels after treatment with ALM201; indicating a reduction in angiogenesis (Fig. 4D). Interestingly, the Kuramochi tumour cells also stained positive for CD31 (Fig. 4D). The *in vivo* limiting dilution assay is the gold standard for assessing agents that target the tumor initiating potential of CSCs. Kuramochi xenografts were treated with ALM201 (0.3 mg/kg/day) until a GMD = 12. Tumors were then disaggregated and implanted into second generation mice at defined cell numbers (2.5 x 10⁶, 1 x 10⁶, 5 x 10⁵, 1 x 10⁵, 1 x 10⁴ cells/mouse; Fig. 4E). The second generation mice did not receive ALM201 treatment and extreme limiting dilution analysis (ELDA) software was used to estimate the frequency of tumor initiating cells in the xenografts (31). There was a greater than 10-fold decrease in the tumor initiating frequency (TIF) in untreated second generation xenografts derived from primary ALM201 treatment mice compared to the PBS controls (TIF; PBS 1.36 x10⁵ v ALM201 1.59 x 10⁶; p=8.77 x 10⁵, n>4) (Fig. 4 F; Supplementary Fig. 5). In addition, there was a dramatic 131.5 day delay in tumor initiation between mice implanted with 2.5 x 10⁶ cells previously treated with ALM201 and mice implanted with 2.5 x 10⁶ cells from PBS treated xenografts (Fig. 4 G). These results strongly indicate that ALM201 is highly effective at

targeting both the CSC subpopulation and angiogenesis in the highly vascularised Kuramochi xenografts.

The Kuramochi cell line displays a pro-angiogenic genotype compared to OVCAR3 cell line

RNA sequencing was performed to investigate gene expression differences between the untreated Kuramochi and OVCAR3 cell lines. The Kuramochi cell line demonstrated a positive correlation to angiogenesis gene regulation, including an upregulation of VEGFA, compared the OVCAR3 cell line (Fig. 5A & B). Other pathways that had differential expression between Kuramochi and OVCAR3 included p38MAPK, TGF β , MTOR and NOD like receptor signalling (Supplementary Fig. 6). This data supports the well-vascularised phenotype observed when Kuramochi cells were grown as xenografts and the distinct lack of angiogenesis when OVCAR3 cells were grown as xenografts.

The OVCAR3 cell line up-regulated inflammatory cytokines *in vivo* which inhibited anti-stem cell activity of ALM201

A previous study, using unsupervised hierarchical clustering of HGSOC patients treated with bevacizumab, a VEGF inhibitor, identified three major subgroups; two with angiogenic gene upregulation and one subgroup with immune gene upregulation (38). The OVCAR3 and Kuramochi *in vitro* monolayers had similar mRNA expression of the pro-inflammatory cytokines IL-6 and IL-8 (Fig. 5C). However, there was a dramatic 150-fold increase in IL-6 and 12.5-fold increase in IL-8 mRNA levels when OVCAR3 cells were grown as xenografts (Fig. 5C). Notably,

there was no change in IL-6 and IL-8 mRNA levels between the Kuramochi cell line cultured as a monolayer or as xenografts (Fig. 5C). The levels of both mouse and human IL-6 and IL-8 (Kc) in the OVCAR3 and Kuramochi xenografts were measured by ELISA. Mouse IL-6 or IL-8 was undetectable, indicating that the source of the cytokines was tumor derived rather than being from stromal tissue. The Kuramochi xenografts had low levels of IL-6 (17 pg/ μ g) while the OVCAR3 xenografts had 51-fold more IL-6 (871 pg/ μ g; Fig. 5D). The OVCAR3 xenografts also had approximately 6-fold more IL-8 (Kc) protein than the Kuramochi xenografts (Fig. 5D). This suggests that the OVCAR3 cell line is more representative of an immune subgroup whilst the Kuramochi cell line is representative of an angiogenic subgroup of HGSOc.

We had previously shown that ALM201 targets the CSC subpopulation in the OVCAR3 cells in *in vitro* assays (Fig. 2) but had no anti-CSC activity in *in vivo* OVCAR3 xenografts (Fig. 3). We decided to evaluate whether the increased IL-6 and IL-8 in OVCAR3 xenografts could explain the lack of response to ALM201. Recombinant IL-6 and IL-8 was added to *in vitro* OVCAR3 tumorsphere assays in the presence of ALM201. IL-6 significantly abrogated the ability of ALM201 to decrease tumorsphere formation at concentrations > 10 ng/ml (Fig. 5E). However, ALM201 was still able to reduce tumorsphere formation in the presence of IL-8 (Fig. 5F), suggesting that IL-6, a known antagonist of other anti-CSC and anti-angiogenic drugs, might be responsible for the lack of ALM201 anti-CSC efficacy in OVCAR3 xenografts (39,40).

ALM201 decreases phosphorylation of STAT3 in OVCAR3 cells

The principle signalling mechanism for IL-6 is via the JAK/STAT pathway. Here we addressed whether IL-6 could abrogate ALM201 activity via inhibiting this pathway. We first investigated

whether ALM201 could inhibit phosphorylation of STAT3; a pathway also associated with CD44 signalling (41). Indeed, ALM201 decreased p-STAT3(Tyr705) in OVCAR3 cells, whilst recombinant IL-6 abrogated its activity post treatment with ALM201 (Fig. 5G, H). We have previously reported that FKBPL and its peptide derivatives might exert their activity through the cell surface receptor, CD44. STAT3 forms a complex with CD44 in the cytoplasm and acts as a linker molecule to NF κ B signalling to promote the CSC phenotype (41,42). Therefore, to further support a role for FKBPL in this pathway, we demonstrate that transient knockdown of *Fkbpl* in OVCAR3 cells resulted in the transient upregulation of *Nfkb1* and the pluripotency factor *Nanog* (Fig. 5I; Supplementary Fig. 7).

High FKBPL expression in ovarian cancer is associated with an increase in progression free survival

A meta-analysis of five breast cancer TMA cohorts has previously indicated that FKBPL is an independent marker of good prognosis in breast cancer (19); not surprising given its anti-angiogenic and anti-CSC activity in this setting (19,21,23). Here we have demonstrated that the FKBPL peptide mimetic, ALM201, has anti-tumor activity in HGSOC and therefore postulated that FKBPL might also be a prognostic marker in this setting. The association of FKBPL expression with overall survival was assessed within publically available data sets. Analysis of 1582 ovarian cancer patients of all subtypes and treatments demonstrated that low FKBPL expression was significantly associated with reduced overall survival ($p=0.021$) (Fig. 6A). This preliminary data suggested a significant correlation between reduced mRNA FKBPL expression and reduced overall survival; correlating with what was observed in breast cancer (16). We then

used four TMA cohorts from HGSOC patients to determine if FKBPL levels were associated with prognosis in this tumor type. The patient clinic-pathological variables for all four cohorts are shown in Supplementary Table 2. Receiver Operative Characteristics (ROC) analysis was carried out on cohort I and II and a histoscore of 190 was determined to be the optimum cut-off (Supplementary Fig. 8). A histoscore of 190 was also previously used as the cut-off in five breast cancer TMAs (19) and was therefore considered a suitable cut-off for this analysis. In cohort I, there was a significant association between high FKBPL and progression free survival (PFS; $p=0.03$, HR=1.44, 95% CI=1.04-2.00; Fig. 6B). However, whilst there was a trend for high FKBPL levels demonstrating improved PFS, this was not significant in cohorts II (Fig. 6C), III (Fig. 6D) and IV (Fig. 6E). An individual patient meta-analysis of the four cohorts ($n= 649$) was performed and there was no heterogeneity between the cohorts ($\chi^2= 3.5$, $p=0.32$). Patients with higher FKBPL levels had a significantly longer PFS from diagnosis (HR= 1.22, 95% CI 1.03 – 1.44, $p=0.02$) in the unstratified analysis (Fig. 6F), but significance was not reached in the stratified analysis ($p=0.07$). The median FKBPL histoscore value over the four cohorts was 165 (interquartile range 146 – 186) however cohort III had a significantly higher median at 190 and had a large number of censored events and thus was considered an outlier. Therefore, a second meta-analysis of cohort I, II and IV was conducted ($n=550$). There was a significant association between higher FKBPL levels and PFS from diagnosis in both the stratified (HR=1.23, 95% CI 1.02, 1.47, $p = 0.03$) and unstratified analysis (HR=1.27, 95% CI 1.06, 1.52, $p = 0.009$; Fig. 6G).

Discussion

The majority of ovarian cancer patients relapse after standard treatment and this has been partially attributed to the CSC subpopulation. HGSOC therefore remains a disease of unmet clinical need and here, for the first time, we evaluate a FKBPL peptide fragment, ALM201, to dual target HGSOC stem cells and tumor angiogenesis.

One of the challenges with studying new therapeutics for HGSOC is determining the histopathological origin of the most commonly used cell lines. OVCAR3 and Kuramochi cells contain the major oncogenes and tumor suppressor genes associated with HGSOC and are most likely to resemble the disease and were therefore used for the majority of the *in vitro* and *in vivo* experiments (33). ALM201 clearly demonstrated *in vitro* anti-CSC efficacy, using both tumorsphere assays and flow cytometry in the OVCAR3 cell line monolayer (Fig.1D; Fig. 2A-D). The Kuramochi cell line did not form tumorspheres or contain a CD44⁺/CD117⁺ cell population and also had a reduced ALDH⁺ subpopulation, thus indicting a lower CSC subpopulation *in vitro*. However, following treatment with ALM201 there was a decrease in spheres produced from PGCCs *in vitro* and a significant reduction in the ALDH⁺ population in the Kuramochi cell line (Fig.1G; Fig. 2E).

FKBPL and its peptide derivatives have previously shown potent anti-angiogenic activity resulting in a tumor growth delay in a range of xenografts studies, potentially through the cell surface receptor CD44 (20,22,24). However, for the first time, we observed no tumor growth delay in the OVCAR3 xenografts after treatment with ALM201 (Fig. 3B). *Ex vivo* analysis of the xenografts

by IHC revealed limited blood vessels and extensive vasculogenic mimicry (Fig. 3 G - I). *In vitro* tubule formation assays suggested that ALM201 had no effect on inhibiting VM channels in the OVCAR3 cells (Fig. 3J). In summary, the paucity of blood vessels and the high level of vasculogenic mimicry within the OVCAR3 xenografts, is a likely explanation for ALM201's lack of anti-angiogenic efficacy in this xenograft model.

Angiogenesis is regarded as an essential hallmark of cancer, however non-angiogenic tumors have been reported to occur in brain (43), liver metastasis (44,45) and lymph node metastasis (46,47). Gene expression analysis in angiogenic and non-angiogenic non-small lung cancer (NSLC) samples suggests that in non-angiogenic tumors, hypoxia leads to an increased activation of the mitochondrial respiration chain and rapid tumor growth (48). Indeed, the OVCAR3 xenografts had a more rapid tumor growth, compared to the angiogenic Kuramochi xenografts (Fig. 3B; Fig 4A). Moreover, there is emerging evidence in the literature that the non-angiogenic growth of tumors is responsible for both the intrinsic or acquired resistance to anti-angiogenic treatment (49,50). Here, for the first time, we describe an ovarian cancer xenograft that is dependent upon VM as opposed to classical angiogenesis. On the other hand, the Kuramochi cell line formed well-vascularised xenografts *in vivo* and treatment with ALM201 resulted in a significant tumor growth delay (Fig. 4C). Analysis of the xenografts showed an extensive blood vessel network consistent with high expression of angiogenesis related genes in Kuramochi cells (Fig. 5A). Kuramochi xenografts treated with ALM201 had decreased CD31⁺ blood vessels (Fig. 4D); in line with our previous studies with recombinant FKBPL and AD-01 (24,26). Furthermore, the *in vivo* gold standard limiting dilution assay clearly demonstrated that ALM201 significantly decreased the tumor initiating potential by 10-fold in Kuramochi xenografts (Fig. 4F). This result has significant

clinical relevance since therapies against CSCs are a very active area of research and there are comparatively very few agents that specifically target HGSOC stem cells. Overall, ALM201 had a potent anti-CSC efficacy in the Kuramochi cells *in vivo* and no effect on the OVCAR3 CSC population. This suggests micro-environmental components are drastically different between the two different tumor xenografts; not surprising given the dramatic differences in tumor vascularization in these tumor types.

These results further highlight the clinical need to stratify patients even within the same subtype of ovarian cancer. Four molecular subtypes within the umbrella of HGSOC (C1/mesenchymal, C2/immune, C4/differentiated and C5/proliferative) have been identified by gene expression profiling (51). Survival is statistically different between the subtypes; best in the immunoreactive type and worst in the proliferative or mesenchymal subtypes (52). The OVCAR3 and the Kuramochi cell lines are both indicative of the HGSOC subtype although the main drivers of *in vivo* tumor growth are clearly very different. In the clinic, treatment with ALM201 or any other anti-angiogenic therapy, in patients with HGSOC tumors with similar properties to the OVCAR3 subtype are likely to be ineffective. On the other hand, highly vascularized tumors, similar to the Kuramochi xenografts, are more likely to respond well to anti-angiogenic therapies and encouragingly, ALM201 also exhibited a potent anti-CSC effect. Bais *et al* recently demonstrated that higher microvessel density was predictive for response to bevacizumab in a Phase 3 clinical trial (GOG-0218) (53). This may prove to be a simple and effective way to stratify patients likely to respond to anti-angiogenic therapy in HGSOC.

Intriguingly, ALM201 inhibited OVCAR3 CSCs *in vitro*, however, there was no effect on the CSC subpopulation in the OVCAR3 xenograft (Fig.1D; Fig. 2A-D; Fig. 3C-D). Analysis of the Kuramochi and OVCAR3 xenografts showed there were substantial differences in IL-6 and IL-8 both mRNA and protein level. Analysis of the xenografts using mouse and human ELISA revealed only human IL-6 and IL-8 could be detected thus suggesting their source is tumor-derived, rather than being from the endogenous mouse microenvironment. IL-6 and IL-8 levels were significantly elevated *in vivo* in the OVCAR3 xenografts compared to the monolayer and no difference was observed between Kuramochi cells grown as monolayers or xenografts (Fig. 5 C-D). We hypothesise that enrichment of the cytokines in OVCAR3 xenograft is a possible contributing factor to the inability of ALM201 to decrease stemness *in vivo*, whilst being effective *in vitro* where levels were substantially lower. Indeed, addition of recombinant IL-6 to *in vitro* OVCAR3 tumorsphere assays abrogated the ability of ALM201 to decrease TFE (Fig. 5E). The principle signalling pathway of IL-6 is STAT3, and for the first time we demonstrated that ALM201, reduces activation of STAT3 in OVCAR3 cells. Future studies using fresh clinical samples are required to further investigate the role of IL-6 mediated resistance to FKBPL based therapies.

We have provided evidence that FKBPL's clinical peptide, ALM201, is a novel anti-CSC agent and a potent angiogenic inhibitor in vascularized HGSOC via STAT3 signalling. The current study will greatly enhance the clinical utility of this agent during its subsequent clinical development. In particular, we would suggest that well-vascularised tumors, with low IL-6, might be most responsive to its dual anti-angiogenic and anti-CSC activity. Although this will need to further validated in fresh clinical samples. Furthermore, we have demonstrated that high FKBPL levels were associated with an increase in PFS. This data indicates that FKBPL has potential as a novel

prognostic biomarker in HGSOC; a cancer with no universally accepted biological prognostic biomarkers. Finally, we have provided further evidence that a number of different subtypes exist under the remit of HGSOC; with Kuramochi xenografts displaying extensive vascularization and the OVCAR3 xenografts representative of 'immune' subtypes.

Additional Information

Declarations

Ethics approval and consent to participate

Solid ovarian and omental samples were removed during cytoreduction surgery and were collected from ovarian cancer patients with fully informed consent (NIB13-0073; Northern Ireland Biobank). In vivo experiments were carried out in Balb – c severe compromised immune deficient (SCID) mice in accordance with the Animal (Scientific Procedures) Act 1986 and conforming to the current UK Coordinating Committee on Cancer Research (UKCCCR) guidelines. The experiments were completed under the Project License Number 2794, and Personal License Number 1598. All TMA tissue used as part of this study was acquired ethically, with the appropriate material transfer agreements (MTAs) and import licenses completed, in line with QUB and RCSI policy. Only persons with Human Tissues Act training handled the tissue. Tissue was received, stored, handled and disposed of according to the Human Tissue Act, 2004 and all data shared between collaborators was password protected. Pseudo -anonymised individual patient data was obtained including survival and relapse information, treatment, tumour size and grade where available.

Consent for publication

Not applicable

Availability of data and material

All data generated or analyzed during this study are included in this published article [and its additional files].

Competing interests

TR is an inventor and patent holder for ALM201. TR has received research funding from ALMAC Discovery. AdeF has received a grant from AstraZeneca (unrelated to work in this manuscript).

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Figure Legends

Figure 1: FKBPL and its clinical peptide derivative, ALM201, reduce tumorsphere formation in ovarian cancer cell lines and high grade serous patient samples.

The effect of ALM201 treatment on the primary TFE in the (A) PE01, and (B) PE04, and (C) OVCAR3, and (D) OVCAR4 after treatment with 1 nmol/L and 100 nmol/L ALM201 treatment. (E) Protein expression of FKBPL, USP19, RBCK1 was analysed in ovarian cell lines (OVCAR3, OVCAR4, Kuramochi, PE01, PE04, A2780) were by western blot (n=3) (F) The kuramochi cell line does not form tumorspheres (top picture) and Kuramochi PGCCs were isolated by incubation with cobalt chloride (450 μ M) for 72 h (bottom picture). (G) The Kuramochi PGCCs were trypsinised and seeded into Matrigel and tumorsphere media and treated with PBS or ALM201 (100 nmol/L). A representative image of spheroid formed from a PGCC (inset). Cells were incubated for 3 weeks, with fresh ALM201 added weekly and the number of spheroids > 50 μ M counted manually. (H) Tumorsphere formation of cancer cells derived from primary chemo-naïve high grade serous ovarian tumors (n= 3) and (I) primary high grade serous ovarian tumors which received neo adjuvant chemotherapy (n = 3). Data points are mean \pm SEM. $n \geq 3$. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (one-way ANOVA or two way student t test). TFE, tumorsphere forming efficiency

Figure 2 ALM201 reduces the CD44⁺/CD117⁺ and ALDH⁺ cell subpopulation by differentiating the CSCs to more 'mature' cancer cells

(A) Representative flow cytometry images demonstrating a reduction in the CD44⁺/CD117⁺ subpopulation following 72 h ALM201 treatment of OVCAR3 monolayers. (B) Percentage CD44⁺/CD117⁺ OVCAR3 cells after treatment with ALM201 compared to PBS treated controls. (C) Representative flow cytometry images demonstrating a shift in the ALDH⁺ cell population in OVCAR3 cells after treatment for 72 h with ALM201 (1 and 100 nmol/L). (D) Percentage reduction in the ALDH⁺ cell population was quantified in OVCAR3 and (E) Kuramochi cell lines following treatment for 72 h with ALM201 (1 and 100 nmol/L). (F) ALM201 treatment does not affect total number of colonies formed. (G) Representative images of OVCAR3 colonies; holoclones, meroclone and paraclones; Different colonies were manually counted and expressed per 100 cells seeded. (H) A reduction in the number of holoclones formed and a concomitant increase in the number of more differentiated, meroclone and paraclone colonies, following ALM201 treatment was observed in OVCAR3 cells. Data points are mean \pm SEM. $n \geq 3$. *, $P < 0.05$; **, $P < 0.01$ (one-way ANOVA or two way student t test). SSC, side scatter.

Figure 3 ALM201 does not target CSCs or inhibit angiogenesis in OVCAR3 xenografts.

(A) Tumor initiation *in vivo* assay following implantation of OVCAR3 cells and treatment with PBS or ALM201 (0.3 mg/kg/d) subcutaneous from day 1 (inset experimental design; $n = 5$). (B) OVCAR3 cells were implanted into mice and established until 100 mm³ then treated with PBS or ALM201 (0.3 mg/kg/d) for 30 days (inset experimental design; $n = 5$). (C) OVCAR3 tumour xenografts were excised and dissociated and the CSC subpopulation analyzed by tumorsphere assay ($n=3$) or (D) flow cytometry by quantifying CD44⁺ CD117⁺ cell population ($n=3$). (E)

OVCAR3 monolayers were treated with ALM201 (1 nmol/L and 100 nmol/L) for 24 h and expression levels of the pluripotency transcription factors (SOX2, OCT4, NANOG) analyzed by q-PCR. (F) Expression of pluripotency transcription factors in OVCAR3 xenografts following treatment with PBS or ALM201 (0.3 mg/kg/d) for 30 days. (G) OVCAR3 xenografts were sectioned and immunohistochemistry staining for CD31⁺ blood vessels conducted. A small number of blood vessels (red arrow) were observed at 2.5 x magnification and no CD31⁺ vessels (H) in the majority of the xenograft at 20 x magnification (I) CD31⁺/PAS dual immunohistochemistry staining of OVCAR3 indicated extensive vasogenic mimicry networks in OVCAR3 xenografts. (J) Treatment with ALM201 (100 nmol/L) does not inhibit OVCAR3 tubule formation (representative image in inset; $n=3$). Each dot represents a single mouse. Data points are mean \pm SEM. $n \geq 3$. *, $P < 0.05$; **, $P < 0.01$ (one-way ANOVA or two way student t test).

Figure 4 ALM201 targets CSCs and angiogenesis in the Kuramochi xenografts

(A) *In vivo* tumor initiation assay following implantation of Kuramochi cells into SCID mice and subcutaneous treatment with PBS or ALM201 (0.3 mg/kg/d) from day 1; days to tumor initiation was calculated (inset experimental design; $n = 5$) (B) and tumor growth monitored. (C) Kuramochi cells were implanted into mice established until 100 mm³ and treatment with PBS or ALM201 (0.3 mg/kg/d) for 56 days (inset experimental design; $n = 5$). ALM201 treatment increased survival; as determined by time to tumor quadrupling. (D) Kuramochi xenografts were sectioned and immunohistochemistry staining for CD31⁺ blood vessels conducted and ALM201 significantly decreased the number of blood vessels (E) Tumor cells dissociated from ALM201

or PBS treated Kuramochi xenografts were reimplanted in a limiting dilution assay into second generation mice. The second generation mice did not receive further treatment and were observed for tumor initiation . (F) The number of mice that developed tumors after six months observation is tabulated. Tumor initiating frequency (TIF) was calculated using ELDA software ($p = 8.77 \times 10^{-5}$; $n > 4/\text{group}$) (G) 2.5×10^6 cells were reimplanted into second generation mice and tumors from first generation ALM201 treated mice demonstrated a 118 day delay in tumor initiation. Each dot represents one mouse. Data points are mean \pm SEM. $n \geq 3$. *, $P < 0.05$; **, $P < 0.01$ (Two way, unpaired t test or One way ANOVA).

Figure 5 OVCAR3 xenografts upregulate inflammatory cytokines and ALM201 anti-CSC activity is abrogated by IL-6

(A) Heat map and (B) enrichment plot of angiogenesis-related genes upregulated (red) in Kuramochi cell line compared to the OVCAR3 cell line by RNAseq analysis. (C) IL-6 and IL-8 mRNA is upregulated *in vivo* compared to *in vitro* in the OVCAR3 cells but not in the Kuramochi cells ($n > 3$). (D) Human IL-6 and IL-8 protein is significantly higher in the OVCAR3 xenografts compared to the Kuramochi xenografts; mouse IL-6 and IL-8 (Kc) was not detected. (E) Addition of recombinant IL-6 to OVCAR3 tumorsphere assay abrogated the ability of ALM201 to decrease tumorspheres ($n = 3$). (F) ALM201 decreases OVCAR3 tumorspheres in the presence of IL-8. (G) Representative western blot demonstrating that ALM201 decreases phosphorylation of STAT3 and this effect is abrogated by addition of IL-6. (H) Densitometric analysis of western blots using ImageJ $n \geq 3$ (I) Diagram summarizing effect of ALM201 on OVCAR3 cancer cells. Data points are mean \pm SEM. $n \geq 3$. *, $P < 0.05$; **, $P < 0.01$ (Two way

ANOVA).

Figure 6 High FKBPL expression increased progression free survival

(A) FKBPL expression was analyzed using microarray data from publically available data sets (<http://www.kmplot.com/ovar>). Kaplan-Meier survival curves of ovarian cancer patients were generated, showing that those with low mRNA FKBPL expression showed a significantly reduced overall survival ($p < 0.05$). FKBPL expression Kaplan-Meier estimates of HGSOc PFS from diagnosis in cohort I (n = 177;B), cohort II (n = 193;C), cohort III (n = 99 ;D) and cohort IV (n = 180; E). Kaplan-Meier estimates were determined with average FKBPL score for PFS, where FKBPL protein expression has been separated by histoscore of 190; high >190 (blue) and low <190 (red). (F) Hazard ratio plot of high grade serous ovarian cancer progression free survival from diagnosis against FKBPL levels by cohorts I, II, III, IV (n=639) . (G) Hazard ratio plot of HGSOc PFS from diagnosis against FKBPL levels by cohort from cohorts I, II, and IV (n = 549).

Abbreviations

CSC - cancer stem cell

EMA – European Medicines Agency

FDA – Food and Drug Administration

FKBP - FK506 binding protein

GMD – Geometric mean diameter

HGSOC - high grade serous ovarian cancer

MTA - material transfer agreements

PGCC - polyploid giant cancer cell

PFS - progression-free survival

ROC - receiver operator curve

STR - short tandem repeat

SCID - Balb – c severe compromised immune deficient

TIF - Tumor initiating frequency

TFE – tumorsphere forming efficacy

TMA – tissue microarray

VM - Vasculogenic mimicry