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The therapeutic and diagnostic potential of FKBPL; a novel anticancer protein

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The immunophilin family of proteins has a vast number of roles regulating a variety of biological processes through protein–protein interactions. A relatively new and divergent member of this family, FK506-binding protein like (FKBPL), is emerging as a key player in the DNA damage response, steroid receptor signalling and more recently, control of tumour growth where it regulates response to endocrine therapy in addition to acting as a novel antiangiogenic protein. As a new therapeutic peptide based on FKBPL approaches clinical trials, this article highlights a unique approach to targeting tumours that are resistant to current antiangiogenic therapies and supports the role of FKBPL as a novel prognostic and predictive biomarker, distinct from its other family members.

FK506-binding proteins (FKBPs) are members of the immunophilin family; other members include the cyclophilins and parvulins [1,2]. The term immunophilin, relates to the ability of this protein family to bind the immunosuppressive drugs, FK506 and rapamycin, through their peptidyl prolyl *cis/trans* isomerase (PPIase) domain. This interaction leads to inhibition of calcineurin or mammalian target of rapamycin (mTOR) signalling (by FK506 and rapamycin, respectively) which reduces T cell activation and mediates immune suppression; although inhibition of PPIase activity is not involved. Family members are distinguished by their molecular weight and domain structure. FKBP12, a smaller member, consists mainly of the PPIase domain and interacts strongly with immunosuppressive drugs while the larger, non-canonical FKBP members, such as FKBP38, have a PPIase domain that does not bind immunophilin ligands [3]. The larger proteins, FKBP51 and 52, have duplicated PPIase domains, one of which can bind ligand, in addition to tetra-trico-peptide repeat (TPR) domains, which facilitate protein–protein interactions and a C-terminal calmodulin-binding domain [4]. In addition to their roles in immunosuppression, FKBPs have a wider variety of biological functions, such as regulatory or stabilizing components of multi-protein complexes integral to cell function and cell cycle control [3,4]. Immunophilins are most widely known for their biological role as co-chaperones within steroid hormone receptor complexes where they associate with the heat shock protein, Hsp90, [5] through their TPR domain and regulate steroid receptor signalling [6]. In 1997, a novel but clearly divergent member of this family, FK506-binding protein like (FKBPL), was identified [7]. It shares homology with the FKBP family (in particular FKBP52/51 and cyclophilin 40) mostly in the C-terminal TPR domain [8], but lacks the crucial residues within a weakly homologous PPIase domain that are required for enzymatic activity [9]. The sequence of FKBPL and relationship to other FKBPs is shown in Fig. 1. Recent research is now revealing the functional characteristics of FKBPL, highlighting its antitumour activity by several diverse mechanisms.

Discovery of the FKBPL gene and its role in radioresistance

FKBPL was discovered from a screen to identify genes responsible for induced radioresistance [7]. A clone, originally designated '8.6', then subsequently 'downregulated by ionizing radiation' (DIR1) [8], was finally renamed FKBPL, following the identification of its FKBP-like structure. Knockdown of FKBPL using antisense oligonucleotides, resulted in cell cycle checkpoint activation, and enhanced DNA repair and

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(a)

METPPVNTIGEKDTSQPQQEWEKNLRENLDSVIQIRQQPRDPPTETLELEVSPDPASQILEHTQGAEKLVAELEGDSHKS
HGSTSQMPEALQASDLWYCPDGSFVKKIVIRGHGLDKPKLGS.C.CRVLALGFPFGSGPPEGWTELTMGVGPWREETW
GELIEKCLESMCQGEEAELQLPGHTGPPVGLTLASFTQGRDSWELETSEKEALAREERARGTELFRAGNPEGAARCYG
RALRLLLTLPPPGPPERTVLHANLAACQLLLGQPQLAAQS.C.DRVLEREPGHLKALYRRGVAQAALGHNLEKATADLKKV
LAIDPKNRAAQEELGKVVIQGKNQDAGLAQGLRKMFG

(b)

% homology with FKBPL		
Full	PPIase	TPR
23	17	33
21	10	29
14	7	21
100	100	100
17	8	-

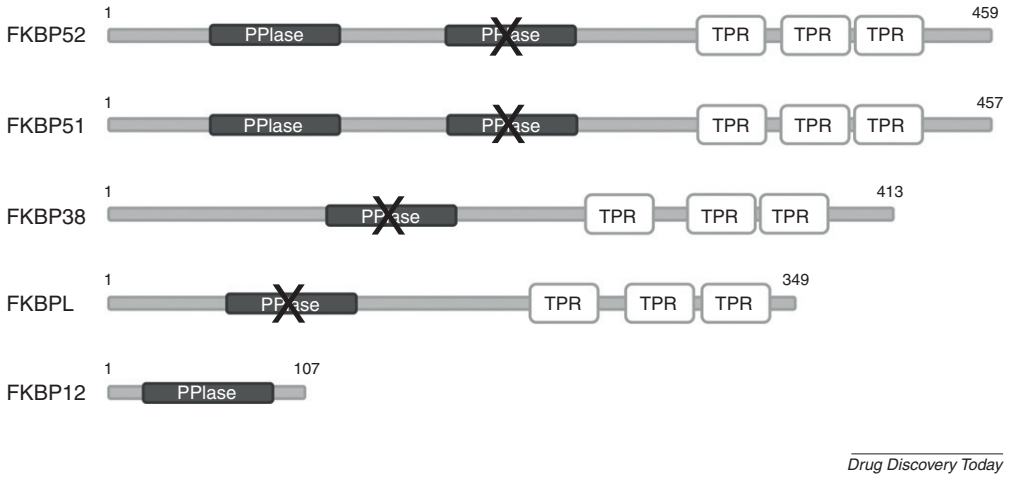

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FIGURE 1

Sequence of FKBPL and alignment with other FKBP family members. **(a)** Amino acid sequence of FKBPL. The antiangiogenic peptide AD-01 is highlighted in red. **(b)** FKBPL protein alignment of the full-length sequence or the PPIase and TPR domains were performed using ClustalW2, a general purpose multiple sequence alignment program for proteins. The table displays the percentage homology of several FKBPs with FKBPL within these regions. Alignment at the TPR regions demonstrates good conservation between FKBPL and the larger FKBPs, FKBP38, FKBP51 and FKBP52 in terms of both quantity and configuration of the TPR domains. Only weak homology was identified at the PPIase domain which is non-functional (X) in FKBPL and FKBP38 in addition to at the C-terminal PPIase domains of FKBP51 and FKBP52. *Abbreviations:* FKBP, FK506-binding protein; FKBPL, FK506-binding protein like; PPIase, peptidyl-prolyl *cis–trans* isomerise; TPR, tetratricopeptide repeat.

clonogenic survival following exposure to ionizing radiation [8,10]. Together the data suggested a role in radioresistance, a phenomenon not previously noted at that time for other immunophilins. More recently, it has been shown that high (rather than low) levels of FKBP51 lead to inhibition of apoptosis and increasing radioresistance [11].

FKBPL was also pulled out of a screen to identify p21-associated proteins [12] where it was described as WISp39 (WAF-1/CIP1 stabilizing protein 39). It was discovered that FKBPL/WISp39 has an important role in a novel multiprotein complex, crucial to a G_2 cell cycle checkpoint following high dose radiation stress. FKBPL binds to newly synthesized p21, in a complex with Hsp90, increasing p21 stability by preventing its proteosomal degradation, enhancing the G2 arrest. Jascur *et al.* suggested that targeting FKBPL in tumours, for which p21 overexpression confers a pro-survival growth advantage and/or chemoresistance/radioresistance, could be therapeutically beneficial. Although our own data [8,10] suggest that FKBPL is downregulated by radiation, which would be inconsistent with the stabilization of p21 observed by Jascur *et al.* [12], this could be due to tissue-specific differences within the cell lines used or because of a dependency of cell lines on a particular pathway. Indeed, in some instances knockdown of p21 results in a decrease in arrested cells and an increase in survival as described by Chu *et al.* [13]; this would be consistent with our own data where FKBPL knockdown, and presumably reduced p21, confers resistance to radiation [8,10].

The available data [7,8,10,12] suggested that the level of FKBPL within a tumour could indeed be important for the control of growth and response to therapy. In this respect, FKBP51 regulates AKT phosphorylation through a scaffolding mechanism and, as a result, can influence response to a variety of antineoplastic agents [14]. The role of FKBPL in mediating resistance and/or sensitivity to chemotherapeutic agents is currently the focus of investigations in our own laboratory. However, others have demonstrated that the association of the FKBPL/Hsp90/p21 complex with high levels of GTSE-1 (G2 and S phase expressed protein 1) increased p21 stabilization and causes resistance to taxane chemotherapy [15], supporting a role for FKBPL in pathways associated with chemoresistance. Furthermore, threonine-193 of FKBPL is phosphorylated in response to DNA damage on consensus sites recognized by ATM (ataxia telangiectasia mutated) and ATR (ataxia telangiectasia and Rad3 related) [16] further highlighting a role for FKBPL in response to therapy.

The role of FKBPL in Hps90 chaperone complexes

The role of FKBPL within Hsp90 chaperone complexes, first described by Jascur *et al.* [12], was not surprising given its homology across the TPR domains to large immunophilins, and the importance of the TPR domain for interaction with Hsp90. The main role of immunophilins within these complexes is to maintain the steroid

receptor in a conformation that enables rapid ligand binding and transactivation of steroid responsive genes [6]. A strong body of evidence suggests that FKBPL has an important role within a host of steroid receptor complexes, including those associated with the glucocorticoid receptor (GR) [17], androgen receptor (AR) [9] and oestrogen receptor (ER) [18]. FKBPL regulates both their levels and transactivation [9,17,18] and facilitates transport of cytoplasmic GR to the nucleus, in response to ligand, through an interaction with the microtubule-associated protein, dynamitin, through the PPIase domain in FKBPL [17]. At present, it is unclear how FKBPL competes with FKBP51/52, because Hsp90 has the ability to bind only one immunophilin at a time [19]. The role of FKBPL in complexes associated with AR correlates with male infertility in patients with mutations in the FKBPL gene; FKBPL appears to control transactivation by AR [9] under normal circumstances.

The association of FKBPL within ER/Hps90 complexes was identified by McKeen *et al.* [18]. In addition to being an oestrogen responsive gene, overexpression of FKBPL inhibited breast cancer cell growth; this is consistent with a previous report demonstrating FKBPL-mediated inhibition of cell proliferation in lymphoma cells [20]. Furthermore, FKBPL overexpression potentiated the cytostatic effects of the ER antagonists, tamoxifen and fulvestrant in addition to rendering cells more sensitive to oestrogen deprivation, supporting a predictive role for this protein in the response to aromatase inhibitors [18]. Increased FKBPL correlated with low ER and high p21 levels, which in turn caused a decrease in ER phosphorylation on Ser118, a mechanism previously linked with sensitivity to endocrine therapies [21]. To date, no other immunophilins have been linked with sensitivity of breast cancer to endocrine therapies, although cyclophilin40 and FKBP52 are oestrogen-inducible genes that are associated with Hsp90/ER heterocomplexes [22]. The influence of FKBPL on other immunophilins and their subsequent response to steroid ligands is currently not known, but it will be important in determining the role of each of these proteins in sensitivity to oestrogen signalling, because the ratio of immunophilins might influence steroid receptor function [23].

FKBPL as a prognostic and predictive biomarker

Prognostic and predictive biomarkers can provide information about a patient's overall cancer outcome, or response to therapy, respectively. Given the role of FKBPL in regulating both ER and p21, proteins both associated with increased breast cancer growth and response to endocrine therapy, it is not surprising that initial data from publically available microarray datasets showed a correlation between high level FKBPL expression and increased survival in untreated breast cancer patients [18]; there was also a trend towards significance in tamoxifen treated patients [18]. This was reproducible in a breast cancer tissue microarray, where FKBPL correlated with prolonged recurrence free survival [24]. FKBPL might therefore have both prognostic and predictive value for endocrine therapies, but this will need to be replicated in larger patient cohorts and randomized trials. Nevertheless, the loss of the chromosome 6p21.32 region containing FKBPL, occurred more frequently in tamoxifen-treated ER-positive breast cancer patients with increased cancer recurrence [25], supporting the growth inhibitory effects mediated by the presence of FKBPL and suggesting tumour suppressive activity.

While other FKBPs are also gaining recognition as potential cancer biomarkers, it is notable that in general most FKBPs are overexpressed in tumours compared with normal tissues and there is a positive correlation with poor outcomes and response to therapy [24]. It is appropriate therefore that FKBPs are seen as exciting new targets for cancer therapy; targeting FKBP38, FKBP12 and FKBP51 led to inhibition of tumour growth in a variety of models [26]. This is the reverse of what has been established for FKBPL, where overexpression appears to decrease tumour growth and response to therapy [18], suggesting further diversity with respect to the functionality of FKBPL. Furthermore, the lack of homology in the PPIase-like domain of FKBPL compared with the catalytic domains of FKBP12, 51 and 38 is an advantage from a structure-based drug discovery perspective, suggesting that it might be possible to target each FKBP selectively if the differences in the PPIase domain were exploited. However, further studies are needed to unravel the complexities of the interplay within this protein family. Nevertheless, rather than targeting FKBPL, the data clearly suggests that its antitumour activity should be harnessed. It is this approach that has led to the development of novel antiangiogenic peptides based on FKBPL [27].

FKBPL as a secreted antiangiogenic protein

Full length recombinant FKBPL (rFKBPL) is a highly potent antiangiogenic protein, inhibiting endothelial cell migration and tubule formation, in addition to microvessel formation from rat aortic rings *ex vivo* or within murine sponges *in vivo* [27]. In mouse xenograft studies twice weekly intratumoral injections of an FKBPL expression construct caused long-lasting inhibition of tumour growth and extensive central tumour necrosis, similar to cavitation reported in clinical trials of angiogenesis inhibitors [28].

The N-terminal region of FKBPL protein is responsible for the antiangiogenic activity (Fig. 1), the sequence is unique, with no homology to other FKBPs or other proteins. Peptides from this region are at least equipotent to full length FKBPL in endothelial cell migration assays. Detailed characterisation of a 24-residue peptide (AD-01) comprising amino acids 34–58 of FKBPL showed inhibition of endothelial cell migration, tubule formation and vessel sprouting from aortic rings with potencies in the picomolar range [27]. In xenograft experiments, daily systemic delivery of AD-01 inhibited blood vessel development and reduced tumour growth.

A growing body of evidence [27] suggests that these antiangiogenic effects are initiated from outside of endothelial cells, because both rFKBPL and AD-01 applied extracellularly are potent in all the models of angiogenesis tested. Consistent with this, endogenous FKBPL is secreted from several cell types including endothelial cells and tumour cells [27]. The mechanism of action is dependent on CD44; AD01 is inactive in cells that

TABLE 1

Comparison of ALM201 with marketed antiangiogenic drugs*

Assay	IC50 (nM)[a]		
	ALM201	**Bevacizumab[b]**	**Sunitinib[c]**
Wound scrape	0.009	59	8.9
Tubule formation	0.082	2400	4.4
Proliferation	No effect at 10 000	158	1300

[a] Data are from three independent experiments.
[b] Humanized monoclonal antibody that inhibits VEGF-A.
[c] Small molecule, multi-targeted receptor tyrosine kinase inhibitor, which targets VEGFR, PDGFR, C-kit and Flt-3.
* The FKBPL derivative ALM201 was compared with bevacuzimab and sunitinib in a range of angiogenic assays *in vitro*.

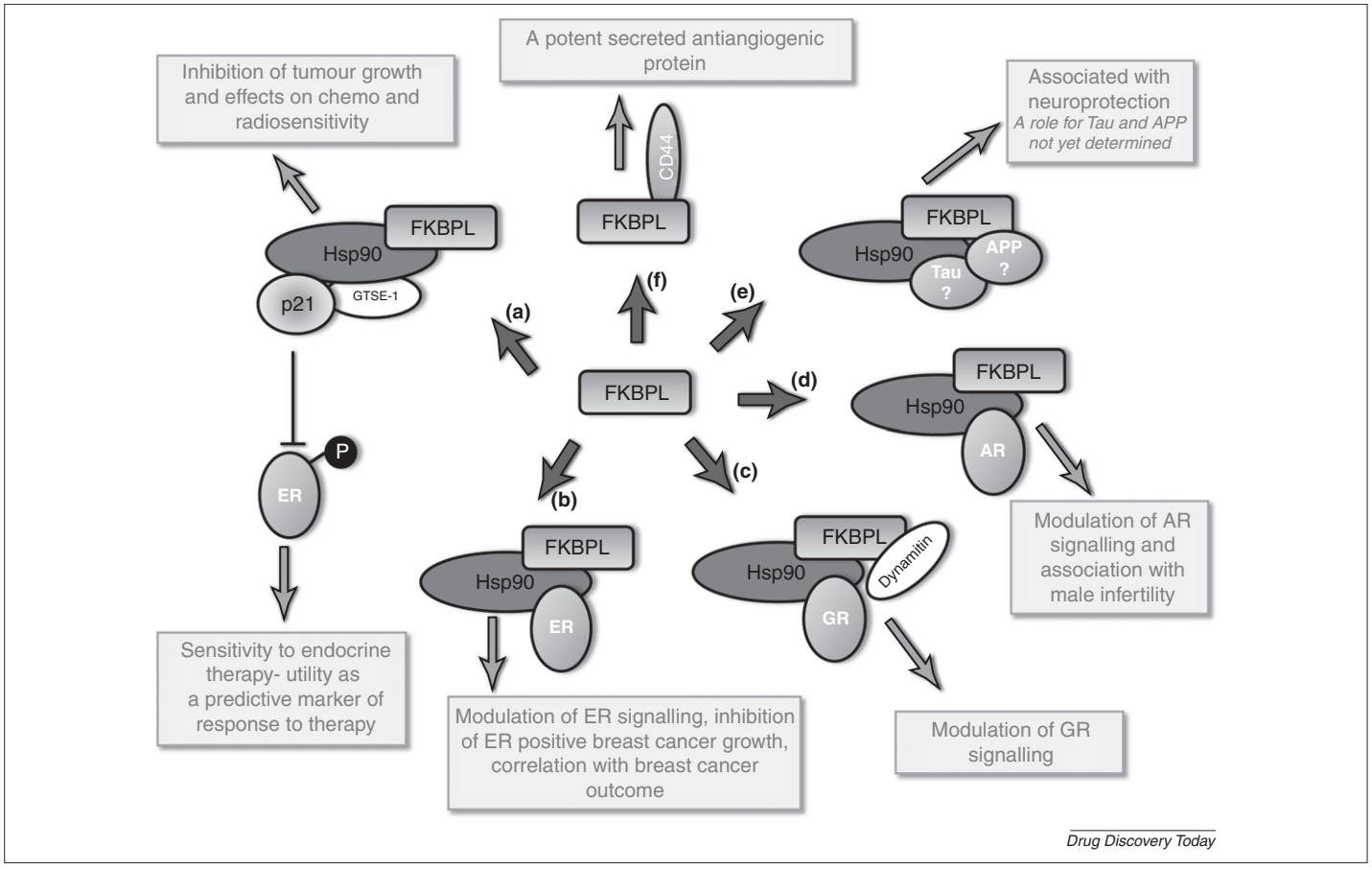

FIGURE 2

Schematic model of FKBPL's functions. **(a)** FKBPL overexpression inhibits tumour growth by stabilizing the cyclin dependent kinase inhibitor, p21, in association with Hsp90 [12,18]. Pathways responsible for the radiosensitizing properties of FKBPL [7,8,10] and possible roles in chemosensitization have yet to be elucidated. The FKBPL-induced stabilization of p21 results in decreased serine 118 phosphorylation of ER mediating sensitivity to endocrine therapy [18]. **(b)** FKBPL modulates ER levels and signalling, reducing the growth of ER positive breast tumours; levels of FKBPL therefore prognosticate for patient outcome [18,24]. **(c)** FKBPL associates with GR and mediates translocation of GR to the nucleus in response to ligand; this is mediated through association with the microtubule-associated protein, dynamitin [17]. **(d)** Mutations in FKBPL are associated with male infertility possibly by perturbation of AR signalling; FKBPL increases the transactivation of AR responsive genes [9]. **(e)** FKBPL is associated with neuroprotection [28]; the role of tau and APP in Hsp90 chaperone complexes associated with FKBPL are yet to be determined. **(f)** Secreted FKBPL and FKBPL-derived therapeutic peptides have potent antiangiogenic activity and are dependent on CD44 for their activity [27]. *Abbreviations:* APP, amyloid-β precursor protein; AR, androgen receptor; CD44, cluster of differentiation 44; ER, oestrogen receptor; FKBPL, FK506-binding protein like; *GR*, glucopcorticoid receptor; GTSE-1, G2 and S phase-expressed protein 1; Hsp90, heat shock protein 90; p21, cyclin-dependent kinase inhibitor 1 encoded by the *CDKN1* gene; Tau, tau proteins are the product of alternative splicing from a single gene that in humans is designated MAPT (microtubule-associated protein tau).

do not express endogenous CD44 and in cells where CD44 in downregulated with siRNA [27]. The role of CD44 is currently under investigation with respect to mediating peptide uptake and the subsequent antiangiogenic signalling.

Development of FKBPL peptide derivatives for clinical trials

The novel mechanism of action and the high potency of AD01 derived from FKBPL make peptides based on AD01 attractive candidates for development as new antiangiogenic drugs. Structure/activity and peptide stability studies using AD01 as the starting point led to the selection of ALM201*, a 23-residue peptide comprising amino acids 35–58 of the FKBPL sequence, as the drug development candidate. ALM201 is much more potent than marketed antiangiogenics in several models and has no effect on cell proliferation (Table 1). Preclinical activities with ALM201 including formulation and toxicology have been completed and the compound is approaching Phase I clinical trials. The clear differentiation from other antiangiogenics in terms of potency, lack of cytotoxicity and novel mechanism of action makes ALM201 an attractive drug candidate with potential roles in many solid tumours and in patients where anti-VEGF (vascular endothelial growth factor) therapy is ineffective or in tumours that develop resistance to current VEGF-targeted therapies.

Concluding remarks

The roles of FKBPL are summarised in Fig. 2. FKBPL is clearly a divergent immunophilin with distinct and important functions in cancer. Emerging evidence is also highlighting activity in other disease states, such as azoospermia [9] and Alzheimer's, where it appears to have neuroprotective properties [29]. Drug development based on FKBPL is being actively pursued in the angiogenesis area, where a clinical candidate is approaching Phase I cancer trials. In addition to its therapeutic potential, the utility of FKBPL as a cancer biomarker, although still in the early stages, might facilitate more tailored cancer therapies with particular attention needed with

* *O'Rourke *et al.* (2011) The anti-tumour efficacy of the novel peptide inhibitor of angiogenesis, ALM201. Poster presented at: 102nd Annual Meeting of the American Association for Cancer Research, 2–6 April, 2011, Orlando, Florida, Abstract No.: 3288.

respect to understanding its role in influencing tumour sensitivity to chemotherapeutic agents. The roles of FKBPL in other disease states are only just emerging, highlighting that it might take another 10 years before the functional and therapeutic significance of this protein is fully realised.

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