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PD-L1 VHH as Functional Antagonists

Charlotte Brookes and Loukia Polycarpou

PD-1 is an immune checkpoint protein expressed on the surface of multiple types of immune cells, including antigen-stimulated T-cells and tumour-specific T-cells[1]. Interaction between PD-1 and its ligands (PD-L1 or PD-L2), is responsible for the regulation of T-cell activation, apoptosis, proliferation and cytokine production[2]. The interaction between PD-1 and PD-L1 can be both beneficial and harmful. On one hand, the PD-1/PD-L1 pathway regulates the immune response, inducing and maintaining immune tolerance, preventing damage to healthy self-tissues and autoimmunity[3]. Conversely, knockout mouse models of PD-1 have disease phenotypes resembling autoimmune disorders. The PD1/PD-L1 axis is a key mechanism of immune evasion in cancer, preventing aberrant cells being targeted by otherwise competent T-cells[4] (Figure 1).

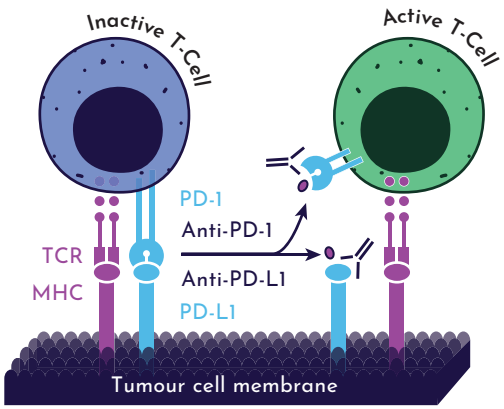


Figure 1: PD-L1 target biology. PD-L1 (CD273) is a transmembrane protein and ligand for PD-1, expressed on the surface of activated T-cells. PD-L1 plays a major role in suppression of the adaptive immune system, allowing tumours to evade immune destruction by "instructing" the T-cells to leave the tumour cells alone. As a marker of cancer, PD-L1 can be targeted therapeutically through numerous mechanisms.

IMMUNE CHECKPOINT PROTEINS & IMMUNE EVASION

PD-1 is strongly expressed on the surface of tumour-infiltrating T-cells; and many advanced tumours overexpress PD-L1 either on the cancer cells themselves or in the tumour microenvironment[5]. Meta-analysis has linked overexpression of PD-L1 to poor prognosis, as well as lower overall disease-free survival, in a variety of different types of cancer[6]. This demonstrates that immune evasion through stimulation of the PD-1/PD-L1 pathway is an important process in the survival and recurrence of cancer.

PD-L1 TARGETED THERAPIES

Inhibition of immune checkpoint proteins is currently an important method in the treatment of a variety of cancers, with multiple approved therapies and many more in development. Monoclonal antibodies (mAbs) specific to PD-1 or PD-L1 have been shown to block the interaction between receptor and ligand, enabling T-cells to initiate an immune response against cancer cells[7,8]. Clinical mAbs against PD-L1 block the PD-1/PD-L1 interaction, facilitating tumour cell killing, as shown in Figure 2. Currently there are three PD-L1 clinically approved antibodies used as frontline treatment of multiple types of cancer[9] (Table 1). Atezolizumab received approval in 2016 for the treatment of metastatic urothelial tract carcinoma. Two additional anti-PD-L1 monoclonal antibodies, avelumab and durvalumab, entered the market in 2017.

Drug name	Brand name	Year of approval	Global Sales (2020) (US $m)	Marketed Indication	Originator	Marketed countries	Antibody type	MoA	Adverse events
avelumab	Bavencio	2017	177.84	Merkel Cell Carcinoma, Metastatic Renal Cell Carcinoma, Metastatic Transitional (Urothelial) Tract Cancer	Merck KGaA	US	Fully natural human IgG1 monoclonal antibody	Inhibits PD-1/PD-L1 interactions whilst maintaining the integrity of the PD1/PD-L2 pathway and enhancing immune activation to tumour cells. Because of its inherrent Fc domain, avelumab retains the cytotoxicity in vitro and is the only therapeutic antibody that uses both immune checkpoint inhibition and ADCC mediated cell killing.	Immune-related adverse reactions
durvalumab	Fidursi; Imfinzi	2017	2042	Non-Small Cell Lung Cancer, Small-Cell Lung Cancer, Metastatic Urothelial Tract Cancer	AstraZeneca Plc (Medimmune)	Global	Highly selective, high affinity fully human IgG1 kappa monoclonal antibody	Blocks binding of PD-L1 with PD-1 and CD80 by binding with PD-L1 and CD80 intead of PD-L2 so that T cells can recognise and kill tumour cells. Potential reduction in immunotoxicity associted with PD-L2 inetraction.	Fatigue, musculoskeletal pain
atezolizumab	Tecentriq	2016	2917.35	Urothelial bladder cancer (second line) metastatic non-small cell lung cancer (NSCLC), Triple Negative PD-L1 expressing Breast Cancer	Genentech USA Inc	Global	Fully human high affinity engineered monoclonal IgG1	Specifically binds to PD-L1 previnting binding to PD-1 and CD80 receptors. Eliminates the inhibitory effect on cytotoxic T cells.	Severe immune-mediated adverse reactions.

Table 1: PD-L1 clinically approved antibodies (Global data). A summary of the mode of action and format of avelumab, durvalumab and atezolizumab. All three antibodies have been approved within the last ten years generating billion-dollar sales, demonstrating the demand for antibodies targeting PD-L1.

Inadequacies of the clinical application of first-generation mAbs targeting PD-1/PD-L1, such as low response rate of patients, severe immune-related adverse reactions, the need for intravenous administration (lack of oral bioavailability) and drug resistance through the development of anti-drug antibodies is reflective of the continuous development of novel therapies targeting this pathway[10]. Despite the long-term, potentially cure-like clinical benefits, therapy resistance remains a significant challenge for the further application of PD-1/PD-L1 blockade therapy[4,6].

	Phase I	Phase II	Phase III
Immunology	1		
Infectious Disease	4	9	
Mouth and Dental Disorders		1	
Musculoskeletal Disorders		1	
Non Malignant Disorders		1	
Oncology	116	123	112

Table 2: Summary of disease areas for PD-L1 therapies in clinical development. Most therapies targeting PD-L1 are oncology focussed (Global data). However, there are other conditions investigated including autoimmune diseases such as psoriasis, viral infections and musculoskeletal disorders[11].

PD-1/PD-L1 BLOCKING ANTIBODIES

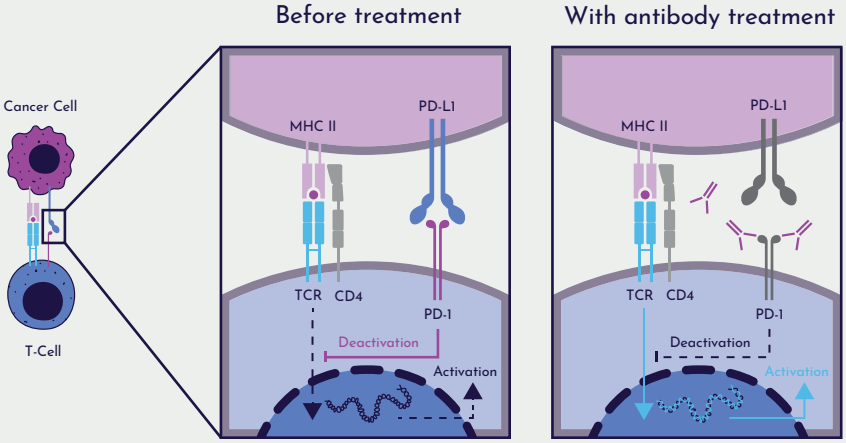


Figure 2: Mechanism of PD-1/PD-L1 antibody inhibitors. Immune checkpoint inhibitors, especially PD-1 and PD-L1 have shown clinical efficacies against many different solid and hematologic malignancies. Binding of PD-L1 to its receptor suppresses T cell migration, proliferation, and secretion of cytotoxic mediators, and restricts tumour cell killing. Inhibitors of PD-1 and PD-L1 disrupt the PD-1 axis thereby reversing T cell suppression and enhancing endogenous antitumour immunity to unleash long-term antitumor responses for patients with a wide range of cancers[10].

PD-L1 IN CLINICAL DEVELOPMENT

Though cancer patients can be resistant to immune checkpoint inhibitors, other immunotherapies (targeting cytokines, tumour-directed antibodies, antibody-drug conjugates, chimeric antigen receptor (CAR) T cells therapy, vaccines, and even genetic payloads) are paving the way for more tumour-specific therapy. Clinically approved PD-L1 antibodies are also in development for other oncology indications or are being trialled in different mAb combinations demonstrating the demand for more effective next-generation therapies, such as VHH. The greatest opportunity for newer and next generation formats are for solid tumours, where the majority of clinical trials are focussed.

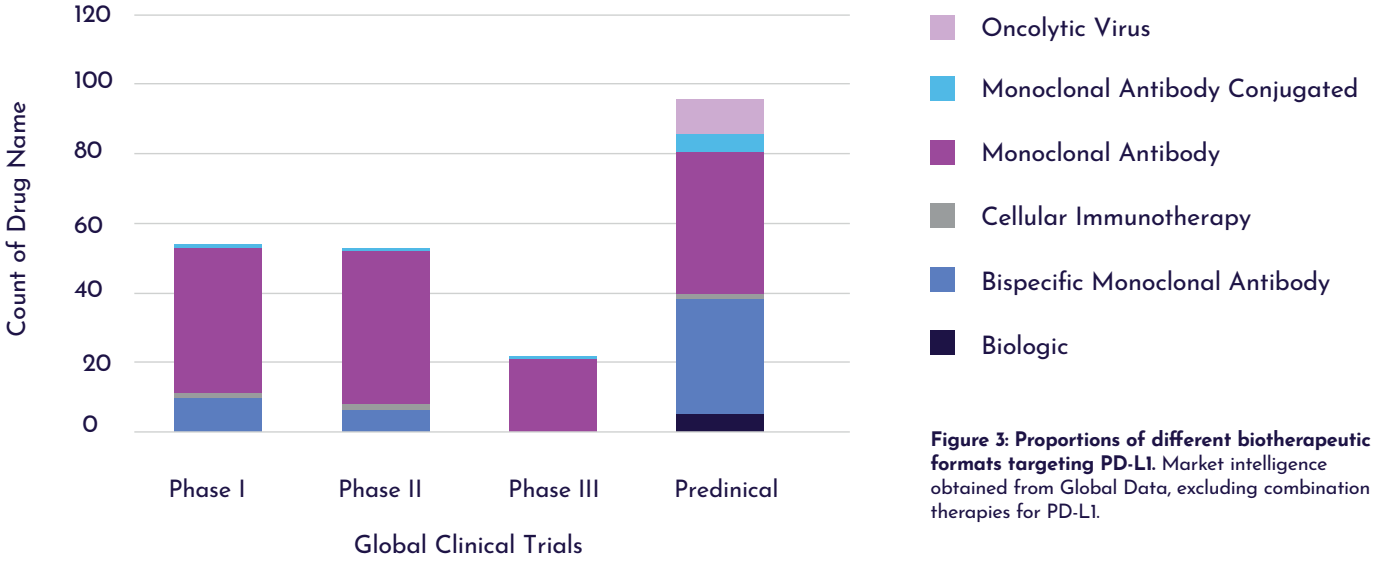


Figure 3: Proportions of different biotherapeutic formats targeting PD-L1. Market intelligence obtained from Global Data, excluding combination therapies for PD-L1.

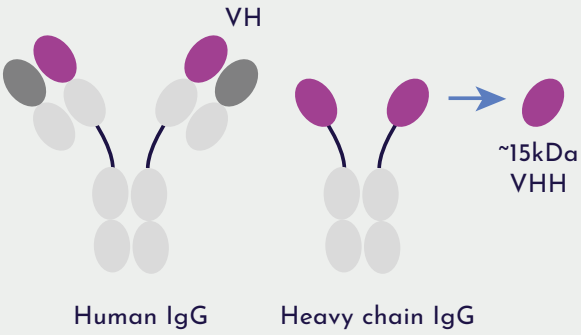
There are a significant number of biotherapeutic molecules in clinical development which target PD-L1, with mAbs comprising the largest proportion currently progressing through preclinical to Phase III studies (Figure 3). The greatest diversity in formats is observed in preclinical studies, ranging from oncolytic viral therapy[9] through to biological modulators such as fusion proteins. Cell therapy represents the smallest proportion of PD-L1 in development, however there are studies demonstrating the successful application of PD-L1 CAR-T for the treatment of solid tumours[12–15]. Although CAR-T therapies have shown success in the treatment of haematological tumours, this has not yet been achieved with solid tumours. A major contributing factor has been the PD-1-mediated suppression which significantly hinders CAR-T cells in the tumour microenvironment (TME). Scientists have recently focused their efforts on a promising new approach of PD-1/PD-L1 blockade which has shown improvement in the effectiveness of CAR-T cells[16]. The opportunity for VHH as an immunomodulator could complement the CAR-T targeting solid tumours in a similar way.

VHH AS AN ATTRACTIVE ALTERNATIVE FOR TARGETING PD-L1

VHH can be engineered to tune their binding selectivity and specificity for antigen targeting whilst their favourable biophysical properties simplify development and manufacturing. Due to their small size and biophysical robustness, VHH single domain antibodies are ideally suited for targeting tumour-associated antigens such PD-L1, as they can be incorporated into a number of therapeutic formats. The dense stroma that surrounds tumours and immunosuppressive TME renders treatment of some solid tumours difficult with current antibody approaches. VHHs represent an attractive alternative. Examples of where this has been demonstrated include studies were an anti-PD-L1 CAR-T caused a delay in tumour growth and improved survival[13]. Another involves the targeted cytokine delivery of fusion proteins to IL-2 or IFN and PD-L1 VHH in pancreatic tumour models, penetrating the TME and reducing the tumour burden by 50%[17].

WHAT ARE VHH?

In the early 1990s researchers discovered that camels and llamas produce heavy-chain only antibodies. VHH, the variable domains of these antibodies, are small fully functional single domain antibodies that exhibit all the features associated with antibody specificity. They have binding capacities similar to those of conventional monoclonal antibodies. However, as a result of their smaller binding region based upon a more probing CDR3 loop and stable, naturally occurring single chain structure they can target antigens and epitopes that are considered difficult or intractable for conventional antibodies. VHH domains are also typically less immunogenic than other single chain constructs such as ScFv because of their high homology with human VH genes and absence of exposed hydrophobic regions making them less potent immune targets. This has identified them as potentially excellent building blocks for novel non- IgG biotherapeutics.



Their small size, approximately 1/10th the size of an IgG molecule, is likely to enable improved tissue penetration in vivo.

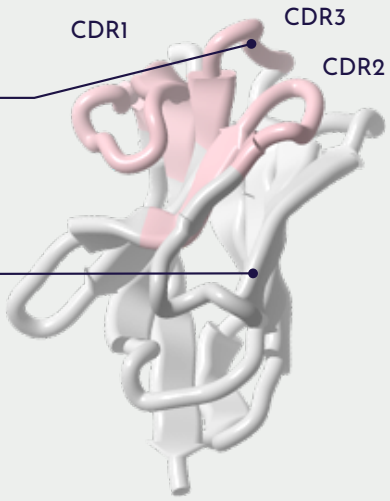
Because they are encoded by only a single gene comprising (approximately) 360 base pairs, VHH can be easily covalently linked to other molecules or pro-drugs. Fusion of VHH that bind different epitopes or have different modes of action allows the creation of multivalent molecules with high affinity or potency.

CDRs are the binding region for target protein epitopes

The CDR3 loop of VHH is long and protruding and has a prolate shape, exposing a convex paratope

Due to their hydrophilic surfaces, VHH can be easily linked to create dimers or higher order multimers

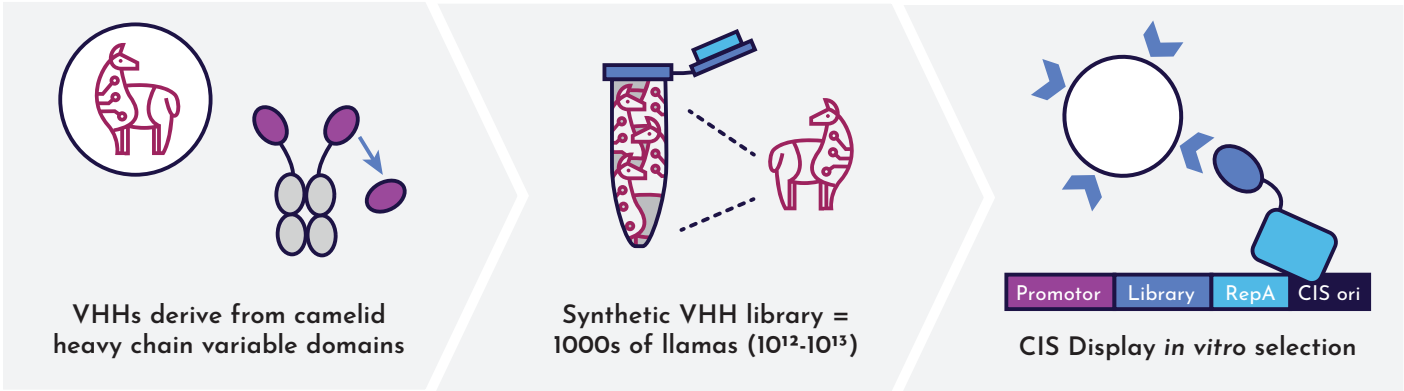
Small stable molecules (15kDa)



CIS DISPLAY OF LLAMDA® VHH LIBRARY TO SELECT ANTI-PD-L1

Rapid isolation of high affinity antibodies to biologically relevant epitopes is critical for biologic discovery. Moreover, developability of the antibody candidates is critical for a molecule to progress through clinical development. Isogenica's LlamdA® library allows the isolation of antibodies pre-screened and depleted for manufacturing liabilities and developability issues, such as glycosylation and isomerisation. This vast, liability-depleted library (>10^{12}) was enriched for PD-L1 binders using Isogenica's cell-free CIS Display technology based on ITT and PCR using an in-house Fc-tagged version of the human PD-L1 extracellular domain.

Figure 4: LlamdA® is a state-of-the-art, highly diverse, synthetic VHH library which, when combined with the power of CIS display, interrogates more than 10^{13} library members: equivalent to the circulating repertoire of one million llamas. LlamdA® offers optimal expression of maximised functional diversity.



SYNTHETIC LIBRARY HTP SCREENING GENERATED POTENT, DEVELOPABLE ANTI-PD-L1 VHH MONOMERS

Unique hits from primary ELISA screening were purified and titration ELISAs showed a range of EC_{50}s in the lead panel from 14.4 to 0.5 nM (Figure 5). Size-exclusion chromatography (SEC) allowed identification of clones within the panel showing extremely strong monomeric peaks with little to no aggregation or fragmentation (Figure 6).

Testing of the lead panel in a PD-1/PD-L1 functional ELISA identified subsets of clones capable of either full or partial antagonism of this interaction (Figure 7), with full antagonists more effective than a durvalumab (Imfinzi) Fab fragment (Figure 8). As well as being of therapeutic relevance, this highlights the targeting of multiple epitopes on PD-L1.

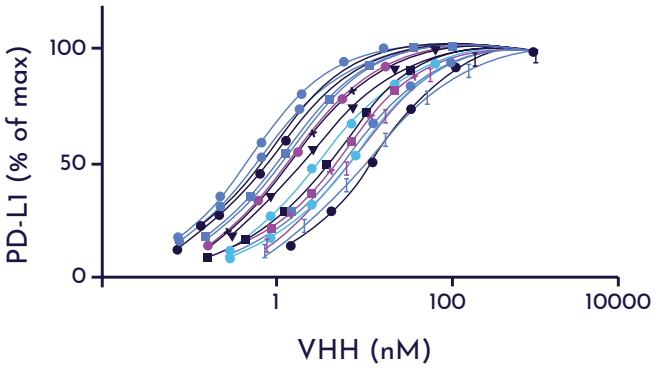


Figure 5: PD-L1 ELISA binding of initial VHH panel. Biotinylated PD-L1 was captured on an ELISA plate via streptavidin. Serially diluted FLAG-tagged VHHs were added to the plate and detected via anti-FLAG-HRP. Mean +/- SD, n=2.

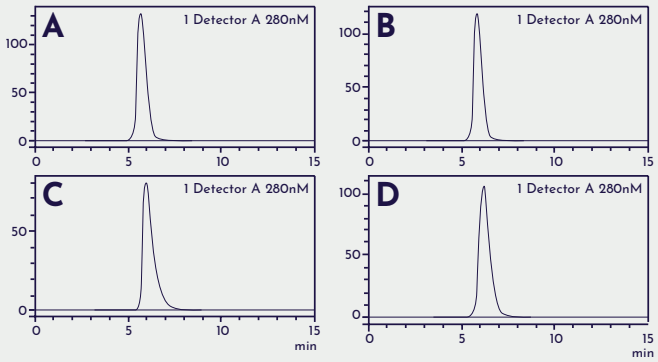


Figure 6: Size exclusion chromatography (SEC). VHHs (A= clone 01, B = clone 04, C = clone 13, D = clone 36) with C-terminal 6His-3FLAG affinity tags were expressed from *E. coli* and purified on Ni-NTA resin followed by preparative SEC into PBS. Analytical SEC was performed using a Superdex 75 5/150 GL column (Cytiva) at 0.3 ml/min.v

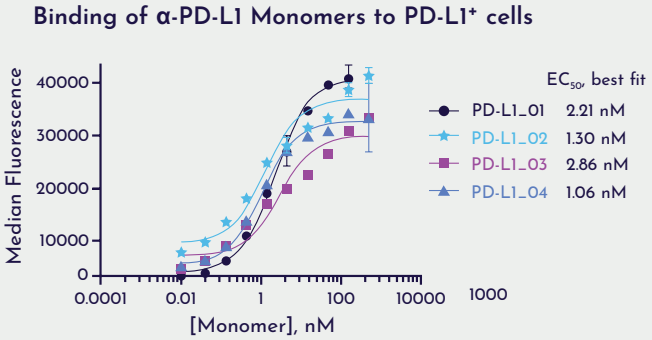


Figure 7: Binding of VHH panel to MDA-MB-231 cells. Serially diluted VHHs were incubated with PD-L1-positive MDA-MB-231 cells, then stained with anti-FLAG-PE. Events were captured on a Novocyte flow cytometer in PE channel and MFI was used to determine EC50s. n=2.

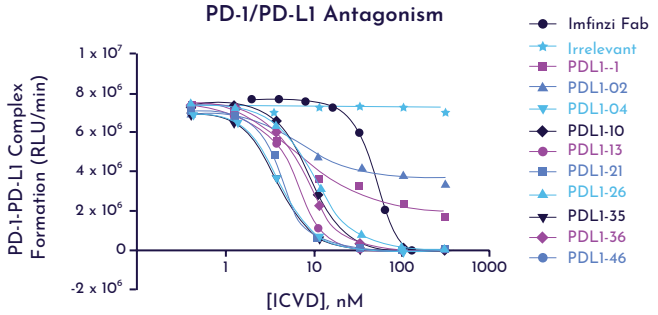


Figure 8: PD-1/PD-L1 antagonism. VHH mediated inhibition of PD-1/PD-L1 complex formation was measured by functional ELISA. The Imfinzi (durvalumab) Fab fragment and an irrelevant VHH were included as positive and negative controls, respectively. Lower signals indicate a greater degree of inhibition. Mean +/- SD, n=2. The results showcase a spectrum of partial and full antagonists, indicating that a variety of epitopes are targeted.

The interaction between PD-1 and PD-L1 is crucial for the regulation of many biological processes, including T cell activation. However, in a pathophysiological context, interaction between PD-L1-overexpressing tumour cells and PD-1-expressing immune cells enables immune evasion and tumour progression. Therefore, blockade of this interaction has therapeutic effects. Additionally, it was proposed that VHH, due to their superior biophysical characteristics, could enhance this therapeutic effect, becoming a more desirable format for future therapeutics. In fact, as shown, the Isogenica LlamdA® VHH can be generated rapidly and at sub-nanomolar affinities with functional activity equivalent to that of a Fab fragment of durvalumab (Imfimzi). Therefore, Isogenica LlamdA® VHH are highly manufacturable alternatives to scFv as targeting agents in the next generation of biologic cell and gene therapies.

READY TO START YOUR NEXT PROJECT?

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T +44 1799 533 680

E bd@isogenica.com

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