Generation and Characterisation of Formatted Affimer® Biotherapeutics

POS008

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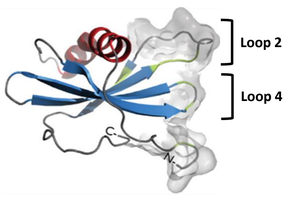
Introduction

Affimer Technology

- The Affimer biotherapeutic protein scaffold is based on human Stefin A
- Two surface loops have been engineered into the scaffold backbone



Binding region: two randomised 9 amino acid loops

- Phage display compatible – large Affimer phage libraries ($1x10^{11}$)

Benefits of Affimer Therapeutics

- **Small size:** 14 kDa, $1/10^{th}$ the size of an antibody
- **Fc-formatted Affimer:** provides half-life extension – 80 kDa, half the size of an antibody – production in Expi293F cells
- **Multimers**: dimer, trimer, tetramer production at 100's mg/L in *E. coli*
- **No post-translational modifications on Affimer proteins**: ease of manufacturing and improved stability
- **Improved tissue penetration:** small size gives greater potential for increased efficacy

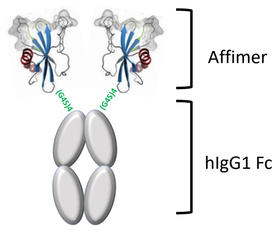
Objectives

- Programmed death-ligand 1 (PD-L1) plays an important role in the modulation of the immune system and has been clinically validated as a target for a number of human cancers with monoclonal antibodies (mAbs)
- Protein scaffold technologies offer an alternative to mAbs as therapeutics due to their flexibility in formatting options
- The objective was to demonstrate that Affimer antagonists of PD-L1 can be formatted for half-life extension (Fc-fusions) or as multimers (in-line fusions) to demonstrate avidity effects

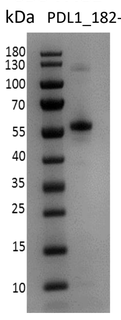
Affimer Protein Formatting – Fc-fusion

- Anti-mouse PD-L1 Affimer protein fused to human IgG1 Fc (PDL1_182-hFc1) using $(Gly_4Ser)_4$ linker for half-life extension
- Protein expressed in Expi293F cells and purified using Protein A and SEC using an ÄKTA FPLC system (GE Healthcare)

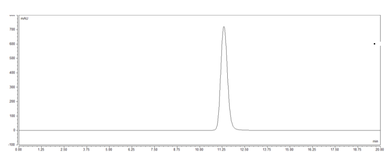
PDL1_182-hFc1 representation



SDS-PAGE



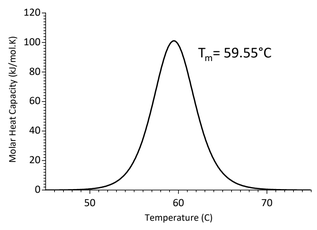
SEC-HPLC



Run on Acclaim SEC-300 (ThermoFisher Scientific)

Note: The Affimer His protein used for these studies is naturally present as a dimer in solution.

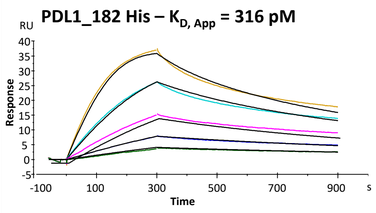
DSC

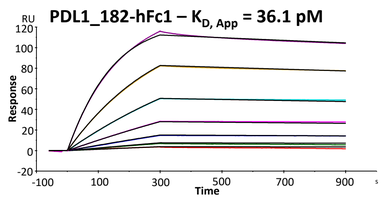


Run on Nano DSC (TA Instruments)
Data fitted using a two state scaled model

Biacore sensorgrams

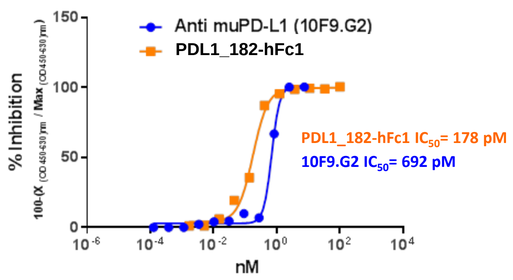
Run on Biacore T200 (GE Healthcare) using a CM5 sensor chip – data is blank-subtracted and fitted to a 1:1 binding model



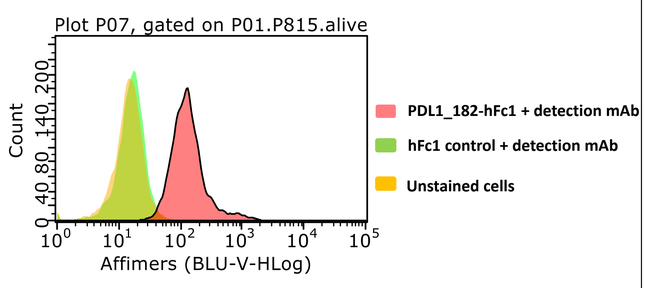


Protein	k_a (1/Ms)	k_d (1/s)	Apparent K_D (M)	Chi² (RU^2)
PDL1_182 His	8.99E+06	2.30E-03	3.16E-10	0.325
PDL1_182-hFc1	3.39E+06	1.23E-04	3.61E-11	0.558

PD-1/PD-L1 blockade ELISA – PDL1_182-hFc1 vs tool mAb



Flow cytometry histogram – PDL1_182-hFc1 binding to P815 cells surface



Affimer-hFc1 key results:

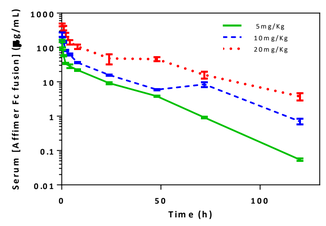
- **Transiently expressed from Expi293F cells and purified to yields >100 mg/L**
- **Has an apparent K_D 10-fold lower than the parent Affimer protein**
- **Competes against PD-1 for binding to mouse PD-L1 (IC_{50} = 178 pM)**
- **Shows specific binding to mouse mastocytoma cells (93.94% of cells) compared to the hFc1 negative control (1.54% of cells)**

Pharmacokinetics of PDL1_182-hFc1 in Mouse

- C57BL/6 mice dosing – single i.v. injection of PDL1_182-hFc1 at 5, 10, or 20 mg/kg
- Blood samples were collected over 7 days
- The concentration of the Affimer-hFc1 in serum was measured by a fluorescent ELISA

Product	Dose (mg/kg)	Cmax (µg/ml)	AUC min*µg/mL	Terminal Half-life (h)
PDL1_182-hFc1	5	155±10.5	3313.8	20.9±1.3
	10	241±36	5964.6	19.2*
	20	462±46	9852	59.9±5.3

PK parameters of the Affimer-hFc1 – Parameters were calculated with a non linear fit at 2 phases decay. *error could not be determined.



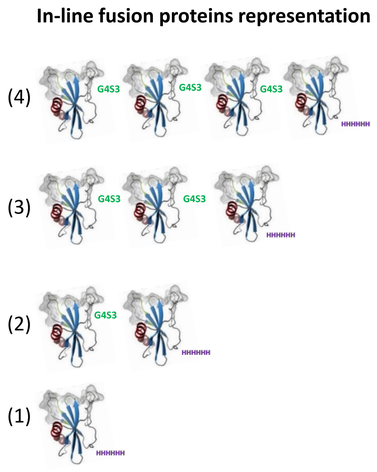
Key results:

- **The serum half-life of PDL1_182-hFc1 was successfully extended**
- **PDL1_182-hFc1 was well tolerated at all doses administered in mouse**

Affimer Protein Formatting – In-line Fusions

- Human Affimer proteins (PDL1_141) fused together by a $(Gly_4Ser)_3$ linker and carrying a C-terminal 6X His tag
- Expressed in *E. coli* and two-step purification performed using affinity and SEC

In-line fusion proteins representation



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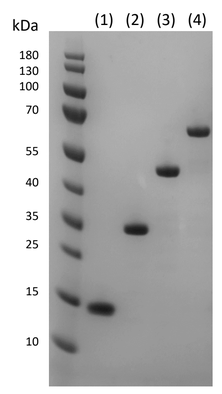
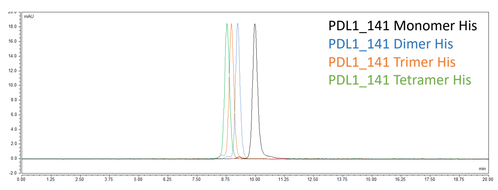
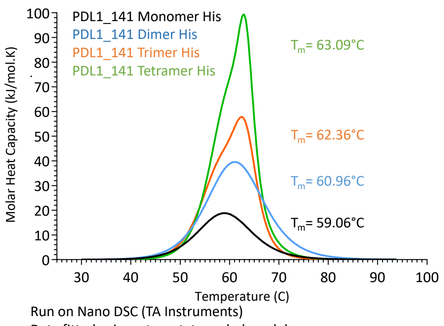


SEC-HPLC



Run on Acclaim SEC-300 (ThermoFisher Scientific)

DSC

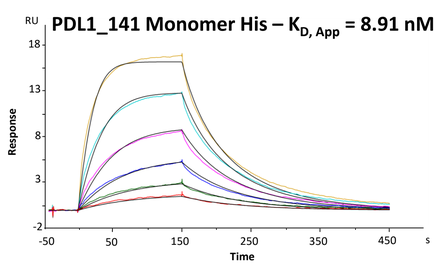


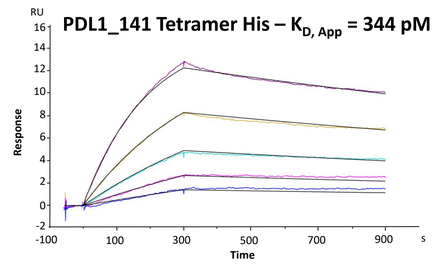
Run on Nano DSC (TA Instruments)
Data fitted using a two state scaled model

Format of the Affimer protein	Expected MW (kDa)	Yield after one-step purification (mg/L)	Yield after two-step purification (mg/L)
PDL1_141 Monomer His	14	270	56
PDL1_141 Dimer His	25	278	116
PDL1_141 Trimer His	42	212	37
PDL1_141 Tetramer His	56	205	47

Biacore sensorgrams

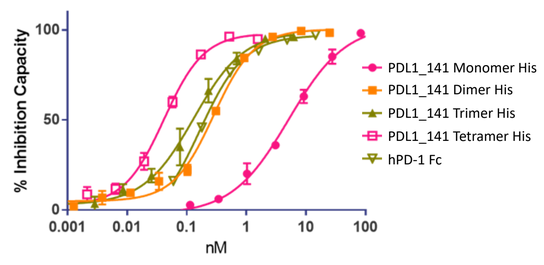
Run on Biacore T200 (GE Healthcare) using a CM5 sensor chip – data is blank-subtracted and fitted to a 1:1 binding model





Protein	k_a (1/Ms)	k_d (1/s)	Apparent K_D (M)	Chi² (RU^2)
PDL1_141 Monomer His	2.81E+06	2.51E-02	8.91E-09	0.0740
PDL1_141 Dimer His	1.12E+06	5.35E-04	4.79E-10	0.0251
PDL1_141 Trimer His	1.13E+06	4.73E-04	4.18E-10	0.0153
PDL1_141 Tetramer His	1.01E+06	3.48E-04	3.44E-10	0.0292

Blockade ELISA – In-line fusion Affimer proteins vs hPD-1 Fc



IC_{50} (nM)				
hPD-1 Fc	PDL1_141 Monomer His	PDL1_141 Dimer His	PDL1_141 Trimer His	PDL1_141 Tetramer His
0.17	2.5	0.28	0.13	0.04

Key results:

- **Successful expression and purification of in-line fusion proteins to yields ~40-120 mg/L**
- **In-line fusion Affimer proteins become more stable as the number of Affimer units in the oligomer increases: T_m (PDL1_141 Tetramer His) > T_m (PDL1_141 Trimer His) > T_m PDL1_141 (Dimer His) > T_m (PDL1_141 Monomer His)**
- **Affimer proteins formatted as in-line fusion display an apparent K_D up to 25-fold lower than the monomer Affimer protein**
- **In-line fusion proteins' ability to compete with hPD-1 Fc increases as the number of Affimer units in the oligomer increases: IC_{50} (PDL1_141 Monomer His) > IC_{50} (PDL1_141 Dimer His) > IC_{50} (PDL1_141 Trimer His) > IC_{50} (PDL1_141 Tetramer His)**

Conclusions

Fc-fusion:

- **Affimer proteins can be successfully formatted and expressed to high yields as hFc1-fusion proteins in mammalian Expi293F cells**
- **The Affimer-hFc1 showed a 10-fold increase in apparent K_D and specifically bound to PD-L1 expressed on a mouse cell line surface. Fusion to hFc1 resulted in half-life extension and the Affimer-hFc1 protein was well tolerated in mouse**

In-line fusion:

- **Affimer proteins can be successfully formatted and expressed to high yields as in-line fusion proteins (up to a tetramer) in *E.coli***
- **In-line fusion Affimer proteins display improved affinity and stability compared to the parent Affimer protein**

This work demonstrates that the Affimer technology has the necessary properties for developing a therapeutic platform

Acknowledgements: Anna Tang and Darren Tomlinson, Astbury Centre for Structural and Molecular Biology, University of Leeds, UK