Generation and Formatting of an Affimer® Biotherapeutic for the Inhibition of the PD-L1/PD-1 Pathway: Proof of Concept in Mouse.

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Introduction

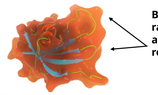
Monoclonal antibodies e.g. Ipilimumab, Atezolizumab, have successfully shown that blocking cellular interactions that negatively regulate T-cell immune responses can amplify pre-existing immunity to cancer. Programmed death-ligand 1 (PD-L1) is a clinically validated oncology target shown to play an important role in down-regulating the immune system allowing tumour cells to evade detection and metastasize.

Affimer proteins were selected against human and mouse PD-L1 (AVA04 programme) using phage display. Affimer AVA04-251 has been selected as a lead human PD-L1 antagonist. AVA04-182, a lead mouse PD-L1 antagonist, has been identified as a surrogate for *in vivo* mouse studies. Both Affimer proteins were fused to the Fc moiety of a human IgG1 (AVA04-251 hFc1 & AVA04-182 hFc1) for half life extension and ADCC effector function.

Affimer Technology

Affimer Technology

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



Binding loops: Two randomised 9 amino acid loop regions

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

Benefits of Affimer Therapeutics

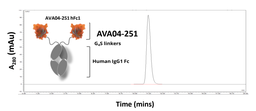
- **Small size:** 14 kDa, $1/10^{th}$ the size of an antibody
- **High expression**: >100 mg/L in flasks (*E. coli*)
- **No post translational modifications**: ease of manufacturing and improved stability
- **Ease of formatting:** Fc format and in-line fusions, potential to generate multi-specific drugs to blockade multiple disease pathways
- **Tissue penetration:** small size gives greater potential of tissue penetration for increased efficacy

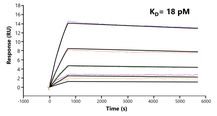
Affimer Discovery Process: Phage Selections

Synthetic Library	• Affimer library with diversity of 10^{11} • 9 amino acids in two loops with equal diversity at each position (no Cys, Met or Stop codons) • Loop regions made from trinucleotide blocks
Phage Display	• Solution selections performed using biotinylated hPD-L1 • Enrichment after each round monitored and confirmed by polyclonal ELISA
Primary Screening	• 2 rounds delivered 20 % hit rate to hPD-L1 and 38 unique sequences • 3 rounds showed 70 % hit rate and increased panel to 64 unique clones
Protein Production	• Binding clones were subcloned into expression vector and purified using Ni-NTA resin
Characterisation	• Affimer proteins were characterized by SEC-HPLC, Biacore, binding & competitive ELISA and cell based blockade assay

Affimer Therapeutic Targeting hPD-L1

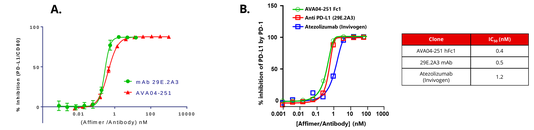
AVA04-251 hFc1 Production and Formatting





AVA04-251 formatted as a N-terminal Fc fusion. Expression in Expi293F cells, purification by Pr-A affinity chromatography and size exclusion chromatography.

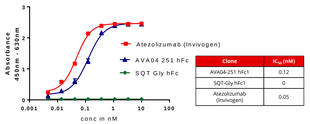
AVA04-251 Competitive ELISA



Clone	IC_{50} (nM)
AVA04-251 hFc1	0.4
29E.2A3 mAb	0.5
Atezolizumab (Invivogen)	1.2

(A.) Anti-hPD-L1 Affimer AVA04-251 disrupts hPD-L1/CD80 interaction with similar affinity to anti-hPD-L1 antibody clone 29E2A3. (B.) Anti-hPD-L1 Affimer AVA04-251 hFc1 disrupts hPD-L1/PD-1 interaction with similar affinity to anti-hPD-L1 antibody Atezolizumab (Invivogen) and 29E2A3.

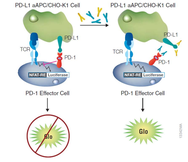
AVA04-251 hFc1 Cynomolgus Cross Reactivity ELISA



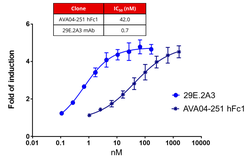
Clone	IC_{50} (nM)
AVA04-251 hFc1	0.12
SQT-Gly hFc1	0
Atezolizumab (Invivogen)	0.05

AVA04-251 hFc1 cross reactivity with cynomolgus PD-L1. Cynomolgus PD-L1 was coated onto the plate. Affimer, Atezolizumab (Invivogen) and negative control were detected by a secondary antibody anti human Fc.

AVA04-251 hFc1 Promega Cell based Assay

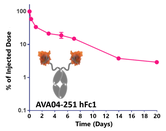


Potency of the AVA04-251 hFc1 Affimer protein was determined in a Promega hPD-1/hPD-L1 blockade cell based assay which measured T-cell signalling through NFAT-mediated luciferase activity.



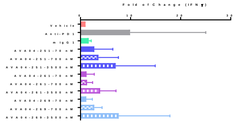
Clone	IC_{50} (nM)
AVA04-251 hFc1	42.0
29E.2A3 mAb	0.7

PK Study of AVA04-251 hFc1 in Mouse



- AVA04-251 formatted on human IgG1 for ADCC activity
- AVA04-251 does not bind mouse PD-L1
- AVA04-251 hFc1 injected at 10 mg/kg IV
- Serum half life of Affimer Fc fusion: ~120 hrs (estimate)

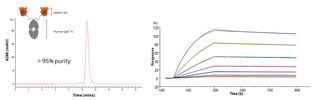
MLR culture: IFN-γ production



A range of anti-PD-L1 Affimer proteins demonstrated potent activity in an MLR assay : CD14+ cells isolated from healthy donors were differentiated into immature monocyte-derived dendritic cells (Mo-DCs) for 7 days. T-cells isolated from different donors were co-cultured with Mo-DCs in a 10:1 ratio for 4 days. Lymphocyte activation was measured based on IFN-γ secretion.

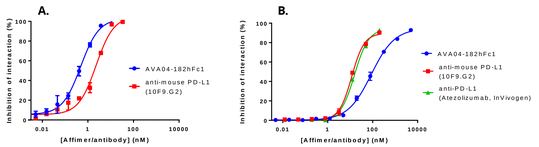
Affimer Protein Targeting mPD-L1

AVA04-182 hFc1 Production, Formatting and Characterisation



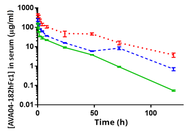
Affimer	k_a (1/Ms)	k_d (1/s)	App K_D
AVA04-182hFc1	$3.39x10^{5}$	$1.23x10^{-4}$	36.1pM

AVA04-182 hFc1 SEC-HPLC and Biacore® SPR Kinetics
Affimers can be formatted as Fc fusions to add effector functions, improve half-life and enhance affinity. AVA04-182 was formatted as a hIgG1 Fc fusion (AVA04-182 hFc1). Purified yields from transient expression in Expi293F cells reach >250 mg/L with purity >95%.



Effect of AVA04-182 hFc1 on mPD-L1/PD-1 interaction and mPD-L1/Atezolizumab interaction by ELISA
(A). Anti-mPD-L1 Affimer AVA04-182 hFc1 disrupts mPD-L1/PD-1 interaction with similar affinity to anti-mPD-L1 antibody clone 10F9.G2. (B). The competitive ELISA showed AVA04-182 hFc1 to recognise the same epitope region as the anti-mPD-L1 antibody (clone 10F9.G2) and Atezolizumab (InVivogen).

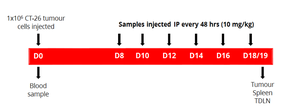
PK study of AVA04-182 hFc1 in Mouse



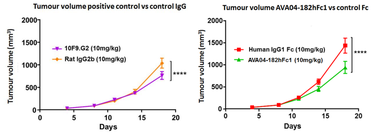
Measurement of serum concentration over time by ELISA
AVA04-182 hFc1 was dosed as a bolus IV injection, 3 mice per time point. PK was followed for 7 days and serum levels of AVA04-182 hFc1 determined by sandwich ELISA. AVA04-182 hFc1 was well tolerated *in vivo* at all doses.

CT-26 Efficacy Study

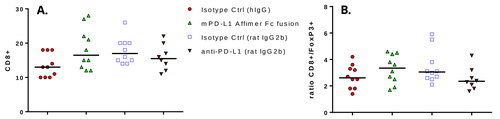
Study dosing regime



Measurement of tumour volume



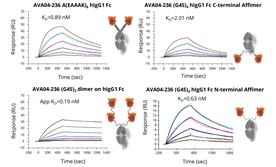
Tumour T cell infiltrate



- Increase of CD8+ T-cell recruitment
- Improvement of $CD8^+/FoxP3^+$ ratio

BALB/c mice were engrafted with CT-26 tumour cells subcutaneously. Treatment started at D8 with either AVA04-182 hFc1, rat Mab anti-mPD-L1 (10F9.G2), or respective isotype control at 10 mg/kg every other day. AVA04-182 hFc1 and clone 10F9.G2 showed a significant decrease in tumour growth. The effect was associated with an increase of $CD8^+$ T-cells (A) in the tumour micro-environment and an improvement of the $CD8^+$/Treg ratio (B).

Affimer Fc Formatting



- Affimers can be formatted as a fusion to both the N & C terminal of the human IgG1 Fc.
- Affimers were expressed in Expi293 cells (Thermo) and purified using Pr-A affinity chromatography.
- Affimers were formatted using flexible (G_4S) or rigid A(EAAAK) linkers either at the N or C terminus.
- The position of the Affimer fusion on the Fc did not affect the binding to hPD-L1 by Biacore®.

Conclusions

- **The PD-L1 Affimer antagonists can be formatted in a variety of ways both as N and C terminal fusions with a human Fc domain to generate high affinity molecules as determined by Biacore®**
- **The anti-hPD-L1 Affimer protein has been shown to disrupt the interaction between hPD-1/PD-L1 and block PD-1 signalling in the Promega hPD-1/PD-L1 cell-based assay. Furthermore, the anti-hPD-L1 Affimer protein increases IFN-γ production in an MLR assay**
- **The anti-mPD-L1 Affimer protein was shown to be well tolerated in mice, even with repeat dosing at 10 mg/kg in the syngeneic model**
- **The anti-mPD-L1 Affimer protein inhibited tumour growth in the CT-26 syngeneic model and improved the tumour T-cell infiltration**
- **This work demonstrates that the Affimer technology has the necessary properties for a therapeutic platform: generation of high affinity binding proteins that can be formatted to extend the serum half-life and blockade a biologically relevant disease pathway *in vivo***

Acknowledgements
This work was part supported by an Innovate UK grant and the CT-26 syngeneic model was run by Oncodesign.