Poster 1349

Application of the DARPin® technology for specific targeting of tumor-associated MHC class I:peptide complexes

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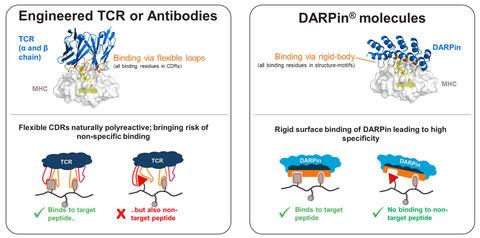
Introduction

Almost all intracellular proteins are processed and presented as peptides on the cell surface by major histocompatibility complexes (MHC) class I molecules, also known as human leukocyte antigens (HLA) in humans. These peptides represent a significant subset of tumor antigens which can be recognized by cytotoxic T-lymphocytes which play a primary role in immune surveillance. Development of biologics targeting MHC class I:peptide (pMHC) complexes is hindered by low target abundance, weak affinity, cross-reactivity or challenging biochemical properties. DARPin® proteins offer the potential to overcome these limitations through their advantageous structural binding characteristics for the small and flat target surface of the pMHC complex in combination with excellent biophysical properties.

Here we present key data on the biophysical properties and in vitro characterization of DARPin® binders specific to the HLA-A2 molecule in association with SLLMWITQC (SLL-peptide), a peptide derived from NY-ESO-1 in the T cell engager format enabling potent re-direction and activation of T cells. Using a number of cellular assays we confirmed high potency and specificity of selected DARPin® proteins to the HLA-A2:SLL complex. Architectural fine tuning and sequence engineering allowed us to further increase potency without compromising specificity. In order to achieve good systemic exposure for optimal anti-tumor response we have established a platform providing long serum half-life with limited impact on potency and specificity. Finally, alanine scan and X-scan mutagenesis analysis demonstrated that interactions with several peptide residues located across the length of the exposed peptide are critical to mediate T cell activation and that binding of similar peptides would not be tolerated, thus providing specificity to the target engagement.

Characteristics for pMHC binding



- The DARPin® rigid binding surface (compared to TCRs and antibodies) mediates highly specific interactions with various protein targets (See ***Fig. 1***)
- The high functional diversity of our DARPin® libraries (~10^{12}) in combination with well-established selection and screening technologies, allowed rapid identification of well-behaved, highly specific pMHC DARPin® binders. Candidates were then re-formatted into DARPin® T cell engagers containing a CD3-binding DARPin® domain.

Figure 1: Schematic illustration of engineered TCR (left side) or DARPin®(right side) binding the pMHC complex.

High throughput screening of DARPin® proteins

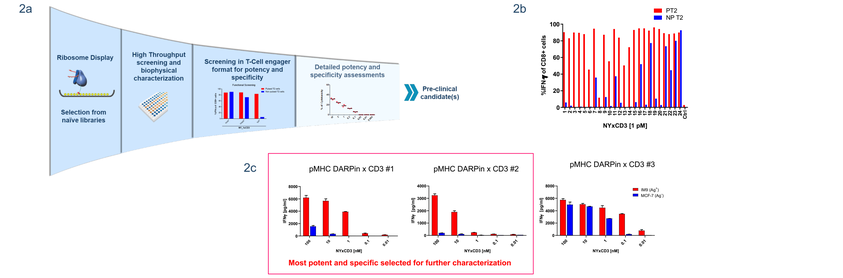


Figure 2. Binding and functional screening of DARPin® proteins (**a**) *Schematic illustration of DARPin® proteins generation and selection process* (**b**) *Functional screening of DARPin® proteins cloned in the T-cell engager (TCE) format. Intracellular IFN-γ levels obtained on CD8+ T-cells are shown in the presence of peptide pulsed T2 (PT2) vs. non pulsed T2 (NP T2) cells* (**c**) *HLA-A2+/ NY-ESO-1+ or HLA-A2+/ NY-ESO-1- tumor cell lines (IM9 and MCF-7 respectively) were incubated with PBMCs in the presence of selected DARPin® binders in the TCE format. Depicted are IFNγ levels obtained in the supernatant. Ag: antigen/NY-ESO-1.*

Results: The screening allowed us to select the most potent and specific DARPin® candidates. Further characterization of two promising candidates, NY_1xCD3 and NY_2xCD3, is shown in figures 3, 4 and 7.

Highly stable and high affinity DARPin® proteins

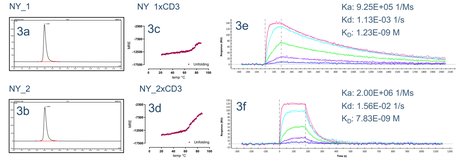
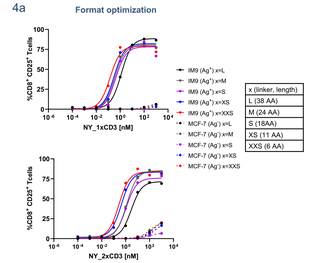


Figure 3: Biophysical characterization of NY_1 (upper panel) and NY_2 (lower panel) (**a** and **b**) HPLC-SEC traces for both candidates demonstrate monodispersity without signs of aggregation or oligomerization, (**c** and **d**) Protein unfolding in Circular Dichroism proves very high temperature *stability up to 70 ° C (NY_1xCD3) and 60 ° C (NY_2xCD3)* (**e** *and* **f**) *Surface plasmon resonance (SPR) multi-concentration measurements of the bispecific DARPin®* candidates reveals different binding kinetics despite comparable K_D values.

Architectural & sequence tuning of DARPin® proteins



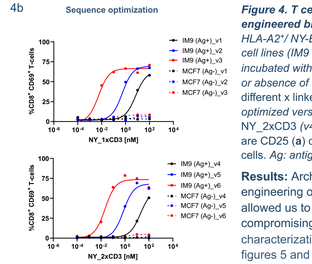


Figure 4. T cell mediated activation with engineered bispecific DARPin® proteins (**a** *and* **b**) *HLA-A2+/ NY-ESO-1+ or HLA-A2+/ NY-ESO-1- tumor cell lines (IM9 and MCF-7 respectively) were incubated with PBMCs for 48 hours in the presence or absence of* (**a**) NY_1xCD3 or NY_2xCD3 with different x linker lengths (L to XXS) *or* (**b**) *sequence optimized versions (v) of* NY_1xCD3 (v1 to v3) or NY_2xCD3 *(v4 to v6)* DARPin® proteins. Depicted are CD25 (**a**) or CD69 levels (**b**) obtained on CD8+ T cells. *Ag: antigen/NY-ESO-1.*

Results: Architectural fine tuning and sequence engineering of bispecific DARPin® candidates allowed us to further increase potency without compromising specificity. Further characterization of NY_1xCD3_v3 is shown in figures 5 and 6.

DARPin® proteins mediate specific T-Cell Activation leading to tumor cell killing

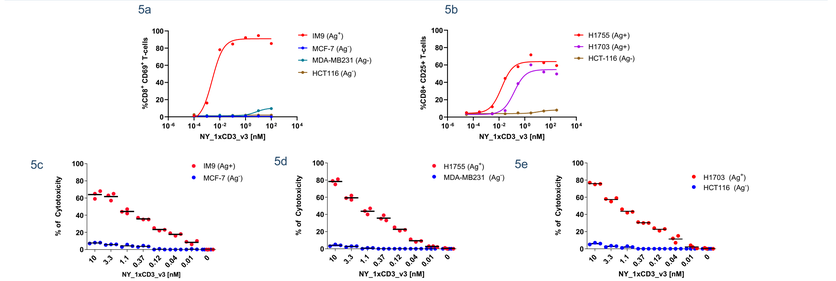


Figure 5: DARPin® mediated potent and specific T-cell activation and cytotoxicity. (**a**-**e**) *HLA-A2+/ NY-ESO-1+ or HLA-A2+/ NY-ESO-1- tumor cell lines (IM9, NCI-H1755, NCI-H1703 and MCF-7, MDA-MB231 and HCT-116, respectively) were incubated with PBMCs (***a*** and* **b**) *or effector CD8+ T-cells (**c**-**e**) in the presence or absence of NY_1xCD3_v3. Depicted are CD25 levels obtained on CD8+ T-cells (**a** and **b**) or the percentage of specific lysis obtained for the different tumor cell lines by the chromium release assay (**c**-**e**). Ag: antigen/NY-ESO-1.*

Results: The NY_1xCD3_v3 DARPin® candidate efficiently mediates potent and highly specific T-cell activation and T-cell cytotoxicity in the presence of various tumor cell lines presenting the SLL:HLA-A2 complex.

Prolonged systemic exposure of DARPin® proteins

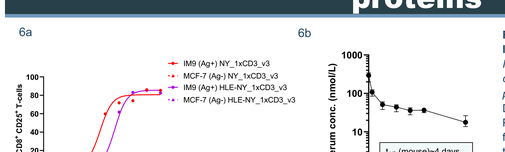
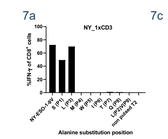


Figure 6. Potency, specificity and systemic exposure of half-life extended (HLE) bispecific *DARPin® proteins* (**a**) *HLA-A2+/ NY-ESO-1+ tumor cells lines (IM9) or HLA-A2+/ NY-ESO-1- tumor cell lines (MCF-7) were incubated with PBMCs for 24 hours in the presence or absence of* NY_1xCD3_v3 or HLE-NY_1xCD3_v3. Depicted are CD25 levels obtained on CD8+T cells. (**b**) Pharmacokinetic of the HLE-NY_1xCD3_v3 was determined in female BALB/c mice (two groups, n=3 per time point, n=6 at last time point, mean +/- max/min) following single i.v. administration of DARPin in PBS.

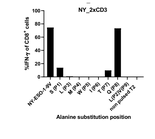
Results: The HLE-pMHC x CD3 DARPin® molecules provide good systemic exposure with limited impact on potency and specificity.

Specificity Assessment (Alanine/X-Scan)



7c NY_1xCD3

Position	WT	A	C	D	E	F	G	H	I	K	L	M	N	P	Q	R	S	T	V	W	Y
P1	S	89.7	94.3	0.5	27.4	72.5	103.1	43.0	69.0	13.3	30.1	79.3	77.9	9.3	69.2	76.3	100.0	107.5	113.8	114.4	111.7
P2	L	53.0	81.8	2.5	0.3	12.1	0.2	0.3	101.3	6.5	100.0	105.4	0.1	0.4	59.9	0.4	0.9	15.6	90.6	0.3	0.3
P3	L	96.1	52.9	96.7	1.5	70.4	39.0	70.0	98.7	2.8	100.0	87.0	93.9	44.2	95.9	0.1	37.7	86.8	75.4	102.6	4.6
P4	M	0.3	1.3	0.1	19.0	34.3	0.4	0.0	95.7	3.0	82.9	100.0	1.1	0.3	13.8	11.2	0.1	98.3	29.3	24.5	2.2
P5	W	0.1	0.2	0.1	0.2	16.4	0.1	0.1	0.3	0.3	0.4	1.0	0.4	0.1	0.1	0.0	0.1	0.2	0.0	100.0	0.2
P6	I	0.1	1.3	0.0	0.0	84.1	0.1	0.3	100.0	0.6	45.4	54.7	0.0	1.0	0.1	0.0	0.4	17.4	113.8	0.2	6.7
P7	T	5.5	32.1	0.1	24.8	114.6	0.3	13.0	95.3	1.0	93.3	102.4	14.1	1.6	78.1	82.3	75.2	100.0	74.8	7.3	52.7
P8	Q	23.1	0.2	0.0	17.8	0.1	20.2	0.7	0.2	0.6	3.4	0.9	0.1	0.1	100.0	0.1	12.6	57.1	3.7	0.3	0.2
P9	V	89.4	39.7	0.4	0.7	0.3	11.2	0.7	94.6	1.0	21.0	1.0	1.0	1.1	0.4	0.7	5.3	42.3	100.0	0.8	0.4



7d NY_2xCD3

Position	WT	A	C	D	E	F	G	H	I	K	L	M	N	P	Q	R	S	T	V	W	Y
P1	S	78.1	73.6	27.7	29.5	68.3	2.0	57.2	69.6	0.6	13.1	81.1	109.9	64.9	97.9	35.2	100.0	110.7	108.4	106.8	93.2
P2	L	0.4	0.8	0.2	0.3	39.4	0.2	0.5	105.1	0.1	100.0	52.5	0.1	2.2	0.3	0.6	0.3	1.8	79.9	57.2	0.2
P3	L	0.3	0.1	0.4	0.2	0.6	0.3	0.1	110.2	0.0	100.0	113.4	0.1	2.5	48.6	0.5	0.6	0.2	71.3	20.2	0.1
P4	M	0.4	0.5	0.2	0.3	2.0	0.1	0.4	65.5	0.3	16.8	100.0	0.3	1.1	1.0	0.5	0.5	0.6	0.5	0.5	0.4
P5	W	0.3	0.1	0.4	0.3	13.7	0.6	0.4	1.3	1.5	1.0	2.1	0.7	0.3	0.3	0.3	0.3	0.3	0.5	100.0	0.7
P6	I	2.1	4.8	0.1	0.2	74.6	1.6	0.8	100.0	1.3	102.3	75.0	1.2	107.4	0.1	0.6	4.8	0.2	98.5	36.6	0.0
P7	T	57.7	93.2	0.3	11.4	106.5	107.8	0.4	96.0	0.3	108.5	124.0	76.2	15.1	108.5	102.2	92.0	100.0	100.8	19.6	9.4
P8	Q	122.6	95.6	120.6	122.2	43.9	126.9	124.2	13.4	123.8	98.1	107.1	100.8	113.3	100.0	51.9	113.0	120.7	104.6	108.9	99.6
P9	V	104.1	86.1	16.8	0.4	76.4	56.3	1.9	104.0	1.8	95.1	24.5	27.4	4.6	10.3	0.4	56.8	56.2	100.0	0.9	0.1

Figure 7. Analysis of DARPin® interactions with the SLL:HLA-A2 complex. (**a**-**d**) *Results of Alanine (**a** and **b**) and X-scan (**c** and **d**). Each amino acid of the target peptide was sequentially replaced by alanine (**a** and **b**) or by every other amino acid (**c** and **d**). T2 cells were pulsed with each peptide and incubated with effector CD8+ T-cells and each DARPin® TCE at EC90 concentrations. Percentage of IFN-γ positive CD8+ T-cells are shown. For the X-scan analysis (**c** and **d**) values of independent experiments were averaged and normalized to 100% for the index residue in each position (dark blue fields). Values above 30%, indicating notable T-cell activation, are marked in bold and colored light blue.*

Results: The two selected DARPin® lead candidates specifically interact with multiple residues of the target peptide. In both instances, few mutations were tolerated at central positions of the peptide i.e. the most exposed surface of the peptide. An *in silico* analysis based on the generated binding profiles allowed us to identify a small number of potential cross-reactive peptide sequences, further highlighting good specificity.

Discussion / Conclusions

Our results demonstrate rapid and reliable generation of DARPin® binders against pMHC and which were then formatted into bispecific T-cell engagers with a CD3-binding DARPin® domain, and engineer to enable potent and specific re-direction and activation of T-cells. We were able to achieve systemic half-life extension with limited impact on potency.

High levels of specificity were obtained with the selected bispecific DARPin® binders as demonstrated by the lack of activity and cytotoxicity towards antigen negative cells and the low number of potentially cross-reactive peptides identified by X-scan. We believe that the DARPin® technology represents a versatile alternative to specifically target pMHC complexes expressed in a broad range of cancers and/or virology indications. The outstanding biophysical and developability properties, capacity for multi-specific formatting, and level of specificity exhibited by pMHC-specific DARPin® proteins prompts investigations on their broader application for alternative treatment modalities (drug conjugates, radio therapy, CAR-T-cells) and pMHC-specific diagnostics.

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