Engineering and characterization of anti-PSMA humabody-deBouganin fusion proteins

Abstract # 5770

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ABSTRACT

Targeted protein therapeutics or TPTs are single-protein therapeutics composed of targeting moieties genetically fused via peptide linker to cytotoxic protein payloads. TPTs are designed to overcome efficacy and safety challenges inherent within ADCs. For tumor indications only reachable by systemic delivery, we have designed a de-immunized cytotoxic protein payload, deBouganin. DeBouganin is a T-cell epitope-depleted variant of bouganin, a type I Ribosome Inactivating Protein (RIP) that blocks protein synthesis by the deadenylation of ribosome RNA resulting in programmed cell death. DeBouganin's biophysical and biochemical properties, in combination with its unique mechanism of action, make it an attractive cytotoxic payload for targeted drug delivery. Using different protein scaffolds and in vivo matured human V_H domains, we have shown that deBouganin exhibits certain advantages over more conventional small molecule cytotoxins, with respect to cell killing power, including the ability to kill cancer stem cells, circumvent multi-drug resistance, and avoidance of cross-resistance mechanisms.

Crescendo has created a proprietary transgenic mouse devoid of any antibody light chains from which it generates highly diverse fully human V_H domain ('Humabody®') building blocks. *In vivo* maturation optimizes Humabody® potency and develops superior biophysical properties. Their small size and high stability permits Humabody® assembly into multifunctional formats optimally configured for therapeutic effcacy. Such molecules are capable of target engagement that is unachievable using regular mAbs and may include a half-life extending serum albumin-binding binding domain or a cytotoxic payload. Using monovalent and biparatopic PSMA targeting Humabody® formats, we describe the molecular engineering and biological testing of novel anti-PSMA Humabody-deBouganin fusion constructs with or without a V_H half-life extender. The data show that Humabody-deBouganin fusion proteins are expressible as a soluble protein in *E. coli* supernatant. Moreover, biological testing demonstrated the preference of a non-cleavable linker composed of a single G_4S motif over a furin linker so as to ensure serum stability. IC_{50} value of all fusion proteins was approximately 0.2 nM against PSMA-positive LnCAP tumor cells. In vitro data support the potential of Humabody-deBouganin fusion constructs as anti-cancer therapeutics.

BACKGROUND

DeBouganin is a ribosome-inactivating plant protein toxin that has been de-immunized by depletion of T-cell epitopes (1). DeBouganin does not contain a cell membrane binding domain and therefore is an ideal payload for internalizing tumor targeting proteins. DeBouganin chemically conjugated to Trastuzumab was overall more potent than T-DM1 against 3+ breast tumor cell lines but more importantly was also able to overcome the resistance induced by antibody drug conjugates with different small molecule payloads (2). DeBouganin is readily amenable to genetic fusion with a variety of antibody fragment formats such as scFv, Fab or scaffold proteins.

Humabodies® are fully human, single V_H domain building blocks generated using Crescendo's proprietary transgenic mouse. *In vivo* maturation in the absence of light chains optimizes Humabody® potency and develops superior biophysical properties. Small size (13kDa) and high stability permits Humabody assembly into an almost limitless array of multifunctional formats optimally configured for therapeutic efficacy. This fully modular plug & play approach coupled with Humabodies® superior biodistribution and absence of Fc-receptor driven toxicity offers many advantages over traditional mAbs for targeted payload delivery.

Historically, deBouganin potency was optimal with a cleavable peptidic linker containing the furin proteolytic site. Here we show that the molecular engineering and biological testing of two anti-PSMA monovalent V_HA and V_HB humabodies and a biparatopic genetically linked to deBouganin with or without the V_HMSA half-life extender. The monovalent and biparatopic deBouganin fusion proteins can be expressed as a soluble protein in *E. coli* supernatant. Potency testing demonstrated that a non-cleavable linker composed of a single G_4S motif was necessary to ensure serum stability of the fusion protein.

METHOD

Molecular engineering

The *E. coli* codon-optimized PelB-V_HA-G_4S-His, OmpA-V_HB-G_4S-His, PelB-V_HHel4-G_4S, $(G_4S)_6$-V_HMSA-G_4S-His, G_4S-F-deBouganin-His and G_4S-deBouganin-His inserts were used to create various Humabody-deBouganin fusion proteins and cloned under the control of an L-arabinose promoter using the appropriate restriction enzymes. For ease of purification, a Histidine tag sequence was placed at the C-terminal end.

Research-scale expression

Transformed E104 cells containing the anti-PSMA-V_H-deBouganin fusion constructs were induced with L-Arabinose (0.1% final), and incubated at 25° C with constant shaking at 225 rpm. At 16 hours post-induction, the level of expression of anti-PSMA-V_H-deBouganin fusion proteins was estimated by Western blot analysis using a mouse anti-histidine antibody followed by a goat anti-mouse-HRP antibody.

Purification

The supernatant obtained from 4 L of induced culture was concentrated and diafiltrated against 20 mM sodium phosphate pH 7.5 and applied onto a charged nickel chelating column. After washing the column with 20 mM imidazole, bound material was eluted with 20 mM sodium phosphate, 150 mM NaCl, 250 mM imidazole, pH 7.5. After dilution and pH adjustment, the diluted nickel eluate was then applied onto a Q-sepharose column and the flow-through and wash of the Q-sepharose column containing the product were collected and applied onto a SP-sepharose column and bound product was then eluted with 20 mM sodium phosphate, 300 mM NaCl pH 7.5 buffer. After analysis by Coomassie, the fractions containing the V_H-deBouganin fusion protein were pooled, OD_{280} determined and protein concentration calculated with the theoretical extinction coefficient obtained with ExpaSy. Purity was determined by SE-HPLC. To increase purity, the pooled biparatopic fusion protein SP fractions were loaded onto a 100 mL Sephacryl-S200 size exclusion column.

Binding

The binding reactivity of purified V_H domains with or without deBouganin against PSMA-positive LNCaP cells was measured by flow cytometry. The selectivity of the binding was assessed with PSMA-negative cell line, PC-3. Briefly, V_H domains or V_H-deBouganin fusion proteins were incubated at 1 µg/mL molar equivalent with 0.2×10^6 cells on ice for 2 hours and bound material was detected with a mouse anti-Histidine antibody followed by a goat anti-mouse-FITC. Purified V_HA and V_HB proteins were used as positive controls.

Potency

The cytotoxicity of anti-PSMA V_H-deBouganin fusion proteins was assessed with an MTS assay using PSMA-positive LNCaP tumor cells seeded at 5000 cells per well. After 5 days, the IC_{50} was determined and the specificity of the cytotoxicity demonstrated against PSMA-negative cells PC-3.

CH0-PSMA and DU145-PSMA, seeded at a density of 400 and 3000 cells respectively, were treated for 72-hours with deBouganin conjugated Humabodies®. Cell viability was determined using the Cell Titer-Glo Cell Viability assay (Promega) according to the manufacturer's instructions. Percentage cell survival was calculated relative to non-treated cells.

Serum stability

V_H-deBouganin fusion proteins were incubated with 100 µL of mouse or human serum at a concentration of 80 µg/mL and placed at 37ºC. Samples taken at 0, 24, 48, 72, 96 and 120 hours were analyzed by Western blotting under non-reducing conditions using a rabbit anti-deBouganin antibody followed by an anti-rabbit-HRP. V_H-deBouganin fusion proteins and potential truncated bands were revealed using the colorimetric DAB procedure (3-3' Diaminobenzidine substrate).

RESULTS

Monovalent V_H-G_4S-F-deBouganin

- Soluble expression of all Humabody-deBouganin fusion proteins
- Higher expression level of the deBouganin-V_H format (Table 1)
- Similar binding activity of the V_H-deBouganin fusion proteins vs. V_H controls against PSMA-positive LNCaP tumor cells
- Higher potency of the V_H-deBouganin orientation
- Degradation (~20%) detected after incubation in mouse serum (Fig. 1)

Table 1: Yield, potency and binding of deBouganin-V_H fusion proteins

	Yield (mg/L)	IC_{50} (nM)	MFI
V_HA -G_4S-F- deB	0.93	0.26 ± 0.06	11.3 ± 0.3
deB -F-$(G_4S)_2$- V_HA	3.6	1.25 ± 0.25	3.6 ± 0.2
V_HB -G_4S-F- deB	2.2	0.28 ± 0.02	14.7 ± 0.6
Hel -G_4S-F- deB	0.93	> 100	1
V_HA	N.A.	N.A.	13.4 ± 0.4
V_HB	N.A.	N.A.	14.9 ± 0.1

V_HA, V_HB, V_HHel4 and deBouganin are represented in blue, red, purple and green box, respectively. Yield from 4 L shake-flasks. S.E. of 2 independent experiments.

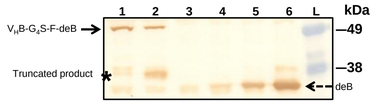

Figure 1: Mouse serum stability. V_HB-G_4S-F-deB was sampled at Time 0 (lane 1) and incubated 24 hours in mouse serum (lane 2) and analyzed by Western blot. Lanes 3 to 6 were loaded with 0.2, 2, 20 and 200ng of deBouganin (deB), respectively, to estimate the degree of truncation. Arrow and star indicate full-length and truncated band, respectively.

G_4S vs. G_4S-Furin linker

- Similar potency and killing efficiency of the fusion proteins with a G_4S linker (L) with or without the furin (F) proteolytic site (Table 2)
- No change of potency with the half-life extender (Table 2 and Fig. 2)
- Similar potency of the biparatopic vs.monovalent fusion proteins for high PSMA copy number tumor cells
- Lower killing efficiency of the biparatopic fusion proteins vs. DU145

Table 2: Potency of various V_H-deBouganin fusion proteins containing a linker (L) comprised of either G_4S or G_4S-Furin (F) against LNCaP tumor

	G_4S, IC_{50} (nM)	G_4S-F, IC_{50} (nM)
V_HB -L- deB	0.21 ± 0.01	0.28 ± 0.02
V_HB 245 -L- deB	0.21 ± 0.01	0.28
V_HA V_HB -L- deB	0.2 ± 0.02	0.18
V_HA V_HB V_HM -L- deB	0.21 ± 0.01	0.18
Hel -L- deB	> 100	> 100
Hel V_HM -L- deB	> 100	> 100

Half-life extender V_HMSA represented with a orange box. S.E. of 2 independent experiments.

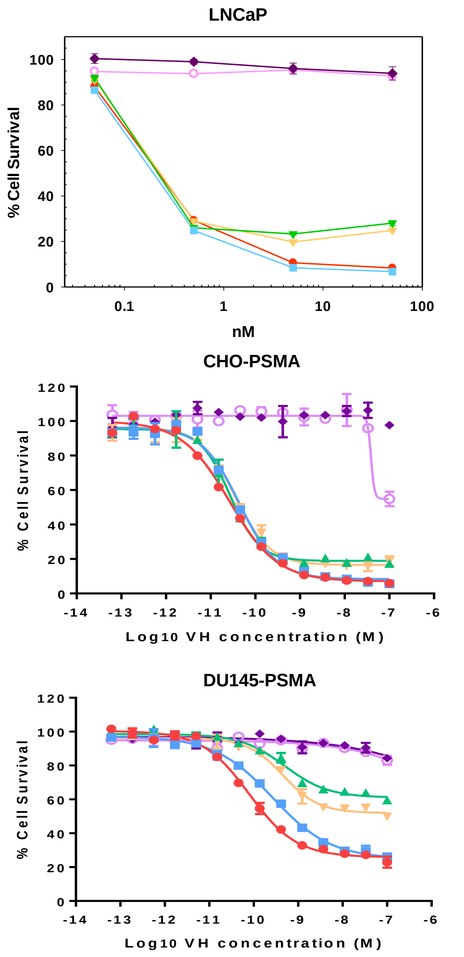

Figure 2: MTS curves of V_HB-G_4S-deBouganin ( ), V_HB-$(G_4S)_6$-V_HMSA-G_4S-deBouganin ( ), V_HA-$(G_4S)_6$-V_HB-G_4S-deBouganin ( ), V_HA-$(G_4S)_6$-V_HB-$(G_4S)_6$-V_HMSA-G_4S-deBouganin ( ), V_HHel4-G_4S-deBouganin ( ) and V_HHel4-$(G_4S)_6$-V_HMSA-G_4S-deBouganin ( ). Representative example of two independent experiments.

- All V_H-G_4S-deBouganin fusion proteins were stable in mouse and human sera for up to 72 hours (Fig. 3)

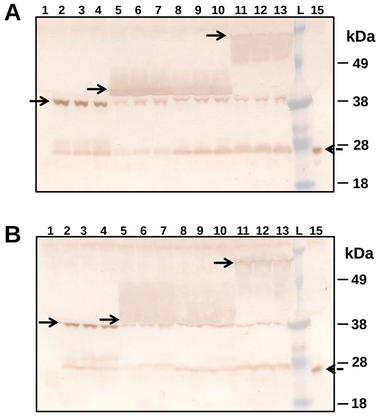

Figure 3: Mouse A) and human B) serum stability. V_HB-G_4S-deBouganin (lanes 2-4), V_HB-$(G_4S)_6$-V_HMSA-G_4S-deBouganin (lanes 5-7), V_HA-$(G_4S)_6$-V_HB-G_4S-deBouganin (lanes 8-10) and V_HA-$(G_4S)_6$-V_HB-$(G_4S)_6$-V_HMSA-G_4S-deBouganin (lanes 11-13) taken after 0, 24 and 72 hours incubation in serum were analyzed by Western blot. Lane 1 was loaded with serum only and L with ladder. Lane 15 was loaded with 200 ng of deBouganin indicated by a dashed arrow. Full-length proteins are indicated by arrows.

CONCLUSIONS

- **DeBouganin and anti-PSMA Humabody® V_H fusion proteins were well expressed in a variety of different formats including monovalent as well as biparatopic molecules**
- **Anti-PSMA-deBouganin molecules exhibited subnanomolar potency**
- **A non-cleavable peptide linker between the Humabody® and deBouganin cytotoxin payload was required over a furin cleavable linker to preserve serum stability**

References

1. Cizeau, J. *et al*. J Immunother 2009, 32(6), 574-584.
2. Chooniedass, S. *et al.* Molecules. 2016, 21(12).

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Poster 5570, American Association for Cancer Research, Session: Antibodies, Fusion Proteins and Related Biologics, Wednesday Apr 18, 2018, 8:00 AM - 12:00 PM.