Abstract # 614

VB4-845 tumor cell killing in a combination with the anti-PD1, Nivolumab

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ABSTRACT

VB4-845 is a Targeted Protein Therapeutic, TPT, that comprises a scFv fragment specific for the Epithelial Cell Adhesion Molecule (EpCAM) genetically fused to a truncated form of *Pseudomonas* exotoxin A, ETA, via a flexible linker. Due to the inherent immunogenicity of the ETA moiety, VB4-845 is only used for the treatment of loco-regionally accessible tumors and is currently in a Phase III clinical trial for the treatment of high grade non-muscle invasive bladder cancer. In a Phase I study in late stage squamous cell carcinoma of the head and neck, direct injection of VB4-845 led to tumor shrinkage of the principal injected tumor as well as non-targeted tumors in some patients, suggesting the activation of a T cell-mediated anti-tumor response through cross-priming. Only cells undergoing immunogenic cell death, or ICD, are capable of stimulating cross-priming. ICD is characterized by distinct "eat-me" signals such as the release of ATP and HMGB1 and cell surface translocation of calreticulin, an endoplasmic reticulum chaperone protein. To evaluate whether VB4-845 cell killing results in ICD events, treated tumor cell lines were assessed for these distinct signaling molecules. In vitro studies using flow cytometry and ELISA showed translocation of calreticulin and release of ATP and HMGB1 by VB4-845-treated tumor cells. Immune T cell activation via an ICD pathway suggests a complimentary outcome when combined with checkpoint inhibitors. A study was performed in PDX tumor-bearing NOG mice reconstituted with a human immune system to assess the combination of intratumoral injection of VB4-845 with an anti-PD1 antibody, Nivolumab, given i.p. VB4-845 showed growth suppression of the injected tumor, whereas the growth delay of the contralateral, non-injected tumor was more pronounced with the addition of Nivolumab. In summary, the data presented suggests that VB4-845 mediates tumor cell killing by an ICD pathway and that the resulting cross-priming effect can enhance the anti-tumoral activity of immune checkpoint inhibitors.

BACKGROUND

VB4-845 is comprised of an EpCAM-specific scFv fragment genetically fused to a truncated form of *Pseudomonas* exotoxin A, ETA. After internalization, ETA translocates into the cytosol and induces apoptosis by irreversibly blocking protein synthesis. In a Phase I study, direct injection of VB4-845 led to tumor shrinkage of the principal injected tumor as well as non-targeted tumors in some patients, suggesting the activation of a T cell-mediated anti-tumor response through cross-priming (Figure 1) (1). Only cells undergoing ICD are capable of stimulating cross-priming. ICD is characterized by distinct signals such as the release of ATP and HMGB1 (High Mobility Group Box 1) and cell surface translocation of calreticulin, an endoplasmic reticulum chaperone protein (2). Nivolumab is a fully human IgG4 antibody specific to PD-1 and is currently approved for the treatment of advanced melanoma as a single agent and in combination with Ipilimumab. As a single agent, Nivolumab is also approved for the treatment of metastatic non-small lung cell carcinoma, renal cell carcinoma, Hodgkin's lymphoma, SCCHN and metastatic urothelial carcinoma (3). The data presented here shows that ICD signaling occurs following VB4-845 exposure. The potential immune T cell anti-tumor activity in response to VB4-845 was further assessed in combination with Nivolumab in PDX tumor-bearing humanized NOG mice.

Figure 1: Response of uninjected tumors after VB4-845 intratumoral treatment

METHODS

Analysis of cell surface calreticulin by flow cytometry

EpCAM-positive SW-480 tumor cells were seeded at a density of 2.5x10^5 in 6 well plates and treated with 10 pM of VB4-845, scFv-PE-cont (an anti-idiotype scFv-PE fusion protein negative control) and 1 μM of mitoxantrone (MTX) (positive control for ICD) the following day. After 18 hours, treated SW-480 cells were collected and washed twice in PBS-2% FBS buffer. Cells were then incubated with an anti-calreticulin antibody for 1 hour on ice followed by a washing step and incubation with an anti-rabbit FITC secondary antibody for 30 minutes on ice. After a washing step, cells were analyzed by flow cytometry in the presence of propidium iodide (PI). The percentage of calreticulin stained cells over the secondary antibody control was obtained on gated PI negative cells.

Assessment of intracellular ATP level by flow cytometry

For the detection of intracellular ATP, cells were seeded and treated as described above. After 9, 18 and 36 hour treatment, treated cells were collected, washed twice in PBS-2% FBS buffer and labelled with 1 μM of quinacrine in Krebs-Ringer solution for 30 minutes at 37ºC. After a washing step in Krebs-Ringer solution, cells were analyzed by flow cytometry in the presence of PI. The fluorescence intensity (FL-1) was measured on PI negative cells.

Assessment of extracellular HMGB1 concentration

Supernatants were collected after 48 hours and centrifuged to remove dying cells. Quantification of HMGB1 was measured by ELISA (IBL) according to the manufacturer's instructions. Results were expressed relative to the untreated cells and normalized to cell number.

Efficacy Study

Female NOG mice engrafted with human CD34+ hematopoietic stem cells were implanted s.c. with tumor fragments of EpCAM-positive human NSCLC patient derived xenograft (PDX) tumor model CTG-0167 (Champions TumorGraft™ model, Baltimore, MD, USA) in the left and right flank of the animal. Tumor growth was monitored twice a week using digital calipers and the tumor volume (TV) was calculated using the formula (0.52 × [length × width^2]). When the TV reached approximately 150-300 mm^3, animals were matched by tumor size and assigned into control or treatment groups (n = 5 per group) and dosing of the left side tumor was initiated on Day 0. The study design is summarized in Table 1. After the initiation of dosing, animals were weighed using a digital scale and TV of the injected and uninjected tumors was determined twice per week. The study was terminated when tumors in the control group reached approximately 1500 mm^3.

Table 1: Design of Efficacy Study in Tumor Bearing Humanized NOG mice

Group	n	Agent	Dose (mg/kg)	Route	Schedule	Total Doses
1	5	Vehicle	-	I.T.	q4dx6	6
2	5	VB4-845	0.005	I.T.	q4dx6	6
3	5	Nivolumab	5	I.P.	q5dx6	6
4	5	VB4-845 + Nivolumab	0.005 5	I.T. I.P.	q4dx6 q5dx6	6 6

I.T.: Intratumoral, I.P.: Intraperitoneal.

At randomization and completion of study, blood was collected from mice for FACS analyses using EDTA tubes and tumors were collected, formalin fixed and paraffin embedded. Tumors < 250 mm^3 were processed as flash frozen samples only.

For IHC staining, slides were deparaffinized and antigen retrieval was performed using citrate buffer. Signal amplification was performed using the avidin-biotin system and slides were developed using DAB as a chromagen with a hematoxylin counterstain.

To assess the peripheral immune response, blood samples were analyzed for the presence of huCD3+, huCD3+CD4+, huCD3+CD8+, huCD4+CD71+ and huCD4+CD25+ T cells using corresponding antibodies by flow cytometry.

RESULTS

VB4-845 and ICD

- **Cell surface translocation of calreticulin with VB4-845 (Table 2)**

Table 2: Cell surface translocation of calreticulin

% calreticulin-positive cells			
N.T.	VB4-845	scFv-PE-Cont.	MTX
22.5 (1.6)	41.6 (1.5)*	16 (0.5)	30.8 (2.6)

Percentage of calreticulin-positive cells was determined over the secondary antibody control. Values in parentheses are the S.E. of 2 replicates from a single representative experiment. * p value < 0.01.

- **Extracellular release of HMGB1 with VB4-845 (Figure 2)**

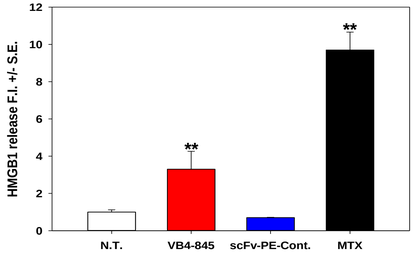

Figure 2: HMGB1 release. Data is expressed as fold-increase (F.I.) over non-treated cells and normalized to cell number. ** p value < 0.01.

- **Loss of intracellular ATP with VB4-845 treatment (Table 3 and Figure 3)**

Table 3: Percentage of cells with lower intracellular ATP level

	N.T.	VB4-845	scFv-PE-Cont.
9 hrs	5.7 (0)	8.8 (0.1)	5.8 (0.3)
18 hrs	10.3 (0.3)	17.05 (0.35)*	10.5 (0.1)
36 hrs	9.75 (0.25)	21.65 (0.55)*	11.5 (0.1)

Values in parentheses are the S.E. of 2 replicates from a single representative experiment. Representative example of three independent experiments. N.T.: nontreated.* p value < 0.005.

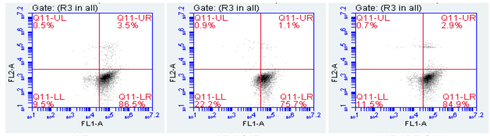

Figure 3: Intracellular ATP measurement. Non-treated (N.T.) and treated cells were stained with quinacrine and loss of intracellular ATP in live cells measured by flow cytometry.

In vivo efficacy

- **Significant anti-tumor activity after intratumoral injection of VB4-845 alone or in combination with Nivolumab (Table 4 and Figure 4A)**
- **Minimal anti-tumor effect with Nivolumab**
- **Superior tumor growth inhibition of the uninjected tumors with the combination of VB4-845 and Nivolumab (Figure 4B)**

Table 4: Anti-tumor activity summary at day 19

Group	Dose (mg/kg)	Mean ± SEM (mm^3)	p-value*	% TGI
Vehicle	-	2092 ± 484	-	-
VB4-845	0.005	324 ± 161	≤ 0.01	94.2
Nivolumab	5	1644 ± 291	n.s.	24.1
VB4-845 + Nivolumab	0.005 5	416 ± 67	≤ 0.01	88.5

* One-way ANOVA followed by Dunnett's Multiple Comparison Test. n.s.: non-significant.

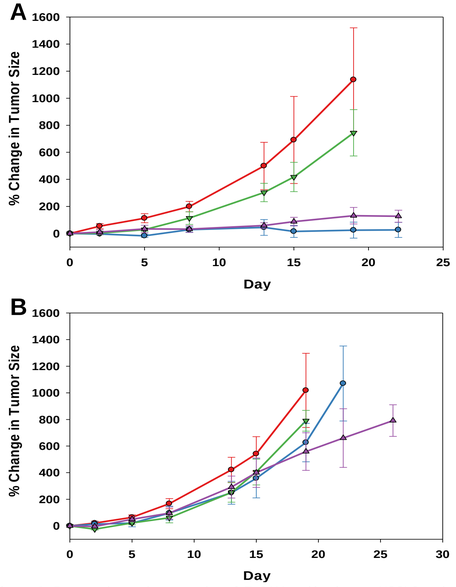

Figure 4: Percent change in tumor size of EpCAM-positive human NSCLC patient derived xenograft (PDX) tumor model CTG-0167 in humanized NOG mice A) treated with vehicle (—), VB4-845 (—), Nivolumab (—) and VB4-845 + Nivolumab (—) B) uninjected tumors on the opposite flank.

Peripheral T cells analysis

- **Higher numbers of CD8+ T cells in circulation after VB4-845 treatment with no change in CD71+ and CD25+ T cells (Table 5)**
- **Lower CD71+ and CD25+ (T-reg) in circulation after Nivolumab treatment and in combination with VB4-845**

Table 5: Flow cytometry of peripheral T cells expressed as live events

Group	CD3+	CD3+ CD4+	CD3+ CD8+	CD4+ CD71+	CD4+ CD25+
Vehicle	707.8 (37.9)	442.4 (33)	277.0 (31.1)	60.2 (9.7)	43.8 (5.6)
VB4-845	824.3 (101)	498.0 (90.3)	325.7 (18.6)*	54.3 (16.5)	41.0 (8.0)
Nivolumab	601.6 (36.5)	342.4 (21.9)	252.8 (15.9)	35.8 (3.7)	35.2 (3.3)
VB4-845 + Nivolumab	603.3 (35.0)	353.3 (43.7)	239.7 (19.9)	35.7 (11.4)	29.7 (7.2)

Statistical analysis of the number of CD8+ cells: VB4-845 vs. Vehicle, t = 1.833 with 6 degrees of freedom, P = 0.116; *VB4-845 vs. PD-1, t = 4.138 with 6 degrees of freedom, P = 0.006; *VB4-845 vs. VB4-845 + PD-1, t = 4.306 with 4 degrees of freedom, P = 0.013.

CD3+ IHC staining

- **Presence of CD3+ positive T cells in the injected and non-injected tumors (Figure 5)**

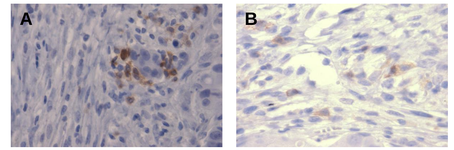

Figure 5: IHC staining of CD3+ T cells in A) tumor injected with VB4-845 and B) non-injected tumor.

CONCLUSIONS

- **The cell surface translocation of calreticulin and extracellular release of HMGB1 and ATP suggest that VB4-845 induces immunogenic cell death.**
- **In vivo efficacy study suggests that intratumoral injection of VB4-845 does not negatively impact Nivolumab activity.**
- **Data support the concept that VB4-845 killing of tumor cells can facilitate and augment checkpoint inhibitor anti-tumor activity.**

References

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Space for qpR

Author disclosures

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Poster 614, American Association for Cancer Research, Session: T-cell Immunity to Cancer: New Progress, Sunday Apr 2, 2017 from 1:00 to 5:00 PM.

Presenting and corresponding author:, email

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