VB6-845d Tumor Cell Killing Elicits Biologic Features of Immunogenic Cell Death

Abstract # 2788

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ABSTRACT

VB6-845d is a Targeted Protein Therapeutic, TPT, that comprises a Fab fragment specific for the Epithelial Cell Adhesion Molecule (EpCAM) genetically fused to deBouganin via a furin protease sensitive linker. DeBouganin (deB) is a de-immunized variant of bouganin, a ribosome inactivating protein (RIP) that when internalized blocks protein synthesis thereby leading to cell death. While all cytotoxic molecules induce tumor cell killing, only some are capable of inducing biological manifestations indicative of immunogenic cell death (ICD). ICD is characterized by the collective appearance of distinct cellular changes termed Damage Associated Molecular Patterns, or DAMPs, which play a role in immune cell activation by triggering pro-inflammatory processes. Key DAMPs necessary for defining ICD are cell surface translocation of calreticulin, an endoplasmic reticulum chaperone protein, ATP secretion and release of HMGB1 (High Mobility Group Box 1). To evaluate whether VB6-845d cell killing elicits the hallmark features of ICD, VB6-845d treated tumor cell lines were assessed for the presence of these distinct signaling molecules. *In vitro* studies showed that VB6-845d cytotoxicity induces the translocation of calreticulin to the cell surface as well as the release of ATP and HMGB1. The expression of other potential immune regulators following VB6-845d treatment was also examined. In summary, the data presented suggests that VB6-845d mediates tumor cell killing by an ICD pathway. The potential cross-priming effect initiated by VB6-845d-induced ICD, suggests the use of VB6-845d in combination with immune checkpoint inhibitors may enhance their effectiveness in EpCAM-positive epithelial cancers.

BACKGROUND

DeBouganin (deB) is a de-immunized variant of bouganin, a type 1 Ribosome Inactivating Protein (RIP) isolated from *Bougainvillea spectabilis willd*. DeBouganin mediates cell killing by deadenylating ribosomal RNA, thereby blocking protein synthesis which leads to cell death. *In vitro* assays have previously demonstrated that deBouganin is unaffected by mechanisms of resistance commonly shown to affect small molecule drugs such as multidrug resistance (MDR) pumps and anti-apoptotic protein expression (1-2). Thus, deBouganin is an attractive cytotoxic payload for antibody-directed drug delivery.

The ability of tumor cells to evade the innate and adaptive immune response plays a major role in cancer development and progression. Altered expression of immune modulators in cancer cells is a key mechanism responsible for tumor escape. Therapeutic drugs capable of killing cancer cells but also eliciting or facilitating tumor specific immune responses are desirable characteristics for establishing a durable therapeutic response. While all cytotoxic molecules induce tumor cell killing, only some are capable of inducing immunogenic cell death (ICD) and 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 (3).

Recent studies have shown that exposure of cancer cells to chemotherapeutic drugs results in the induction of immunosuppressive mechanisms (4). Cell surface expression of CD47 that blocks phagocytosis is one such outcome that can aid the evasion of cancer cells from innate immune mechanisms. In addition, enrichment of PD-L1 positive tumor cells was observed after treatment with chemotherapeutic agents which may prevent T-cell activation. Therefore, while some small molecule drugs elicit features of ICD, they can also foster an immunosuppressive environment.

Here, we show that, as an anti-EpCAM Fab fusion protein, VB6-845d, deBouganin mediated cytotoxicity elicits the hallmark features of ICD. Furthermore, unlike small molecule chemotherapeutic treatment, the cytotoxicity of deBouganin chemically conjugated to trastuzumab (T-deB) does not promote the enrichment of tumor cells expressing either CD47 or PD-L1, biomarkers associated with immunosuppressive activity.

METHODS

VB6-845d and T-deB

VB6-845d was purified from transformed *E.coli* cells grown in glycerol minimal media in a 15 L fermentor and induced with the addition of L-arabinose. The culture supernatant was microfiltered, concentrated and buffer exchanged for binding onto a CM sepharose column. The CM eluate was then flowed-through a Q-sepharose column and VB6-845d and truncated products captured on a SP-sepharose column. The SP eluate was then applied to a size exclusion column and the peaks were fractionated. Based on the SEC chromatograms, the product peak fractions were pooled and applied to a SP-sepharose column. The bound protein was then eluted and filter sterilized. The purity and identity of VB6-845d was confirmed by SEC-HPLC and Western blot analysis.

Trastuzumab-deBouganin (T-deB) conjugate was prepared by chemically conjugating trastuzumab and deBouganin after random insertion of sulfhydryl groups on lysine residues. The purity and identity of the T-deB conjugate was confirmed by SEC-HPLC and Western blot analysis. To determine the drug to antibody ratio, T-deB conjugate was reduced with 2-mercaptoethanol and analyzed by SEC-HPLC. The number of deBouganin molecules per trastuzumab antibody in the reduced conjugate was interpolated from a standard curve generated from the HPLC profiles for trastuzumab and deBouganin combined in 1:1, 1:2 and 1:3 molar equivalents of trastuzumab:deBouganin.

Analysis of cell surface calreticulin by flow cytometry

EpCAM-positive SW-480 tumor cells were seeded at a density of $2.5x10^5$ cells per well in 6 well plates and treated with 100 nM of VB6-845d and VB6-Cont (an anti-idiotype Fab-deBouganin fusion protein negative control). After 48, 72 and 96 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 48, 72 and 96 hour treatment, treated cells were collected, washed twice in PBS-2% FBS buffer and labelled with 1 mM 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 loss in fluorescence intensity (FL-1) was measured on PI negative cells relative to that of untreated (NT) cells.

Assessment of extracellular HMGB1 concentration

Supernatants were collected after 48, 72 and 96 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.

Potency

The cytotoxicity of taxol, T-deB, cisplatin, DM1, MMAE, duocarmycin and doxorubicin was assessed with an MTS assay using HER2-positive HCC1569 tumor cells. Briefly, tumor cells were seeded at 5,000 cells per well in a 96 well plate and allowed to adhere for 3 hours at 37 ºC. Indicated drugs were added over a range of concentrations. After 5 days, the IC_{50} values were determined relative to the non-treated control cells.

Analysis of CD47 and PD-L1 expression by flow cytometry

HER2-positive HCC1569 tumor cells were seeded at a density of $1.5x10^5$ cells per well in 6 well plates and treated with 10 nM taxol, 0.1 nM T-deB, 20 µM cisplatin, 50 nM DM1, 6 nM MMAE, 0.15 nM duocarmycin or 60 nM doxorubicin. After 48 and 72 hours, cells were collected and incubated with an anti-CD47 antibody conjugated to FITC or an isotype-matched control for 40 minutes on ice. Alternatively, cells were incubated with an anti-PD-L1 antibody conjugated to PE or an isotype-matched control for 40 minutes on ice. After a washing step, cells were analyzed by flow cytometry in the presence of propidium iodide (PI). The percentage of CD47-positive or PD-L1-positive cells was obtained on gated PI negative cells.

RESULTS

Immunogenic cell death

- **VB6-845d mediated tumor cell killing induces:**
  - **Calreticulin translocation to the cell surface (Table 1)**
  - **Extracellular release of HMGB1 (Fig. 1)**
  - **Loss of intracellular ATP (Table 2 and Fig. 2)**

Table 1: Translocation of calreticulin in response to VB6-845d cytotoxicity

% Calreticulin positive cells			
	NT	**VB6-845d**	**VB6-Cont**
48 hrs	**11.75 (0.55)**	**27.5 (3.7)**	**12.55 (1.65)**
72 hrs	**21.4 (2.1)**	**38.35 (0.45)***	**15.25 (1.15)**
96 hrs	**14.25 (0.15)**	**25.5 (0.7)***	**13.65 (0.55)**

Percent calreticulin positive SW-480 tumor cells calculated as a function of the 2° antibody control. Values in parentheses are the S.E. of 2 replicates from a representative experiment. * p value < 0.05.

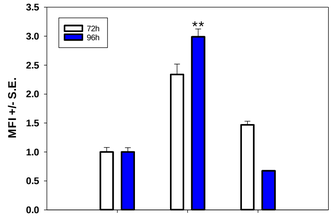


Figure 1: HMGB1 release. Data is expressed as fold-increase (F.I.) over non-treated cells (NT) and normalized to cell number. ** p value < 0.01.

Table 2: Percentage of cells with lower intracellular ATP level

	NT	**VB6-845d**	**VB6-Cont.**
48 hrs	**7.1 (0.3)**	**9.9 (0.3)**	**9.9 (0.4)**
72 hrs	**16.65 (8.65)**	**18.35 (2.45)**	**17.9 (7.4)**
96 hrs	**10.75 (0.15)**	**45.9 (0.9)***	**14.75 (0.45)**

Values in parentheses are the S.E. of 2 replicates from a single representative experiment. Representative example of three independent experiments. NT: non-treated. * p value < 0.005.

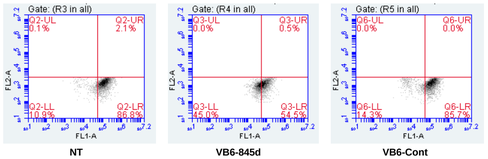
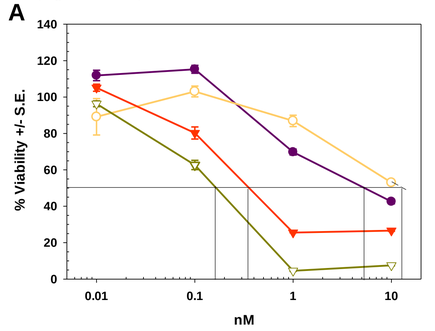


Figure 2: Intracellular ATP measurement. Non-treated (NT) and cells treated for 96 hours were stained with quinacrine and loss of intracellular ATP in live cells measured by flow cytometry.

Potency

- **T-deB is potent vs. HCC1569 cells (Fig. 3A)**
- **Disparate potencies of small molecule drugs vs. HCC1569 cells (Fig. 3A and 3B)**



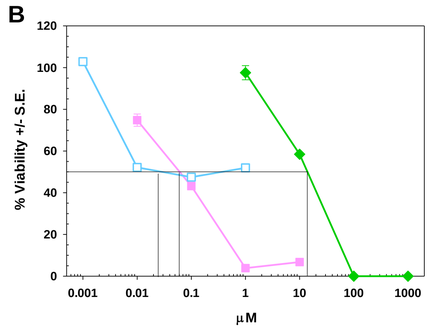


Figure 3: A) MTS curves of T-deB ( ), MMAE ( ), DM1 ( ) and duocarmycin ( ), **B)** doxorubicin ( ), taxol ( ) and cisplatin ( ). Representative example of two independent experiments.

CD47 and PD-L1 positive cells

- **No CD47 positive HCC1569 cell enrichment with T-deB (Fig. 4A)**
  - **2 to 4 fold increase in CD47 positive cell population following treatment with most small molecule drugs tested including the known ICD inducer doxorubicin**
- **No PD-L1 positive HCC1569 cell enrichment with T-deB (Fig. 4B)**
  - **Increase in PD-L1 positive cell population with taxol and cisplatin**

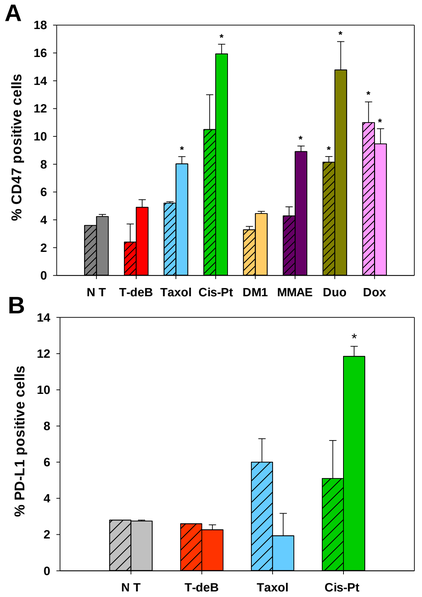


Figure 4: Percentage of **A)** CD47 positive **B)** PD-L1 positive HCC1569 cells treated with T-deB, taxol, cisplatin (Cis-Pt), DM1, MMAE, duocarmycin (Duo) and doxorubucin (Dox) for 48 (hatched bars) or 72 hours (plain bars) was determined by flow cytometry. NT: non-treated. * p value < 0.05. Representative example of two independent experiments.

CONCLUSIONS

- **Cell surface translocation of calreticulin and extracellular release of HMGB1 and ATP suggest that targeted deBouganin killing induces immunogenic cell death.**
- **Targeted deBouganin cytotoxicity does not result in an enrichment of cells expressing the immunosuppressive biomarkers CD47 or PD-L1.**
- **Data support the concept that deBouganin mediated killing of tumor cells could facilitate and augment checkpoint inhibitor anti-tumor activity.**

- **References**
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Poster 2788, American Association for Cancer Research, Session: Therapeutic Antibodies, Including Engineered Antibodies 2, Monday Apr 16, 2018 from 1:00 to 5:00 PM.

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