VB7-756: a HER2-specific Diabody Armed with deBouganin, a Plant Toxin with a Distinct MOA

Abstract #2963

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ABSTRACT

VB7-756 is a Targeted Protein Therapeutic (TPT) comprised of a de-immunized form of bouganin (deBouganin), a potent, plant-derived, type I ribosome-inactivating protein (RIP), genetically linked to the C6.5 anti-HER2 diabody via a furin protease sensitive linker. To engineer the optimal diabody TPT format, several constructs were generated to assess the best diabody-deBouganin orientation. All constructs were expressed as a soluble protein in *E. coli* supernatant and compared with respect to expression level, stability and potency. The optimal configuration consisted of deBouganin genetically linked to the N-terminus of the V_H-V_L diabody via a furin protease-sensitive linker and was termed VB7-756. VB7-756 potency was analyzed against a panel of breast cancer cell lines with disparate levels of HER2 expression and compared to that of Trastuzumab chemically linked to either DM1 (T-DM1) or MMAE (T-MMAE). Overall, VB7-756 was more potent than T-DM1 and T-MMAE with high HER2 expressing tumor cell lines. In contrast to T-DM1, VB7-756 potency was unaffected by the HER2/HER3 dimerization mediated by heregulin. As opposed to T-DM1 or T-MMAE, which showed only minimal cytoxicity, VB7-756 was highly potent *in vitro* against tumor cells with cancer stem cell properties. To further differentiate the RIP mechanism of action of deBouganin from tubulin inhibitor reagents, tumor cells that escaped T-DM1 and T-MMAE treatment were incubated in the presence of VB7-756. Results revealed that, VB7-756 was cytotoxic against cancer cells surviving T-DM1 or T-MMAE treatment suggesting that deBouganin can overcome mechanisms of resistance developed against small molecule agents. Moreover, VB7-756 was also cytotoxic against tumor initiating cells evading T-DM1 or T-MMAE toxicity by preventing tumorosphere formation. Overall these results demonstrate that deBouganin's distinct mechanism of action (MOA) could overcome mechanisms of resistance affecting the efficacy of small molecule drugs.

INTRODUCTION

Antibody drug conjugates (ADCs) consist of antibodies specifically targeting tumor–associated antigens conjugated to small molecule toxins. The development of ADCs has offered new approaches for the clinical management of cancer. For HER2 positive breast cancer, treatment with T-DM1 (ado-trastuzumab emtansine) has shown encouraging survival benefits. However, not all patients respond to therapy. In addition, the majority of patients who initially respond eventually relapse. Lowered HER2 expression, decreased intracellular trafficking and increased recycling to the cell surface are thought to affect T-DM1 potency. Furthermore, mechanisms of resistance known to affect small molecule cytotoxicity such as multidrug resistance (MDR), disruption of the apoptotic pathway as well as the recurrence of tumors from treatment refractory cancer stem cells (CSCs) are additional factors likely affecting T-DM1 efficacy. Payloads that offer an alternative MOA to the small molecule chemotherapeutic agents could therefore address some of these limitations.

Bouganin is a type I Ribosome Inactivating Protein (RIP) that functions as an *N*-glycosidase and deadenylates the 28S ribosomal RNA ultimately blocking protein synthesis and thereby leading to cell death. Bouganin lacks a cell binding domain and is only cytotoxic following internalization via a targeting ligand such as an antibody. As such, bouganin has an excellent safety profile with an LD_{50} exceeding 32 mg/kg in mice. Furthermore, Bouganin has been de-immunized (deBouganin) through the removal of T-cell epitopes which allows for repeated systemic administration. The activity of deBouganin conjugated to trastuzumab (T-deB) was compared to that of T-DM1. Results suggest that deBouganin could be highly effective against tumor cell phenotypes evading small molecule therapeutics cytotoxicities.

Antibody fragments offer several advantages over full-length mAbs including enhanced therapeutic effect through better tumor penetration and economical biomanufacturing. C6.5 diabody is a scFv-based non-covalent dimer specifically targeting HER2. DeBouganin was genetically fused to C6.5 anti-HER2 diabody via a furin protease sensitive linker. DeBouganin C6.5 diabody in the optimal configuration was selected and designated VB7-756. The potency of VB7-756 versus that of T-DM1 and T-MMAE was assessed. VB7-756 potency was also assessed against cells and cancer stem cells evading T-DM1 or T-MMAE treatment.

METHODS

Molecular engineering and expression

The constructs were assembled by the Splice Overlapping Extension Polymerase Chain Reaction (SOE-PCR) method using specific primers. Each construct contained a PelB leader sequence that targeted the expressed proteins into the supernatant *via* the periplasmic space. Transformed E104 *E. coli* cells containing the various constructs were propagated and induced with 0.1 % L-arabinose in 30 mL of TB media shake flask. Subsequently, the supernatants were collected by centrifugation and expression assessed by Western blot analysis. Fed batch fermentation of clones selected through small scale expression was performed in a 20 L CHEMAP fermenter and expressed fusion proteins purified. Purity was confirmed by coomassie staining and protein concentration determined by BCA. The stability of purified samples stored at 4 ºC was regularly monitored by SEC-HPLC.

Biological activity

The binding activity of VB7-756 and C6.5 diabody was tested against SK-BR-3 cells using flow cytometry. Binding was detected using a mouse anti-His antibody followed by a goat anti-mouse (H+L) biotin antibody followed by PE-Cy5 streptavidin.

The potency was measured by an MTS assay against a panel of HER2 positive tumor cells. Briefly, tumor cells were seeded at 5000 cells per well in a 96 well plate and allowed to adhere for 3 hours at 37 ºC. VB7-756 (or its variants), T-DM1 or T-MMAE were added to the cells over a range of concentrations and incubated for 5 days. For the heregulin assays, a fixed concentration of 20 nM was used in combination with VB7-756, T-DM1 or T-MMAE. The potency of VB7-756, T-DM1 and T-MMAE against cells surviving one T-DM1 or T-MMAE treatment was measured as follows. Briefly, 150000 cells were seeded per well in a 6 well plate and 10 nM VB7-756, T-DM1 or T-MMAE was added. Following a 5 day treatment, surviving cells were seeded at 5000 cells per well in a 96 well plate. VB7-756, T-DM1 or T-MMAE was added over a range of concentrations and incubated for 5 days. Viability was measured and the IC_{50} interpolated from the resulting plot.

The tumorosphere forming efficiency of cells pre-treated with 10 nM VB7-756, T-DM1 or T-MMAE was assessed. Briefly, 150000 cells were seeded per well in a 6 well plate, allowed to adhere for 3 hours at 37 ºC and 10 nM VB7-756, T-DM1 or T-MMAE was added. Following a 5 day treatment, surviving cells were trypsinized, placed in tumorosphere media and 10000 cells were plated per well in ultra-low attachment 6 well plates. At this time, cells were either left untreated or treated with 10 nM VB7-756, T-DM1 or T-MMAE. After 10 days, all tumorospheres greater than 50 µm in diameter were counted using an inverted microscope fitted with a graticule.

RESULTS

VB7-756: anti-Her2 diabody armed with deBouganin

- Optimal configuration: VB7-756, deBouganin genetically linked to the N-terminus of the V_H-V_L diabody via a furin protease-sensitive linker and stable up to 1 year at 4 ºC (**Table 1**)
- No change in the binding specificity between VB7-756 and C6.5 diabody (**Figure 1**)

Table 1 : Comparison of VB7-756 and its variant

	Expression	IC_{50} (nM)*	Stability
Diabody V_H-V_L-deB	++	0.30	+
deB-V_L-V_H Diabody	+++	0.04	-
deB-V_H-V_L Diabody	+++	0.06	+

*IC_{50} was measured against BT-474. Stability data after 3 weeks at 4 ºC.

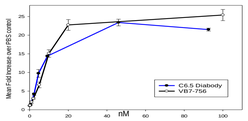

Figure 1: VB7-756 and C6.5 diabody binding reactivity against SK-BR-3 cells.

VB7-756 potency

- VB7-756 potent against all 10 HER2 3+ cell lines with IC_{50} values in the double digit picomolar to sub nanomolar range (**Table 2**)
- No clear association between HER2 expression and T-DM1 or T-MMAE potency
- On average 7000-fold differential between VB7-756 and deBouganin potency (**Figure 2.C**) as opposed to 33-fold between T-DM1 and DM1 and 5-fold between T-MMAE and MMAE (**Figure 2.A and B**)

Table 2: VB7-756, T-DM1 and T-MMAE potency vs. tumor cell lines

		IC_{50} (nM)		
Cell Line	**HER2 Expression**	**VB7-756**	**T-DM1**	**T-MMAE**
BT-474	3+	[a]0.047 (0.005)	0.715 (0.025)	0.040 (0.002)
Calu-3	3+	[a]0.041 (0.000)	1.400 (0.400)	0.086 (0.006)
HCC1419	3+	0.155 (0.015)	1.900 (0.900)	5.705 (5.095)
HCC1569	3+	[a, b]0.195 (0.025)	10.200 (0.200)	1.045 (0.155)
HCC1954	3+	[a, b]0.043 (0.005)	0.320 (0.000)	0.076 (0.005)
HCC2218	3+	0.265 (0.065)	0.290 (0.042)	0.250 (0.050)
NCI-N87	3+	0.032 (0.016)	0.265 (0.177)	0.091 (0.055)
OE-19	3+	0.043 (0.008)	0.037 (0.008)	0.042 (0.007)
HCC202	3+	0.022 (0.010)	0.100 (0.049)	0.165 (0.055)
SK-BR-3	3+	0.330 (0.020)	[c]0.047 (0.004)	[d]0.037 (0.003)
MDA-MB-361	2+	0.685 (0.035)	[c]0.320 (0.000)	[d]0.051 (0.001)
MDA-MB-453	2+	0.225 (0.085)	0.440 (0.060)	0.255 (0.035)
MCF-7	1+	>10	>10	>10
T-47D	1+	>10	8.000 (2.000)	>10
MDA-MB-231	0	>10	>10	>10

Values derived from a minimum 3 representative experiments with 3 replicates per dilution. [a]VB7-756 IC_{50} significantly better than T-DM1 ($p < 0.05$, Student's *t* test). [b]VB7-756 IC_{50} significantly better than T-MMAE ($p < 0.05$). [c]T-DM1 IC_{50} significantly better than VB7-756 ($p < 0.05$). [d]T-MMAE IC_{50} significantly better than VB7-756 ($p < 0.05$). Values in parentheses indicate the SE.

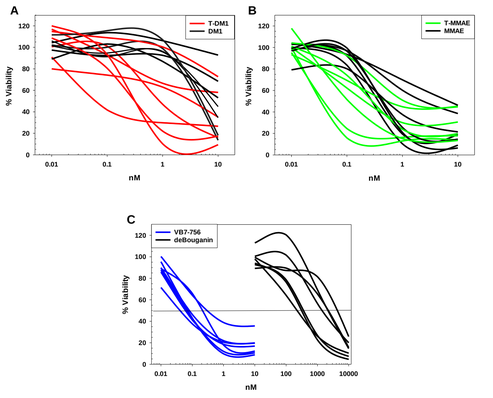

Figure 2: MTS curves of (A) T-DM1, (B) T-MMAE or (C) VB7-756 versus their respective payload (DM1, MMAE or deBouganin) against all HER2 3+ breast cancer cell lines tested.

VB7-756 and heregulin

- VB7-756 potency unaffected by concomitant heregulin treatment (**Table 3 and Figure 3**)
- Reduced T-DM1 and T-MMAE potency upon concomitant heregulin treatment

Table 3: VB7-756, T-DM1 and T-MMAE potency in the presence of heregulin

	IC_{50} (nM)					
Cell line	**VB7-756**	**VB7-756 + heregulin**	**T-DM1**	**T-DM1 + heregulin**	**T-MMAE**	**T-MMAE + heregulin**
BT-474	0.23 (0.05)	0.08 (0.02)	0.29 (0.11)	4.90 (3.10)	0.04 (0)	0.08 (0.03)
ZR-75-30	0.28 (0.13)	0.19 (0.09)	0.12 (0.08)	10 (0)	0.40 (0.28)	5.31 (4.69)

Values derived from a minimum 2 representative experiments with 3 replicates per dilution. Values in parentheses indicate the SE.

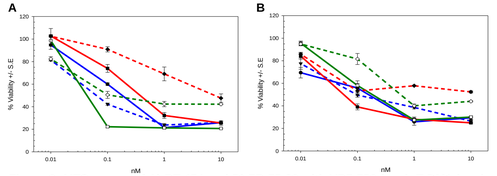

Figure 3: MTS curves of A) BT-474 and B) ZR-75-30 with VB7-756 (——), T-DM1 (——), T-MMAE (——) with (dashed lines) or without (solid lines) concomitant heregulin treatment.

VB7-756 against T-DM1 and T-MMAE treated cells

- VB7-756 cytotoxic against T-DM1 or T-MMAE treated cells (**Table 4 and Figure 4**)
- T-DM1 or T-MMAE treated cells resistant to further T-DM1 or T-MMAE exposure

Table 4: VB7-756, T-DM1 and T-MMAE potency against T-DM1 and T-MMAE treated cells. Values are expressed as IC_{50} (nM).

	BT-474			HCC1419		
	NT	**T-DM1 treated**	**T-MMAE treated**	**NT**	**T-DM1 treated**	**T-MMAE treated**
VB7-756	0.07 (0.02)	0.33 (0.27)	0.11 (0.01)	0.15 (0.02)	0.19 (0.01)	0.17 (0.05)
T-DM1	0.85 (0.25)	>10	>10	1.9 (0.9)	>10	>10
T-MMAE	0.04 (0.01)	>10	>10	5.7 (5.1)	>10	>10

Values derived from a minimum 3 experiments with 3 replicates per dilution. NT represents non-treated. Values in parentheses indicate the SE.

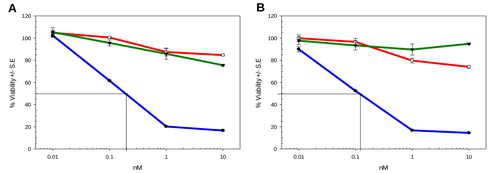

Figure 4: MTS curves of VB7-756 (——), T-DM1 (——) or T-MMAE (——) against HCC1419 cells evading (A) T-DM1 or (B) T-MMAE cytotoxicity.

VB7-756 and CSCs

- Over 95% inhibition in the tumorosphere forming efficiency of VB7-756 treated cells as compared to non-treated cells (**Table 5 and Figure 5.A**)
- No change in the tumorosphere forming efficiency between T-DM1 or T-MMAE treated and non-treated cells
- VB7-756 is more effective than T-DM1 or T-MMAE at inhibiting sphere formation of T-DM1 or T-MMAE treated cells (**Table 6 and Figure 5.B**)

Table 5: Effect of VB7-756, T-DM1 and T-MMAE on tumorosphere forming efficiency

Treatment	**BT474**	**HCC1419**
VB7-756	0	2.9 (2.9)
T-DM1	91.7 (4.8)	91.3 (11.7)
T-MMAE	87.8 (5.8)	90.4 (13.6)

Values derived from 2 representative experiments. Tumorosphere forming efficiency expressed as % relative to non-treated control. Values in parentheses indicate the SE.

Table 6: Effect of VB7-756, T-DM1 and T-MMAE on tumorosphere forming efficiency of T-DM1 and T-MMAE treated cells

Treatment	**BT-474**		**HCC1419**	
	T-DM1 treated	**T-MMAE treated**	**T-DM1 treated**	**T-MMAE treated**
VB7-756	0.14 (0.14)	0.6 (0.6)	2.1 (0.4)	1.8 (0.5)
T-DM1	42.8 (29.4)	72.6 (6.9)	110.6 (2.1)	83.6 (10.1)
T-MMAE	60.1 (28.0)	70.6 (8.1)	114 (6.1)	90.9 (12.9)

Values derived from 2 representative experiments. Tumorosphere forming efficiency expressed as % relative to non-treated control. Values in parentheses indicate the SE.

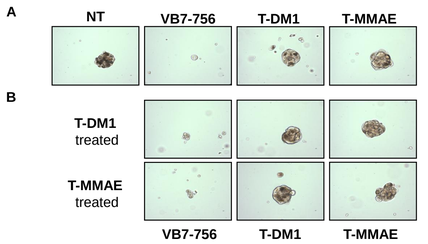

Figure 5: Representative images of tumorospheres from (A) HCC1419 cells non-treated (NT) or treated with 10 nM VB7-756, T-DM1 or T-MMAE or (B) T-DM1 or T-MMAE treated HCC1419 cells subsequently incubated under tumorosphere forming conditions with 10 nM VB7-756, T-DM1 or T-MMAE.

SUMMARY

- **VB7-756 is highly potent against HER2 3+ tumor cells.**
- **VB7-756 potency is not affected by HER2/HER3 dimerization.**
- **VB7-756 is cytotoxic against T-DM1 or T-MMAE treated cells and CSCs, highlighting deBouganin's distinct MOA.**