Abstract #2961

deBouganin Conjugated to Trastuzumab Overcomes Multiple Mechanisms of T-DM1 Drug Resistance

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ABSTRACT

DeBouganin is a T-cell epitope-depleted variant of the type I Ribosome Inactivating Protein (RIP) plant toxin Bouganin. DeBouganin binds and deadenylates the Sarcin/Ricin loop of the 28S subunit of the ribosomal RNA leading to protein synthesis inhibition and apoptosis. To demonstrate the potential advantages of deBouganin over current small molecule payloads, deBouganin was randomly chemically conjugated to trastuzumab with a DAR of approximately 2 and compared to T-DM1 both *in vitro* and *in vivo*. The trastuzumab-deBouganin conjugate (T-deB) demonstrated a tighter IC_{50} range of killing and an overall greater potency *in vitro* against most cells lines with high levels of HER2 expression as compared to T-DM1. In addition, T-deB, unlike T-DM1, was unaffected by inhibitors of multidrug resistance (MDR) and overexpression of Bcl-2 family members. Moreover, T-deB potency was unchanged by HER2-HER3 dimerization in the presence of heregulin. Contrary to T-DM1 which showed only minimal cytotoxicity, T-deB was highly potent *in vitro* against tumor cells with cancer stem cell (CSC) properties by preventing the formation of tumorspheres. Furthermore, in a BT-474 xenograft study, T-deB was more efficacious than T-DM1 resulting in increased survival of the T-deB treated mice. Overall, the results demonstrate the potency and efficacy of deBouganin and emphasize the importance of using payloads with different MOAs. The data suggest that deBouganin used alone or in combination with other payloads could be a highly effective cancer therapeutic that would provide prolonged clinical benefit.

INTRODUCTION

ADCs (Antibody Drug Conjugates) combine the potency of small molecule payloads with the targeting specificity of an antibody. One class of small molecule drugs commonly used for ADCs are the potent anti-microtubule agents, which include monomethyl-auristatin E (MMAE) and a derivative of mertansine (DM1). Although ado-trastuzumab-emtansine (T-DM1) has substantially improved survival rates, most patients eventually relapse. Lowered HER2 expression, decreased intracellular trafficking or increased recycling to the cell-surface may reduce the drug load per cell and hence 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 CSCs could explain some relapses associated with T-DM1 therapy. Given the inherent refractory nature of tumors, payloads that offer an alternative mechanism of action (MOA) to the small molecule chemotherapeutic agents represent an important ADC drug design approach for improving patient benefit.

Plant toxins are highly potent proteins that inhibit translation leading to cell death. Bouganin is a type I Ribosome Inactivating Protein (RIP) that functions as an *N*-glycosidase to deadenylate the 28S ribosomal RNA, ultimately blocking protein synthesis. deBouganin (deB), a variant of Bouganin which has been de-immunized through the removal of T-cell epitopes permits repeated systemic administration of targeted protein therapeutics containing deB as a payload. To highlight the different MOAs between plant toxins and small molecule drugs, deBouganin was conjugated to trastuzumab (T-deB) and assessed against a panel of HER2-positive cells and compared to T-DM1. Moreover, the potency of T-deB versus T-DM1 was assessed in the presence of MDR and Bcl-2 inhibitors, heregulin and the cytotoxicity against CSCs determined *in vitro*. *In vivo* efficacy studies were performed to confirm the potency of T-deB.

METHODS

Antibodies, toxins and conjugates

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, respectively. 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.

Biological activity

The ability of T-deB to inhibit protein synthesis was assessed *in vitro* using the TnT® T7 quick coupled transcription/translation system and compared to unconjugated deBouganin.

The binding activity of T-deB was determined by flow cytometry against breast tumor cell lines and compared to T-DM1 and trastuzumab at equimolar concentrations. Bound antibody was detected using a fluorescein isothiocyanate labeled goat anti-human H&L chain antibody. To determine the functional affinity of T-deB, the linear range of the saturation curve obtained with T-deB and trastuzumab against SK-BR-3 cells was used to calculate the dissociation constant, K_D, by the Lineweaver-Burk method.

The potency was measured by an MTS assay against a panel of HER2-positive 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 °C. Conjugated antibodies or free drugs were added to the cells over a range of concentrations and incubated for 5 days. The maximal concentration of inhibitor that had no effect on cell proliferation on its own was used for the potency assays (ABT-737: 0.25 µM for HCC1419, 0.075 µM for HCC1569; MK571: 30 µM for HCC1419 and HCC1569). The inhibitor was held at a fixed concentration in combination with a range of concentrations of the conjugated antibodies. For the heregulin assays, a fixed concentration of 2 nM was used in combination with a range of concentrations of the conjugated antibodies. All treated cells were incubated for 5 days and the IC_{50} interpolated from the resulting plot.

To assess tumorsphere forming efficiency, BT-474 cells were trypsinized, placed in mammosphere media and resuspended as single cells using a 25 gauge needle. Cells were plated in ultra-low attachment six well plates at a density of 10,000 cells/well and T-DM1 or T-deB added at the time of plating. After 10 days, all tumorspheres greater than 50 µm in diameter were counted using an inverted microscope fitted with a graticule. Each well was counted twice independently. Results are representative of two independent experiments.

In vivo efficacy study

Female CB.17 SCID mice were implanted subcutaneously in the flank with 1 mm^3 BT-474 tumor fragments. Animals were assigned to 4 treatment groups (n=6) when tumor volumes reached an average size of 100 mm^3. Group 1 mice were vehicle treated controls. Groups 2 and 3 were treated with either 1 mg/kg or 2 mg/kg T-deB administered by intraperitoneal (i.p.) injection. Animals in Groups 2 and 3 received either 4 doses of 1 mg/kg of T-deB on days 1, 6, 16 and 21 (Group 2) or 2 doses of 2 mg/kg T-deB on days 1 and 21 (Group 3).

For T-DM1 (Group 4), dosing was performed by intravenous (i.v.) injection of 1.5 mg/kg on Days 1 and 21 for a total dose of 3 mg/kg (molar equivalent to 4 mg/kg of T-deB). Dosing volumes for i.p. and i.v. injections were 10 mL/kg and 5 mL/kg, respectively, scaled to the body weight of each animal. Animals were monitored for tumor size twice weekly using caliper measurement and study endpoint was a tumor volume of 1000 mm^3 or day 42. Toxicity was defined as a weight loss of > 20 % of total starting body weight.

RESULTS

T-deB binding activity and potency

- 100 % purity of T-deB conjugate with an average of 1.9 deBouganin molecules per trastuzumab
- Similar affinity and rank order of T-deB binding vs. trastuzumab and T-DM1 (**Table 1**)

Table 1: Cell surface reactivity of trastuzumab, T-deB and T-DM1 against breast cancer epithelial cell lines

	MFI		
Cell Line	Trastuzumab	T-deB	T-DM1
HCC1419[a]	106.0 (0.7)	47.9 (1.2)	92.8 (1.8)
SK-BR-3[a]	48.7 (6.0)	23.0 (0.7)	40.7 (0.6)
BT-474[a]	24.6 (0.3)	13.0 (0.0)	25.5 (0.0)
MDA-MB-453[b]	14.2 (0.0)	8.7 (0.0)	13.1 (0.1)
MDA-MB-231[c]	1.3 (0.0)	1.1 (0.0)	1.3 (0.0)

Binding was determined as the mean fold increase (MFI) in median fluorescence over the PBS control. Values in parentheses are the SE of 2 replicates from a single representative experiment. [a]Tumor cell lines expressing high levels (3+) of HER2, [b]moderate levels (2+) of HER2 and [c]HER2-negative.

- Greater T-deB potency against 8 of the 10 HER2 3+ tumor cell lines vs. T-DM1 (**Table 2**)
- No clear association between HER2 expression and T-DM1 potency
- Higher level of T-deB killing for most breast cancer cell lines tested (**Figure 1**)

Table 2: Potency of T-deB and T-DM1 against carcinoma cell lines

		IC_{50} (nM)	
Cell Line	HER2 Expression	T-deB	T-DM1
BT-474	3+	***0.082 (0.019)**	0.715 (0.025)
Calu-3	3+	***0.105 (0.005)**	1.4 (0.4)
HCC202	3+	**0.055 (0.015)**	0.101 (0.049)
HCC1419	3+	***0.086 (0.025)**	1.900 (0.900)
HCC1569	3+	***0.210 (0.080)**	35.500 (13.500)
HCC1954	3+	**0.045 (0.009)**	0.064 (0.009)
HCC2218	3+	**0.245 (0.095)**	0.290 (0.042)
NCI-N87	3+	**0.090 (0.012)**	0.185 (0.075)
OE-19	3+	0.050 (0.009)	0.044 (0.005)
SK-BR-3	3+	0.275 (0.005)	****0.047 (0.004)**
MDA-MB-361	2+	1.795 (0.524)	****0.358 (0.034)**
MDA-MB-453	2+	0.335 (0.105)	0.440 (0.060)
MCF-7	1+	>10	>10
T-47D	1+	>10	8.000 (2.000)
MDA-MB-231	0	>10	>10

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

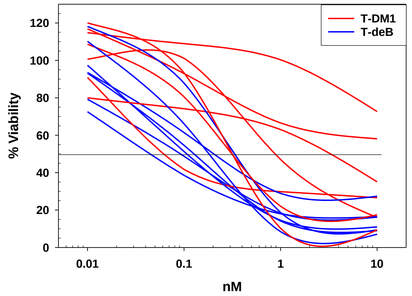

Figure 1: MTS curves of T-deB versus T-DM1 against all HER2 3+ breast cancer cell lines tested.

T-deB and targeting index

- At least a 1000-fold differential between T-deB and deBouganin potency (**Table 3**)
- Much lower TI for T-DM1 vs. DM1 with a range of 1.1 to 101-fold with half of the cell lines lower than 20-fold

Table 3: Comparison of targeting index

	deBouganin (deB)		Mertansine (DM1)	
Cell Line	deB[a]	TI (T-deB)[b]	DM1[a]	TI (T-DM1)[b]
BT-474	240 (10)	2926.8	3.8 (0.3)	5.3
HCC202	217 (12)	3954.5	6.25 (1.75)	41.7
HCC1419	500 (10)	5814.0	83.0 (29.0)	43.7
HCC1569	745 (65)	3547.6	6.7 (0.3)	0.19
HCC1954	245 (65)	3828.1	4.30 (0.45)	95.6
HCC2218	1950 (250)	7959.2	5.35 (0.55)	18.4
SK-BR-3	2100 (0)	7636.4	3.45 (0.35)	73.4
MDA-MB-361	2250 (350)	25280.9	6.05 (0.15)	17.8
MDA-MB-453	495 (55)	1477.6	5.00 (0.20)	11.4
Calu-3	145 (15)	1381.0	9.5 (3.5)	6.8
NCI-N87	850 (0)	9444.4	8.7 (0.5)	47.0
OE-19	>1000	>20000	4.45 (0.25)	101.1

[a]Indicates IC_{50} values (nM) generated with payload. Values derived from a minimum 2 representative experiments with 3 replicates per dilution. Values in parentheses indicate the SE. [b]Targeting Index, TI, defined as the IC_{50} of the payload divided by the IC_{50} of the respective ADC . TI over 3 logs are indicated in bold.

T-deB and resistance mechanisms

- No change in T-deB potency in the presence of ABT-737 or MK571, a Bcl-2 family inhibitor and MRP inhibitor, respectively (**Table 4**)
- Increased T-DM1 potency in the presence of ABT-737 or MK571 inhibitor

Table 4: T-deB potency in the presence of ABT-737 and MK571

	IC_{50} (nM)			
Cell Line	T-deB	T-deB + ABT-737	T-DM1	T-DM1 + ABT-737
HCC1419	0.10 (0.03)	0.11 (0.03)	1.30 (0.29)	0.36 (0.03)
HCC1569	0.11 (0.03)	0.09 (0.07)	35.5 (13.5)	4.69 (4.40)
	T-deB	T-deB + MK571	T-DM1	T-DM1 + MK571
HCC1419	0.14 (0.03)	0.14 (0.02)	1.30 (0.29)	0.39 (0.09)
HCC1569	0.20 (0.02)	0.17 (0.02)	35.5 (13.5)	7.54 (1.89)

Values are the mean IC_{50} of 2 representative experiments with 3 replicates per dilution. Values in parentheses indicate the SE.

T-deB and heregulin

- No change in T-deB potency in the presence of heregulin in contrast to T-DM1 (**Figure 2** and **Table 5**)

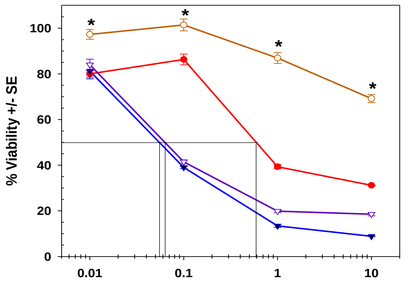

Figure 2: MTS curves of BT-474 treated with T-deB (—), T-deB + heregulin (—), T-DM1 (—), and T-DM1 + heregulin (—). *$p < 0.05$, Student's *t* test.

Table 5: T-deB and T-DM1 potency in combination with heregulin

	IC_{50} (nM)			
Cell Line	T-deB	T-deB + heregulin	T-DM1	T-DM1 + heregulin
BT-474	0.05 (0.01)	0.06 (0.02)	0.70 (0.11)	> 10.00 (0)
Calu-3	0.32 (0.03)	0.29 (0.09)	1.05 (0.15)	3.75 (1.45)
ZR-75-30	0.49 (0.09)	0.49 (0.19)	0.54 (0.16)	> 10.00 (0)

Values are the mean IC_{50} of 2 representative experiments with 3 replicates per dilution. Values in parentheses indicate the SE.

T-deB and CSCs

- Complete inhibition of tumorsphere formation by T-deB at 10 nM whereas only partial effect with T-DM1 (**Figure 3**)
- No growth of T-deB treated cells after reculture

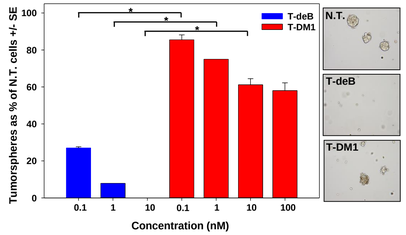

Figure 3: Tumorsphere-forming efficiency of T-deB and T-DM1 treated BT-474 cells relative to non-treated (N.T.) cells (*$p < 0.001$, Student's *t* test). Representative images of tumorspheres in N.T. and 10 nM treatment.

T-deB *in vivo* efficacy

- Increased tumor growth delay with T-deB vs. T-DM1 at equimolar concentration (**Figure 4**)
- Optimal T-deB potency by fractionating the 4 mg/kg total dose leading to a survival rate of 83 % at end of study

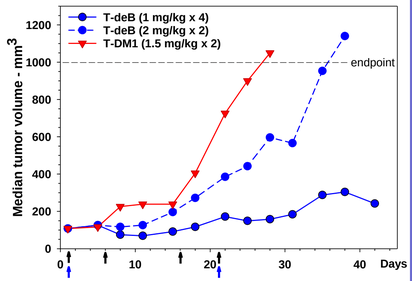

Figure 4: *In vivo* efficacy of T-deB and T-DM1 in a subcutaneous BT-474 tumor xenograft model in SCID mice.

SUMMARY

- **T-deB is highly potent against HER2 3+ tumor cells and more efficacious on a per-mole basis than T-DM1 in a tumor xenograft model.**
- **T-deB potency is unaltered by MDR, the upregulation of Bcl-2 family members, or by HER2-HER3 dimerization.**
- **deBouganin payload represents an alternative to small molecule drugs.**