ABSTRACT # 4065

Intracellular trafficking of VB6-845, an immunocytotoxin containing a de-immunized variant of bouganin

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ABSTRACT

Recombinant immunocytotoxins consist of an antibody-binding domain fused with a cytotoxin. The specificity of the antibody fragment permits the targeting of the cytotoxin to tumor cells which, upon internalization, mediates cell killing. Bouganin is a type I ribosomal inactivating protein (RIP) isolated from the plant *Bougainvillea spectabilis* Willd and lacks a cell-binding domain. The bouganin cytotoxin has been further modified by epitope-depletion to generate a de-immunized variant (de-bouganin). This de-immunized variant of bouganin has been linked to the Fab fragment *via* a furin-sensitive linker creating VB6-845 immunocytotoxin protein. Cytotoxins traffic intracellularly by one of two means, an endosomal/lysosomal pathway or *via* a Golgi/ER route. Once bound to its cognate antigen, EpCAM, VB6-845 internalizes and effects potent cytotoxic activity (IC_{50} = 1 nM). The efficiency of de-bouganin cytotoxicity was correlated with its release from the antibody moiety. To optimize the release of de-bouganin, we have investigated the intracellular trafficking of de-bouganin by confocal microscopy and evaluated cell viability in presence of drugs that affect organelle function. For confocal microscopy, EpCAM-positive CAL-27 cells were treated with a VB6-845 variant containing an inactive de-bouganin and then stained with anti-de-bouganin and organelle specific markers. De-bouganin was shown to co-localize along with the EEA1 endosomal and LAMP-2 lysosomal markers after 15 and 45 minutes in CAL-27 cells, respectively. Only marginal co-localization was detected within the Golgi apparatus after a 3-hour incubation. Treatment with lactacystine, a proteasome inhibitor, or the addition of the KDEL sequence, an ER retrieval sequence, did not improve VB6-845 cytotoxicity. This suggests that de-bouganin does not traffic *via* a Golgi/ER pathway in contrast to other cytotoxic proteins such as *Pseudomonas* exotoxin A and ricin. Treatment with chemical drugs such as chloroquine, ammonium chloride and monensin, which increase the pH of the endosome and lysosome, led to an improvement of the VB6-845 cytotoxicity ranging from 5- to 10-fold. To confirm these results, different constructs containing other proteolytic sites of proteases localized in the endosome and the lysosome compartments were engineered and tested on a variety of tumor cells. VB6-845 cytotoxicity with either a legumain, cathepsin B or D sensitive linker was still in the nanomolar range but less potent than the original molecule. Moreover, minimal additional cytotoxicity was observed when legumain, cathepsin B or D sites were added to the furin linker. Together, these data demonstrate that the de-bouganin traffics *via* an endosome/lysosome pathway to reach the cytosol and that the furin linker is the optimal configuration of the Fab variant for delivering maximal cytotoxic activity.

INTRODUCTION

Cytotoxins are proteins that, upon internalization *via* a receptor, inhibit protein synthesis of eukaryotic cells thereby leading to apoptosis. Cytotoxins, such as *Pseudomonas* exotoxin A (PE), cholera toxin (CT) or diphtheria toxin (DT), have been isolated from prokaryotic cells while others have been isolated from plants e.g. Ribosome Inactivating Proteins (RIPs). RIPs arrest protein synthesis by deadenylation of ribosomal RNA (28S rRNA) leading to the inactivation of the 60S ribosome unit. Classification of RIPs is based on their primary structure. Type I RIPs such as gelonin, saporin, or bouganin are monomeric proteins without a cell binding domain. Type II RIPs like ricin or Shiga toxin (ST), as well as most bacterial cytotoxins, are dimeric proteins consisting of a catalytic A moiety and a cell binding B moiety. Type III RIPs are, similarly to type I RIPs, single chain proteins but are produced by plant cells as a zymogene or proenzyme.

To date, two intracellular pathways used by RIPs and bacterial cytotoxins to reach the cytosol have been characterized. After being endocytosed, cytotoxins, such as saporin and DT and anthrax toxin, traffic *via* the lysosome through a pH-dependent or -independent mechanism and translocate into the cytosol.

On the other hand, upon internalization, ricin, ST, CT and PE reach the endosome and then traffic through the Endoplasmic Reticulum (ER) *via* the Golgi apparatus. The mechanism for transport from the endosome to the Golgi apparatus remains unclear. Once in the Golgi apparatus, CT and PE, which bear a KDEL or KDEL-like retrieval motif (Lys-Asp-Glu-Leu), use the KDEL receptor to traffic to the ER. To translocate from the ER compartment to the cytosol, ricin and CT are known to use the Endoplasmic Reticulum Associated Degradation (ERAD) pathway.

De-bouganin is an epitope-depleted version of bouganin, a type I RIP of 29 kDa isolated from *Bougainvillea spectabilis* Willd, which lacks the T-cell recognition sites of the native molecule, thus, allowing systemic administration as an immunocytotoxin. To that end, de-bouganin was linked *via* a furin-sensitive linker to an EpCAM Fab fragment creating VB6-845, currently in phase I clinical trial. EpCAM is a homophilic calcium-dependent adhesion molecule present during all stages of tumour development and furin is a serine endopeptidase present in the endosome but mostly in the *trans* Golgi network. Using a MTS assay, an IC_{50} of 6.6 nM and 1.5 nM was determined against EpCAM positive cells CAL-27 and OVCAR-3, respectively.

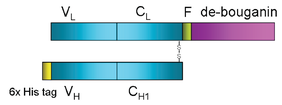


Figure 1: Schematic representation of VB6-845. F: furin linker

To study the intracellular trafficking of VB6-845, colocalization of de-bouganin with endosomal, lysosomal and Golgi apparatus markers was assessed by confocal microscopy. Moreover, VB6-845 cytotoxicity in the presence of chemical drugs that affect properties of various organelles was determined. To confirm these trafficking data, VB6-845 was reengineered with different proteolytic sites and tested by MTS assay.

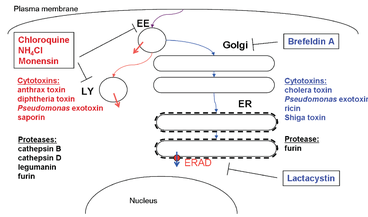


Figure 2: Schematic representation of the intracellular trafficking of cytotoxins. Chemical reagents interfering with organelle properties are indicated in black boxes.
Cathepsin B, cathepsin D, legumanin and furin are proteases found in the lysosome. Furin is also found in the *trans* Golgi network. EE: early endosome; LY: lysosome; Golgi: Golgi apparatus; ER: endoplasmic reticulum; ERAD: ER-associated degradation.

METHODS

Molecular engineering and small-scale expression
The constructs were assembled by the Splice Overlapping Extension Polymerase Chain Reaction (SOE-PCR) method using specific primers to create a dicistronic expression unit containing the different linkers or to insert the Tyr^{70}→Ala^{70} mutation. Each unit contained a PelB leader sequence that targeted the expressed proteins into the supernatant *via* the periplasmic space. In addition, the V_H-C_{H1} unit of each construct contained a 6xHis tag to facilitate purification. Transformed E104 *E. coli* cells containing the VB6-845 variants were propagated in 30 mL of TB media (1% inoculum) in a 250 mL shake flask at 37 ºC, shaken at 225 rpm for approximately 5 hours until the optical density ($OD_{600\ nm}$) reached approximately 2. The culture was then induced with a final concentration of 0.1% L-(+) arabinose for 16 hours and incubated at 25 ºC. Subsequently, the supernatant was collected by centrifugation and VB6-845 variants were quantified by ELISA using rabbit anti-bouganin for the coating and a mouse anti-human IgG Fd, an anti-mouse IgG (H+L) biotin conjugated and streptavidin-HRP for the detection.

Purification
At 16 hours post induction, the culture (6 L) was harvested, centrifuged, concentrated and diafiltered against a phosphate buffer and VB6-845 variants were purified using CM sepharose and chelating sepharose columns followed by a size exclusion column. Purity after the size exclusion column was confirmed by colloidal blue staining.

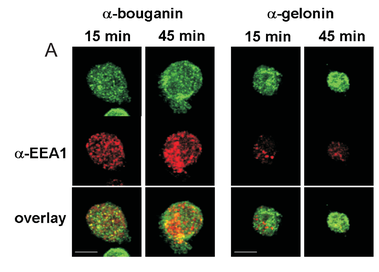
Biological activity
The binding activity of the purified VB6-845 variants was tested on EpCAM-positive cell lines (CAL-27 and MCF-7) and an EpCAM-negative cell line (A375) using flow cytometry. Binding was detected using a rabbit anti-de-bouganin antibody followed by a goat anti-rabbit FITC antibody. MTS assay was subsequently used to determine the IC_{50} values of each variant using a panel of EpCAM positive tumor cell lines.

Immunofluorescence
Adherent CAL-27 cells were grown on coverslips and subjected to different cytotoxin treatments at room temperature (RT). After different incubation times, cells were fixed and incubated with the different primary antibodies at RT. Cells were then washed and incubated with secondary antibodies: goat anti-mouse Alexa Fluor® 594 conjugated and goat anti-rabbit Alexa Fluor® 488 conjugated. Images were captured on an Olympus IX-70 inverted confocal LASER microscope at the University of Manitoba (Winnipeg, Canada). Image analysis was performed with Fluoview version 2.0 software.

RESULTS

VB6-845 colocalization studies

To follow the subcellular trafficking of de-bouganin after internalization, cells were fixed and stained at different time intervals with anti-de-bouganin and different markers specific for intracellular organelles. VB6-845-gelonin and VB6-845-PE colocalization staining was used as endosome/lysosome and Golgi positive controls, respectively.



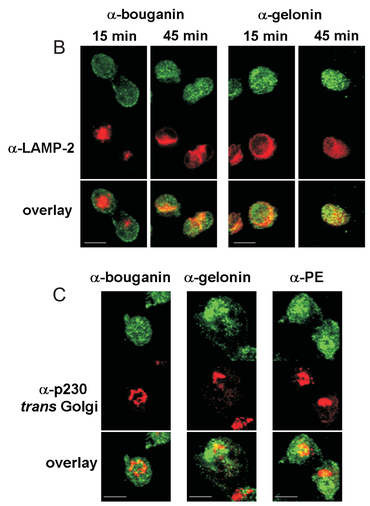


Figure 3: Colocalization of VB6-845 constructs with A) EEA1, early endosome antigen 1; B) LAMP-2, lysosomal associated membrane protein 2; or C) p230 *trans* Golgi after 3 hours incubation.
Scale bars: 10 µm.

- ***VB6-845 and VB6-845-gelonin colocalize with EEA1 and LAMP-2 and show a marginal colocalization with the Golgi apparatus.***

Effect of alkalinisation of endosomal and lysosomal pH on VB6-845 cytotoxicity

To confirm the confocal studies, CAL-27 cells were exposed for 3 days to serial logarithmic dilutions of cytotoxins and in the presence or absence of chemical drugs that affect the endosome and the lysosome and IC_{50} was measured by MTS assay.

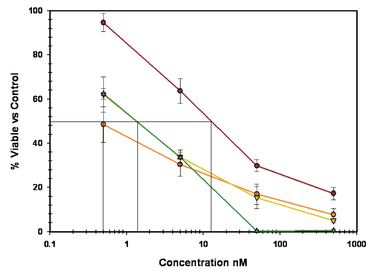


Figure 4: Effect of 6.25 µM chloroquine (orange circles), 10 mM NH_4Cl (yellow cones) or 300 nM monensin (green triangles) on VB6-845 cytotoxicity. Cells treated with VB6-845 only are shown in dark red circles.

Table 1: Summary of the IC_{50} of several cytotoxins in the presence or absence of different drugs: chloroquine, NH_4Cl or monensin.

		Untreated	Chloroquine (6.25 µM)	NH_4Cl (10 mM)	Monensin (300 nM)
IC_{50} (nM)	VB6-845	10	0.5	1	1
	De-bouganin	>500	50	200	100
	VB6-845-gelonin	10	0.5	0.7	1
	Gelonin	>500	100	90	200
	Saporin	100	10	10	4
IC_{50} (pM)	VB6-845-PE	0.4	1	0.9	0.7
	Ricin	0.2	0.09	0.08	0.04

- ***The alkalinisation of endosomal and lysosomal pH improves VB6-845, gelonin and saporin cytotoxicity by 5- to 10-fold.***

Effect of irreversible inhibition of the proteasome degradation on VB6-845 cytotoxicity

To investigate whether or not VB6-845 uses the ERAD pathway, the IC_{50} of MCF-7 cells exposed for 3 days to serial logarithmic dilutions of cytotoxins and in the presence or abcence of 10 µM of lactacystin was measured by MTS assay.

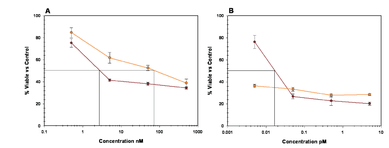


Figure 5: Effect of 10 µM of lactacystin (dark red) on the cytotoxicity of A) VB6-845, B) VB6-845-PE. Cells treated with cytotoxin only are shown in dark red.

- ***Inhibition of the proteasome degradation by lactacystin treatment does not improve VB6-845 cytotoxicity.***

VB6-845 linker studies

To confirm these trafficking data, the VB6-845 furin-sensitive linker was replaced by proteolytic sites targeted by legumain (Leg), cathepsin B (CB) and/or cathepsin D (CD). *E. coli* supernatants were quantified by ELISA and IC_{50} were tested by MTS assay. Leg, CB and CD, overexpressed in tumor cells, are mostly localized in the endosome/lysosome compartment and have a role in mitogenic activities (CD) or metastasis/invasion processes (CB, Leg) of tumors.

Table 2: Amino acid sequences of the different linkers including cleavage sites.

Linker	Leg	CB	CD	F-Leg	F-CBCD	F-CBCD-Leg
Amino acid sequence	AAN↓L	G↓FGSV QFAGF	TF↓FAGF	TRHRQP R↓WEQL AAN↓L	TRHRQP R↓GWEQL G↓FGST F↓FAGF	TRHRQP R↓GWEQL G↓FGST F↓FAF AAN↓L

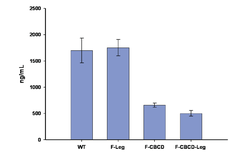


Figure 6: Supernatants of various forms of VB6-845 were quantified by ELISA.

- ***The addition of more than two proteolytic sites on the VB6-845 linker lowers its expression.***

Table 3: Comparison of the IC_{50} of VB6-845 and its variants.

	VB6-845 – IC_{50} (nM)					
	F	Leg	CB	CD	F-Leg	F-CBCD
CAL-27	1.8	10.3	6.9	8.9	1.8	2
HT-29	1.4	19	12	13	1.3	0.65
KATO III	0.14	1.2	0.9	0.71	0.16	0.19
LNCaP	11.2	>50	>50	>50	10.6	1.8
MCF-7	0.44	13	4.4	9.9	0.23	0.4
NCI-H69	1.5	1.7	10.7	0.91	0.31	0.33
NIH:OVCAR-3	0.4	3.8	1.7	1.8	0.44	0.22
SW-480	1.4	4.5	3.9	3.9	1.2	1.1

- ***The IC_{50} of linkers targeting only one proteolytic site (Leg, CB or CD) are equivalent or higher than the furin-sensitive linker.***
- ***The combination of different linkers shows a minimal additional effect except for the LNCaP cell line.***

CONCLUSIONS

- **VB6-845 colocalizes with EEA1 and LAMP-2 and its IC_{50} is lowered with alkalinisation of endosomal and lysosomal pH**
- **VB6-845 trafficking to the cytosol is independent of the Golgi/ER pathway**
- **VB6-845 containing Leg-, CB- or CD- sensitive linkers is cleaved by endosomal and lysosomal enzymes leading to a nanomolar range IC_{50}comparable to VB6-845**

Our data demonstrate that upon internalization via EpCAM, VB6-845 traffics via the endosomal and the lysosomal pathway to inactivate ribosomes and induce apoptosis. Moreover, the furin-sensitive linker seems to be optimal for the VB6 format.