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Development of a GMP Phase III purification process for VB4-845, an immunotoxin expressed in *E. coli* using high cell density fermentation

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ABSTRACT

VB4-845 is a recombinant immunotoxin comprised of an anti-epithelial cell adhesion molecule (EpCAM) scFv fused to a truncated form of the bacterial toxin, *Pseudomonas* exotoxin A. VB4-845, purified from TB fed-batch fermentation, showed clinical efficacy when administered locally to treat non-muscle invasive bladder cancer (NMIBC) and squamous cell carcinomas of the head and neck (SCCHN). Here, we describe the implementation of an *Escherichia coli* high cell density (HCD) cultivation and purification process for VB4-845. HCD cultivation was a prerequisite for achieving higher yields necessary for Phase III clinical trials and commercialization. Using this process, the VB4-845 titer in the supernatant was increased by 30-fold over the original TB fed-batch cultivation. To obtain clinical grade material, a process involving a five-step column purification procedure was implemented and led to an overall recovery of ~40%. VB4-845 purity of >97% was achieved after the first three columns following the removal of low-molecular weight product-related impurities and aggregates. Endotoxins were effectively separated from VB4-845 on the Q-columns and by washing the Ni-column with a detergent buffer while host cell proteins were removed using ceramic hydroxyapatite. Comparability studies demonstrated that the purified product from the Phase III process was identical to the Phase II reference standard produced using TB fed-batch fermentation.

Introduction

Antibodies, unlike most conventional cancer treatments such as radiation and chemotherapy, are able to specifically target tumor cells without damaging normal cells. For this reason, antibody-based therapies continue to be at the forefront in the search for more effective cancer treatments. Although antibody based therapeutics have proved to be commercially successful, recent clinical evidence suggests that antibodies alone lack the potency required to offer long term benefits [1,2]. In order to address the shortfall in potency, antibodies are now being connected to a cytotoxic payload. The payload component can vary from small molecule drugs, radionuclides to toxins [3–5]. Where toxins are concerned, immunotoxins (ITs)[1] are comprised of an internalizing antibody linked to a bacterial or plant toxin. This addition of a toxin provides a highly potent and well-defined mechanism of action for tumor cell killing. The antibody acts as a delivery vehicle for the toxin by targeting antigens on the surface of cancer cells. Once internalized, the toxin payload is released into the cytosol where it mediates cell death by blocking protein synthesis [6].

Historically, ITs have been constructed using different approaches, each with its own unique challenges. Chemical conjugation methods, including disulfide and thioether bonds, have been used to randomly attach several toxin molecules to a binding protein [7]. From a commercial manufacturing perspective, IT production via chemical linkage is complex and costly as two separate production processes in addition to the conjugation step are required. As an alternative, recombinant ITs genetically link the binding protein to the toxin thus foregoing the need for chemical conjugation and its associated costs. Recombinant ITs are typically produced by microbial fermentation where the active forms of the ITs are recovered from inclusion bodies or alternatively, directly

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[1] *Abbreviations used:* EpCAM, epithelial cell adhesion molecule; NMIBC, non-muscle invasive bladder cancer; SCCHN, squamous cell carcinomas of the head and neck; HCD, high cell density; ITs, immunotoxins; scFv, single-chain variable fragment; SOPs, standard operating procedures; GMP, good manufacturing practice; GLP, good laboratory practice; WFI, water for injection; SIP, sterilized in place; MCB, master cell bank; OD_{600}, optical density; WCB, working cell bank; GMM, glycerol minimal medium; DO, dissolved oxygen; TFF, tangential flow filtration; TMP, transmembrane pressure; CIP, cleaned-in-place; CHT, ceramic hydroxyapatite; CV, column volumes; LMWI, low molecular weight impurities; HP, high performance; HCP, host cell protein; CD, circular dichroism; LAL, limulus amebocyte lysate; pNA, p-nitroaniline; TMB, tetramethyl benzidine; ILA, immuno-ligand assay; ssDNA, single-stranded DNA; ZC, zero calibrator; MF, median fluorescence; HMWI, higher molecular weight impurities.

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from the culture supernatant [8,9]. However, in the case of inclusion bodies, the renaturation of ITs is a complex process often leading to low overall recoveries [10,11].

VB4-845 is a recombinant IT that targets the epithelial cell adhesion molecule (EpCAM) antigen, a marker known to be over-expressed in many epithelial carcinomas including colon, breast, prostate and bladder and often associated with disease progression and poor patient prognosis [12–14]. EpCAM is circumferentially expressed on tumor cells but limited to the baso-lateral surface of normal epithelium. The expression of EpCAM along areas of cell-to-cell contact in normal tissue is believed to restrict the accessibility of targeted therapies thus minimizing the potential for off-target toxicities [15–17]. VB4-845 consists of an anti-EpCAM humanized single-chain variable fragment (scFv) linked to a truncated form of *Pseudomonas* exotoxin A ($ETA_{252-608}$) that lacks the cell binding domain [18]. The scFv portion binds to EpCAM on the surface of carcinoma cells and VB4-845 is rapidly internalized, allowing the exotoxin portion of the fusion protein to inhibit translation and induce apoptosis [19]. The immunogenicity of the ETA moiety limits VB4-845 therapy to cancers amenable to local delivery such as transitional cell carcinoma of the bladder where VB4-845 is instilled directly into the lumen. To that end, the safety and efficacy of VB4-845 was explored in Phases I and II clinical trials, respectively, in patients with high grade, BCG-refractory Ta or T1 TCC, or carcinoma *in situ* (TIS) of the bladder [20].

For these early Phase trials, drug product was manufactured at a 1200 L scale using a TB fed-batch microbial fermentation process where the IT was secreted directly into the culture broth after induction with L-arabinose. Soluble VB4-845 expression level was quantified at ~25 mg/L in the culture supernatant, with an overall recovery rate of ~35% after purification. While microbial expression of soluble VB4-845 has clear advantages over other production methods for ITs, the product yield from the TB-fed batch fermentation process was insufficient for a Phase III pivotal trial given the predicted drug demand.

In this report, we describe the GMP clinical production process for the VB4-845 immunotoxin fusion protein using high cell density (HCD) cultivation. In order to accommodate the higher titers, a new purification strategy was developed to effectively purify VB4-845 and reduce the higher host-cell related impurities associated with HCD. In addition, VB4-845 produced with the new HCD process was comparable to that of the TB fed-batch method in terms of structure, biological activity and quality.

Materials and methods

General techniques for performing production and analytical experiments

Clinical production was performed at Viventia Biotechnologies Incorporated using sterile technique, Standard Operating Procedures (SOPs), Good Manufacturing Practice (GMP) and Good Laboratory Practice (GLP) guidelines. Air-filtration (Slim line RSR 24″ × 48″ and 12″ × 48″) and sticky floor mats (VWR, Catalog No. 21924-106) were used. All the reusable glassware were washed, rinsed with water for injection (WFI), dried and baked in an oven at 240 °C for 4 h. The seed media was sterilized in an autoclave for 30 min at 121 °C at 15 psi, and production media sterilized in place (SIP) for 60 min at 121 °C at 15 psi. All buffers were sterile-filtered with disposable 0.2 μM Hydrosart 0.05 m^2 filters (Sartorius, Catalog No. 523 5307 H7-OO-A) and stored in sterile liquid storage bags (Hyclone, Catalog No. 11605). Water used for VB4-845 purification met specifications for WFI.

Preparation of VB4-845-C master and working cell banks

Construction and cloning

The VB4-845 nucleotide sequence was analyzed using Translation Engineering™ and modified to allow optimal expression in *Escherichia coli* (CODA Genomics, Irvine, CA). The codon optimized insert, VB4-845-C, was ligated into the pING3302 plasmid under the transcriptional control of the L-arabinose-inducible promoter, araB, from *Salmonella typhimurium*, using the unique *Eco*RI and *Xho*I restriction sites located at the 5′ and 3′ ends, respectively. The *E. coli* host strain, E104, a derivative of the K12 strain W3110 (XOMA Ltd., Berkeley, CA) was transformed with the recombinant VB4-845-C/pING3302 plasmid and transformants selected on a LB agar plate containing 25 μg/mL of tetracycline (Sigma, Catalog No. T3383).

Preparation of master and working cell banks

To create a Master Cell Bank (MCB), a 50 mL conical tube containing 5 mL of 2xYT medium and 25 μg/mL of tetracycline was inoculated with a single colony and grown to an optical density (OD_{600}) of 2.0 in a shaking incubator set at 25 °C and 220 RPM (New Brunswick Scientific, Model No. Innova 4300). The culture was further expanded by adding 1.25 mL of the culture into a sterile 250 mL Erlenmeyer flask (Fisher Catalog No. C4980250) containing 50 mL of 2xYT medium and 25 μg/mL of tetracycline and grown to an OD_{600} of 1.0–1.5 as described above. The culture was then mixed with 25 mL of sterile 30% glycerol solution (Fisher, Catalog No. 2143-07), dispensed in 1.5 mL aliquots into cryotube vials (Nunc, Catalog No. 277267) and stored at −80 °C to create a MCB. Genetic stability testing of the MCB was performed on three independent vials by assessing plasmid integrity, plasmid retention and sequence identity. Briefly, for plasmid integrity, restriction enzyme digestion using *Eco*RI, *Xho*I and *Eco*RI in combination with *Xho*I and *Bam*HI, was performed with extracted DNA plasmid from MCB cultures grown to an OD_{600} of ~3. To determine the percentage of host cells retaining the plasmid, Tryptic soy agar plates were used to isolate single colonies from the inoculum seed and end of fermentation culture. Subsequently, 50 colonies from each were replated onto Tryptic Soy Agar plates with or without tetracycline. After 24 h incubation at 32 °C, colonies from both plates were counted and the data expressed as a percentage of host cells retaining the plasmid.

For sequence identity, plasmid DNA isolated from three vials of the MCB was sequenced (CEQ Genetic Analysis System, Beckman Coulter, Mississauga, ON) to confirm VB4-845-C nucleotide identity.

Once genetic stability was established, a working cell bank (WCB) was created by inoculating the contents of a vial from the MCB, in a 2 L Erlenmeyer flask (Fisher, Catalog No. C49802L) with 500 mL of 2xYT medium containing 25 μg/mL tetracycline and grown to an OD_{600} of 1.2–1.5 in a shaking incubator set at 37 °C and 200 RPM then, 250 mL of the culture was mixed with 125 mL of sterile 30% glycerol solution and 1.5 mL aliquots were stored into cryovials at −80 °C. The WCB was used in the development of the manufacturing process at the 15 L scale and for clinical production at the 1000 L scale of VB4-845 Phase III drug.

HCD cultivation for expression of VB4-845 at 15 L scale

HCD cultivation in GMM of transformed E104 *E. coli* cells was performed in a 20 L bioreactor (Chemap AG 8604, Volketswil, Switzerland) at 28 °C, airflow and agitation of 10 SLPM and 1000 RPM, respectively, and a pH of 7.0 maintained with ammonium hydroxide, as previously described [21]. Briefly, 15 L of GMM was inoculated with 150 mL of seed culture grown to an OD_{600} of 2.0–2.5 in a shaking incubator set at 26 °C and 200 RPM. In the bioreactor,

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a spike in dissolved oxygen (DO) to ⩾90% triggered feeding with glycerol via the pO2 loop at a 40% DO set-point, to control the growth rate. At an OD_{600} of 50, the culture was induced with a glycerol feed containing 6 g/L of arabinose using the same feeding strategy. After 32 h induction, the supernatant containing VB4-845 was clarified by centrifugation followed by microfiltration, concentrated and diafiltered against 20 mM $NaPO_4$ buffer, pH 8.0. VB4-845 produced at the 15 L scale was purified with a scaled-down version (1/14) of the process described for the 78 g batch size of VB4-845 produced at the 1000 L scale.

Clinical production-1000 L scale

The primary seed culture was prepared as described above and then expanded into 15 L of GMM containing 25 μg/mL tetracycline, to an OD_{600} of 2.0–2.5, in a 35 L B Braun bioreactor set at 26 °C, 200 RPM and 10 SLPM.

In a 1500 L ABEC bioreactor equipped with two DO probes and one pH probe, 900 L of GMM production media and 0.120 L of Polyglycol P-2000 antifoam (Univar, Catalog No. 635824) were SIP. To confirm sterility, the agitation speed, temperature and air flow were set to 53 RPM, 28 °C and 200 SLPM, respectively, and overhead pressure maintained at 3–5 psi, for 12–18 h. Then, filter-sterilized ingredients were added, the vessel inoculated with the seed culture and cells grown as described above until nutrients were depleted. At the onset of the fed-batch stage, feeding was controlled using the pO2 loop to attain an OD_{600} of 50 at which time gradient induction was initiated. To control foaming, Polyglycol P-2000 antifoam was added as required. After 30 h of induction, the temperature was set to 4 °C and the airflow and agitation rates were reduced to 200 SLPM and 53 RPM, respectively. Once the temperature of the broth was less than 15 °C, the culture broth was clarified by centrifugation.

Harvesting of culture supernatant

Initial clarification of the broth was performed at a rate of 800 mL/min using a disc stacked centrifuge (Westfalia Model No. SB7) equipped with a 5 L bowl at an operating speed of 10,000 RPM and backpressure of 75 psi. Approximately, 780 L of supernatant with an OD_{600} < 10 were collected for microfiltration and 220 L of cell slurry were discarded.

Microfiltration

The supernatant was collected in 2 × 300 L batches and a 180 L batch. The individual batches were further clarified to an OD_{600} of <0.5 by tangential flow filtration (TFF) at a transmembrane pressure (TMP) of 15 (P_{Inlet} 35 psi, P_{Ret} 10 psi, P_{Perm} 7.5 psi) using 0.22 μM Hydrosart filter cassettes (10.8 m^2) (Sartorius, Catalog No. 3061860706W-SG). For higher recoveries, the filter cassettes were cleaned-in-place (CIP) between batches by re-circulating 1 N NaOH and 3% H_3PO_4, respectively, for 30 min, then rinsed with RO water until permeate pH was between 7 and 8.

Concentration and diafiltration

Each microfiltered batch was concentrated eightfold followed by a fivefold buffer exchange by diafiltration. Briefly, using TFF at TMP 7.5 (P_{Inlet} 20 psi, P_{Ret} 10 psi, P_{Perm} 0 psi) with 4.2 m^2 of Hydrosart 10 KDa filter cassettes (Sartorius, Catalog No. 30514439E-SG) the concentrated supernatant was diafiltered with 20 mM $NaPO_4$ buffer, pH 8.0 for optimal binding of the VB4-845 protein (pI 5.9) to Q-Sepharose. The filter cassettes were CIP between batches by re-circulating 1 N NaOH for 30 min, rinsed with RO water and equilibrated in diafiltration buffer. Each batch was filtered using a 0.22 μM (1.2 m^2) filter (Sartorius, Catalog No. 5231307H2-SS) into a 50 L storage bag (Hyclone, Catalog No. 114205). The diafiltered batches were pooled and divided into 78 g batches of VB4-845 product based on ELISA quantification. One batch was kept at 2–8 °C for 72 h before purification and the remaining batches stored up to six months at −20 °C prior to purification.

Purification of a 78 g batch size

All packed chromatography columns were depyrogenated by flowing 1 N NaOH through the column for 35 min and washed with WFI until pH of the column effluent was < pH 8.0 except for the ceramic hydroxyapatite (CHT) column which was washed with equilibration buffer.

Primary recovery using Q-Sepharose adsorption

Q-Sepharose Fast Flow resin (19.6 L; GE Healthcare, Catalog No. 17-0510-05) was packed into a Quickscale 450/550 column (Millipore, Catalog No. GA451511) to a bed height of 12.5 cm at 76.8 cm/h. A flow rate of 64 cm/h was used throughout except for sample loading. The resin was charged for 3 column volumes (CV) with charging buffer (20 mM $NaPO_4$, 2 M NaCl, pH 8.0) and equilibrated with 20 mM $NaPO_4$, 100 mM NaCl buffer, pH 7.5 for 5 CV. A volume of diafiltered supernatant, equivalent to 78 g of VB4-845 was loaded onto the column at 17.7 cm/h and the resin washed with equilibration buffer until the UV absorbance of the effluent at 280 nm (A_{280}) reached baseline. VB4-845 was eluted using 20 mM $NaPO_4$, 300 mM NaCl buffer, pH 8.0 and collected in a single peak based on A_{280} absorbance for direct application on the Ni-chelating column.

Affinity purification using Ni-charged chelating Sepharose

VB4-845 contains two 6-mer histidine tags to facilitate purification by immobilized metal affinity chromatography. Chelating Sepharose Fast Flow resin (GE Healthcare, Catalog No. 17-0575-04) was packed into a BPG 300/500 column (GE Healthcare, Catalog No. 18-1103-21) to a bed height of 7.8 cm at 174.4 cm/h. The resin was charged at 61 cm/h for 3 CV with 0.1 M $NiCl_2$, washed for 5 CV with WFI to remove the excess Ni^{2+} ions then equilibrated for 5 CV with 20 mM $NaPO_4$, 150 mM NaCl buffer, pH 8.0. After loading the eluate from the Q-Sepharose column at 61 cm/h, the column was washed at 110 cm/h for 20 CV with 20 mM $NaPO_4$, 150 mM NaCl, 75 mM imidazole buffer, pH 8.0 to specifically elute Low Molecular Weight Impurities (LMWI), followed with 20 mM $NaPO_4$, 150 mM NaCl, 0.5% Triton X-100 buffer, pH 8.0 for 40 CV to further reduce endotoxins, and then equilibrated in 20 mM $NaPO_4$, 150 mM NaCl, pH 8.0. VB4-845 was eluted with 20 mM $NaPO_4$, 150 mM NaCl, 250 mM imidazole buffer, pH 8.0, at 61 cm/h as a single peak based on A_{280} absorbance.

Aggregate removal using Q-Sepharose adsorption

A Q-Sepharose column using differential salt elution separated monomeric VB4-845 from VB4-845 aggregates, a major product-related contaminant at this point in the process. Briefly, Q-Sepharose High Performance (HP) resin (4.1 L; GE Healthcare, Catalog No. 117-1014-04) was packed into a BPG 300/500 column to a bed height of 6.0 cm at 145.3 cm/h. The column was charged as described above and equilibrated for 5 CV with 20 mM $NaPO_4$, 100 mM NaCl buffer, pH 8.0. All operations were performed at a flow rate of 91.7 cm/h except during sample loading. The nickel chelating eluate was diluted fivefold with 20 mM $NaPO_4$ buffer, pH 8.0 and applied onto the column at 76.5 cm/h. After washing the column with 10 CV of equilibration buffer, bound VB4-845

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was eluted with 20 mM $NaPO_4$, 200 mM NaCl buffer, pH 8.0 and collected in a single peak based on A_{280} absorbance.

Host cell protein reduction using ceramic hydroxyapatite

CHT was used to reduce host cell protein (HCP) levels. CHT, (5.5 L; Biorad, Catalog No. 157-4500) was packed into a BPG 300/500 column to a bed height of 8.0 cm at 174.4 cm/h. The resin was equilibrated with 10 mM $NaPO_4$, 200 mM NaCl, pH 7.1 until the pH of the effluent reached 7.1 ± 0.1. The pH of Q-Sepharose eluate was adjusted to 7.1 with 6 N HCl and diluted twofold with 200 mM NaCl during the loading on the column to reduce the phosphate concentration to 10 mM. At this phosphate concentration, the CHT resin only bound HCPs whereas the VB4-845 protein remained in the flow-through.

Concentration step using Q-Sepharose adsorption

Q Sepharose HP (2.1 L) packed in a BPG 200/500 column (GE Healthcare, Catalog No. 18-1103-11) to a bed height of 6.7 cm at 254.8 cm/h, was used as a concentration step prior to formulation. The resin was charged as described above and equilibrated for 5 CV with 20 mM $NaPO_4$, 100 mM NaCl buffer, pH 8.0 at 200.6 cm/h. To reduce the NaCl concentration to 100 mM, which is required for optimal VB4-845 binding, the CHT effluent was diluted twofold with 20 mM $NaPO_4$, pH 8.0 throughout the loading on the Q-column at 200.6 cm/h. After washing for 10 CV with equilibration buffer, VB4-845 was eluted with 20 mM $NaPO_4$, 500 mM NaCl buffer, pH 8.0 at 127.4 cm/h and collected in 700 mL fractions. All fractions with an A_{280} value $\geqslant$ 0.7, corresponding to a protein concentration $\geqslant$ 0.5 mg/mL, were pooled and formulated.

Formulation of VB4-845 drug substance

Diafiltration was performed using a Sartorius TFF system which included a 0.6 m^2, 10 kDa Hydrosart filter cassette (Sartorius, Catalog No. 30514439E–SG). The membrane was depyrogenated by circulating 1 N NaOH for 35 min and equilibrated with 20 mM sodium phosphate buffer, pH 8.0. Polysorbate 80 (Mallinckrodt Baker, Catalog No. 4117-04) was added to a concentration of 0.1% (w/w). The VB4-845 protein solution was diafiltered for five buffer exchanges against 20 mM $NaPO_4$ buffer pH 8.0 at a TMP of 7.5 (P_{Inlet} 12 psi, P_{Ret} 3 psi, P_{Perm} 0 psi). The protein concentration was then adjusted to meet the required release specification (5.0 mg/mL ± 0.5 mg/mL) with 20 mM $NaPO_4$ buffer, pH 8.0, containing 0.1% Polysorbate 80 and filtered with a Sartobran 300, 0.22 μm filter (Sartorius, Catalog No. 523 1307H5-OO-B) into a 10 L sterile storage bag (Hyclone, Catalog No. SH3B6510) for storage at −20 °C.

Fill/finish

The drug substance was thawed at room temperature and filter sterilized using a 0.22 μm filter (Sartorius, Catalog No. 523 1307H5-OO-B) under a class 100 laminar flow hood. The drug substance was filled in 8 mL aliquots using a Flexicon PF6 dispensing pump (Watson-Marlow, DK 4100, Ringsted Denmark) into 10 mL Type I USP clear borosilicate glass vials (Chase Scientific, Vineland, NJ), sealed with gray butyl stoppers (West Pharmaceuticals, Lionville, PA), capped with flip off aluminum caps (West Pharmaceuticals) labeled, and stored at −20 °C. Before use, the vials, butyl stoppers, aluminum flip off caps and forceps were sterilized as per USP <1211> Sterilization and sterility of compendial articles. The sterilized forceps were used to place the stoppers on the vials followed by crimping with a crimper (Genesis Packaging Technologies, Exton, PA). The vials were labeled and stored in a −20 °C freezer. During the fill/finish operations, settling plates and a laser particle counter (Met One Model No. A 2498) were used to monitor both viable and total particle counts for quality control purposes. Sterility was evaluated according to USP <71> standards. Bacteria and yeast growth were monitored for 14 days after inoculation. Testing was repeated 12 months after the initial evaluation for quality assurance and stability.

VB4-845 mass balance

The amounts of VB4-845 in the culture supernatant and in-process purification samples were quantified using a sandwich ELISA. Briefly, 100 μL of sample was incubated in triplicate in a microtiter plate previously coated with affinity purified rabbit anti-PE antibody (Sigma, Catalog No. 2318). After washing, bound VB4-845 was detected with 100 μL of a biotinylated rabbit anti-PE antibody followed with 100 μL of streptavidin-HRP (Fisher, Catalog No. 21126). After washing, TMB substrate (KPL, Catalog No. 50-76-00) was added and the colorimetric reaction measured at 450 nm. The concentration of VB4-845 in the sample was determined by comparison against a standard curve generated with known quantities of VB4-845.

VB4-845 drug product biochemical characterization

Quantity and identity

VB4-845 protein concentration was determined by UV absorbance at 280 nm (Pharmacia Biotech Model Ultrospec® 3000) using a sample diluted in phosphate buffered saline (PBS). The structural integrity of the protein was evaluated using circular dichroism (CD) on a Jasco 715 CD spectropolarimeter instrument fitted with a 1 mL cell. Overlaid CD spectra were obtained in the far UV spectral region (220–250 nm) for the Phase III product and the Phase II reference standard. A comparison of the key spectral signatures was made to ensure comparability of the product.

Purity

VB4-845, at a concentration of 1 μg and 50 ng, was analyzed under reducing and non-reducing conditions on SDS–PAGE gels using an Invitrogen Novex system (Invitrogen Canada Inc, Burlington, ON) and stained with Coomassie Instant Blue (Expedeon Inc., Catalog No. ISB1L) and silver stain (Invitrogen, Catalog No. LC6100), respectively. The gels were analyzed using a Personal Densitometer SI (Molecular Dynamics, Sunnyvale, CA).

For the quantification of protein monomers, aggregates and impurities, size exclusion chromatography was performed using a BioSep-SEC S2000 column (7.8 mm × 30 cm) (Phenomenex, Torrance, CA) on a Waters Alliance HPLC system equipped with a photodiode array detector (Waters, Milford, MA), with a mobile phase consisting of 0.1 M KH_2PO_4/0.1 M K_2HPO_4/0.1 M Na_2SO_4, pH 7.0, at a flow rate of 1 mL/min at 15 L scale versus 0.5 mL/min at the 1000 L scale.

Measurement of host-cell impurities

Endotoxin

Endotoxins were quantified using the BioWhittaker Kinetic-QCL Limulus Amebocyte Lysate (LAL) test kit (Lonza, Catalog No. 50-650H) and following the USP <85> Bacterial Endotoxins Test method. Briefly, 100 μL of sample was incubated with 100 μL of kinetic-QCL reagent in triplicates in a 96-well plate and the presence of gram negative bacterial endotoxins was assessed by the release of p-nitroaniline (pNA). The release of pNA was measured at 405 nm continuously throughout the incubation period using a microplate reader. The concentration of endotoxins in the sample was calculated by comparing its reaction time to that of serially-diluted endotoxin standard curve.

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Host cell proteins

An *E. coli* HCP detection kit (Cygnus Technologies, Catalog No. F410) was used to quantify the amount of HCPs in the VB4-845 samples using an ELISA-based method as per manufacturer's instructions. Briefly, 25 µL of samples in triplicate, were captured on microtiter strips coated with an affinity purified anti-*E. coli* antibody and detected with an anti-*E. coli* antibody coupled to horseradish peroxidase. Excess reactants were removed by washing and tetramethyl benzidine (TMB) substrate added. The amount of hydrolysed substrate was measured on a microplate reader at 450 nm and concentration of HCPs in the sample determined by comparison against a standard curve generated with known amounts of HCP.

Total DNA

The concentration of residual DNA in the drug product was determined by the Threshold Immuno-Ligand Assay (ILA) using the Total DNA Assay Kit, specific for detecting single-stranded DNA (ssDNA) as per instructions (Molecular Devices, Catalog No. R9009). Briefly, 0.5 mL of VB4-845 sample diluted 1:350 with Zero Calibrator (ZC) solution was incubated overnight at 55 °C followed by a denaturation step at 106 °C for 25 min to convert all DNA to single-stranded form. A 1 mL solution containing a mixture of an anti-DNA antibody coupled to Urease, anti-ssDNA binding protein coupled to biotin and streptavidin was added for 1 h at 37 °C. The DNA complex was then captured onto a biotinylated nitrocellulose membrane via one of the free biotin binding sites present on streptavidin. The membranes were analyzed on the Threshold System (Molecular Devices, Sunnyvale, CA) whereby, the enzyme reaction with urea caused a detectable pH fluctuation that was proportional to the amount of DNA present. The amount of DNA in the samples was obtained by interpolating the results from the standard curve generated with the Threshold System software.

Biological characterization

VB4-845 binding was assessed by flow cytometry using the EpCAM positive CAL-27 cell line (ATCC, CRL-2095, American Tissue Type Collection, Manassas, VA). Briefly, tumor cells (0.45×10^6) were incubated with 0.15 µg of VB4-845 drug product for 1.5 h on ice. After washing, bound VB4-845 was detected using a mouse anti-his antibody (GE Healthcare, Catalog No. 27-4710-01) followed by goat anti-mouse FITC (The Binding Site, Catalog No. AF271). Cells were analyzed on a FACS Calibur (Becton Dickson FACScalibur, BD Biosciences, Mississauga, ON) following propidium iodide (Molecular Probes, Catalog No. P1304MP) staining. The results were presented as median fluorescence (MF) compared to PBS.

Cytotoxicity was assessed using WST-1 reagent (Roche, Catalog No. 11 644 807 001). Briefly, 3000–5000 per well of EpCAM-positive and EpCAM-negative cells, CAL-27 and COLO-320, respectively, were seeded in 96-well plates and incubated at 37 °C, 5% CO_2 for 3 h. VB4-845 was then added over a range of concentrations in triplicate. After incubation for 72 h, WST-1 reagent was added and absorbance measured at 450 nm using a microplate reader. Cell viability as a function of concentration was plotted to determine the IC_{50} of the drug product.

Stability

A minimum of three vials were assessed for physical characteristics (appearance, pH), cytotoxic potency (WST-1 cell-based assay), purity (SDS–PAGE), aggregation and impurities (SE-HPLC), binding (flow cytometry), charge variants (isoelectric focusing) and protein concentration (UV spectrophotometry, A_{280}) at time 0, 0.5, 1, 3, 6, 9 and 12 months after storage at 5 °C and −20 °C.

Results

In early phase clinical trials, the upstream microbial fermentation of VB4-845 was based on a low density fed-batch process. Cells were grown in TB and expression of recombinant VB4-845 was triggered at a cell density (OD_{600}) of 20 with a 17% L-arabinose/glycerol feed over a period of 46 h. Using this method, VB4-845 expression in the culture supernatant was ~25 mg/L. As part of the optimization of expression, HCD cultivation using defined medium (GMM) was introduced along with assessment of growth and culture feeding conditions. ELISA analysis of post-induction

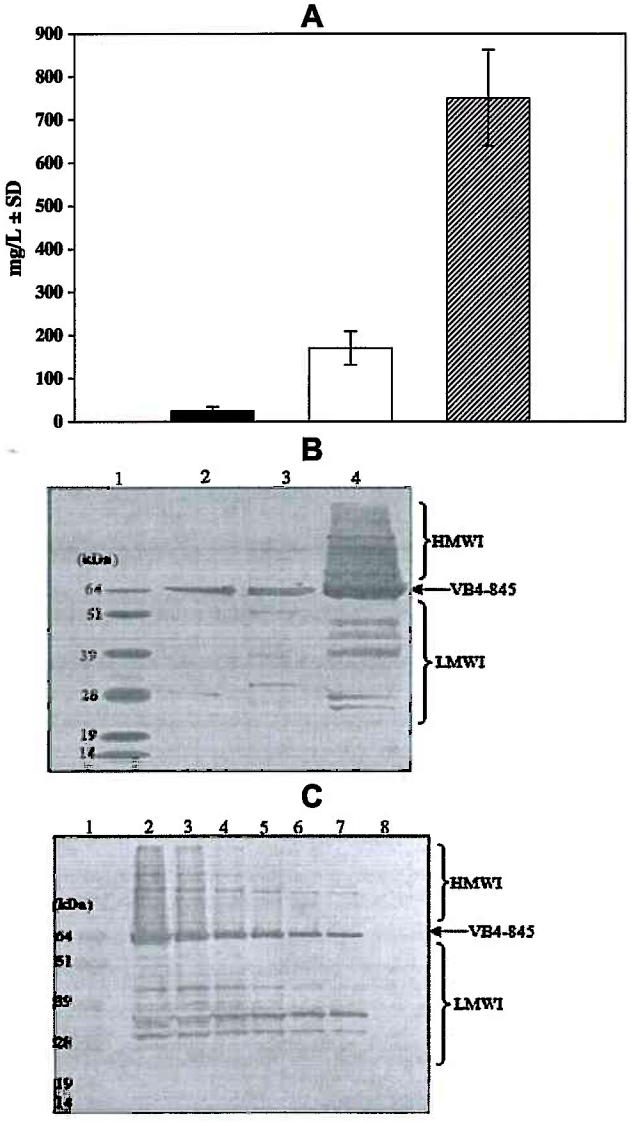

Fig. 1. VB4-845 quantification and Western blot analysis at 15 L scale. (A) Induced supernatants of VB4-845 expressed in TB media (solid bar), VB4-845 expressed in GMM (open bar) and VB4-845-C expressed in GMM (diagonal bar) were collected post-induction and quantified by ELISA in triplicate. (B) Induced supernatant of VB4-845 expressed in TB media loaded neat (lane 2), 1/5 diluted VB4-845 (lane 3) and 1/5 diluted VB4-845-C (lane 4) expressed in GMM and (C) Induced supernatant of VB4-845-C expressed in GMM diluted 1/5 (lane 2), 1/10 (lane 3), 1/20 (lane 4), 1/40 (lane 5), 1/80 (lane 6), 1/160 (lane 7) and neat pre-induced supernatant (lane 8) were loaded under non-reducing conditions and immunoblotted with a rabbit anti-PE antibody followed by a goat anti-rabbit antibody coupled to HRP. Lane 1 was loaded with 10 µL of molecular weight standard. Arrows and brackets indicate the gel bands corresponding to the full-length VB4-845 migrating as a ~64 kDa protein and high and low molecular weight impurities (HMWI and LMWI), respectively.

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samples from a 15 L GMM fermentation run showed an eightfold increase in expression compared to the TB fed-batch process (Fig. 1A). To assess the impact of codon usage on VB4-845 expression, the nucleotide sequence was codon-optimized and pause sequences removed thereby generating a template referred to as VB4-845-C. ELISA analysis of post-induction samples from a 15 L GMM fermentation of VB4-845-C showed a further 4.4-fold increase of titers for an overall 30-fold increase (750 mg/L) over the original TB fed-batch process (Fig. 1A). The reproducibility of the yield for the VB4-845-C was demonstrated over four independent 15 L fermentations and shown to be 763 ± 36 mg/L.

Western blot analysis of the undiluted TB and 1/5 diluted GMM culture supernatants confirmed the increased level of the 64 kDa full-length VB4-845 protein. In addition, concomitant with higher levels of VB4-845-C was the appearance of product-related impurities, including soluble VB4-845 aggregates (Fig. 1B, lane 4). Further analysis revealed that these low and high molecular weight impurities increased with the VB4-845-C product (Fig. 1C). In addition, no bands were detected with the secondary antibody alone demonstrating the specificity of the primary antibody (data not shown).

To ensure reproducibility and consistency in production in accordance with GMP, a MCB and WCB were generated from the parental VB4-845-C clone. Enzyme restriction mapping of the plasmids obtained from the MCB and WCB clones showed no plasmid rearrangements and the DNA templates were identical to the parental clone demonstrating genetic stability of the banks (data not shown). All subsequent development and scale-up studies were done with the qualified WCB.

Downstream process development at the 15 L scale

Primary recovery using Q-Sepharose adsorption – column 1

To capture the VB4-845 protein (pI 5.9), the culture supernatant was loaded onto a strong anion exchanger, a Q-Sepharose column operated at pH 8.0. VB4-845 bound to the column and was eluted with 300 mM NaCl. Of note, the option of capturing VB4-845 on a cation exchanger was ruled out since adjustment of the culture supernatant pH, below the pI of VB4-845 leads to its precipitation. SDS–PAGE analysis (Fig. 2A, lane 3) revealed an enrichment of VB4-845 protein as well as the presence of higher molecular weight impurities (HMWI) and LMWI. Western blot analysis of the Q-eluate with an anti-PE antibody demonstrated that some HMWI and most of the LMWI bands were product-related impurities whereas the non-reacting bands were most likely HCPs (data not shown).

Affinity purification using Ni-charged chelating Sepharose – column 2

The Q-eluate was then loaded on a nickel chelating column which binds VB4-845 via the histidine tags present on both ends of the molecule. To remove the product-related truncated LMWI

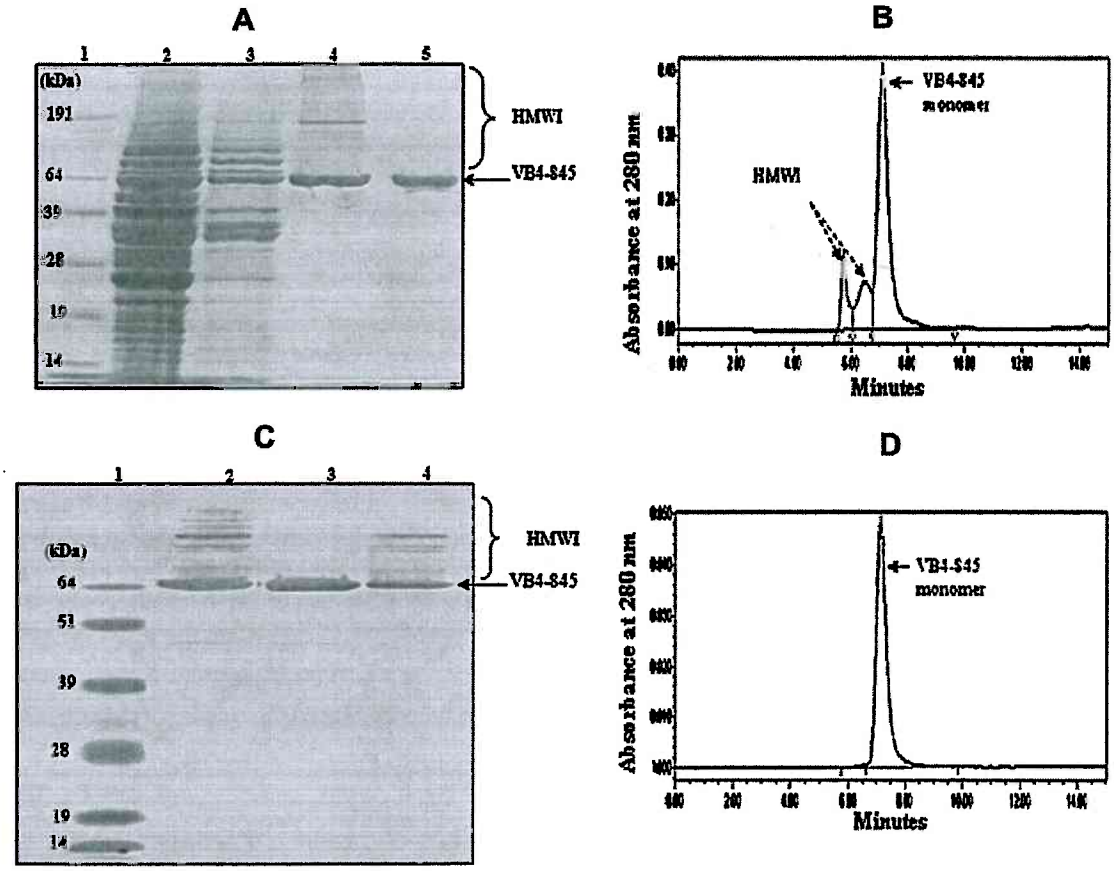

Fig. 2. In-process analysis at the 15 L scale. (A) SDS–PAGE analysis of eluates from columns 1–3. Molecular weight standards (lane 1), diafiltered VB4-845 Phase III supernatant (lane 2), Q-Sepharose eluate, column 1 (lane 3), Ni-chelating eluate, column 2 (lane 4) and Q-Sepharose eluate, column 3 (lane 5) diluted 1/10 in PBS were loaded under non-reducing conditions on an SDS–PAGE gel and Coomassie stained. Arrows and brackets indicate the gel bands corresponding to the full-length VB4-845 protein migrating at ~64 kDa and high molecular weight impurities (HMWI), respectively. (B and D) HMWI detection by SE-HPLC. (B) Ni-chelating eluate (column 2) and (D) Q-Sepharose eluate (column 3) were analyzed using a BioSep 2000 size exclusion column. Solid arrow indicates the VB4-845 monomers and broken arrows indicate HMWI. (C) Western blot analysis demonstrating removal of HMWI on column 3. Molecular weight standards (lane 1), Eluate of the Ni-chelating, column 2, (lane 2), Eluate and strip of the Q-Sepharose, column 3, lanes 3 and 4, respectively, were loaded on an SDS–PAGE gel under non-reducing conditions and immunoblotted with a rabbit anti-PE antibody as described in the materials and methods. The arrow indicates the gel bands corresponding to the full-length VB4-845 protein migrating at ~64 kDa and the bracket shows the HMWI.

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bands likely containing only one histidine tag, the nickel chelating column was washed using a 75 mM imidazole buffer. As expected, SDS–PAGE (Fig. 2A, lane 4) and SE-HPLC (Fig. 2B) analysis of the Ni eluate confirmed the removal of LMWI and most HMWI. Western blot analysis also revealed that the remaining HMWI were product-related aggregates (Fig. 2C, lane 2).

Aggregate removal using Q-Sepharose adsorption – column 3

Separation of the monomeric VB4-845 from its HMWI aggregates was achieved on a third column, a Q-Sepharose column, whereby monomeric VB4-845 protein was preferentially eluted with 200 mM NaCl. SDS–PAGE analysis of this eluate (Fig. 2A, lane 5) and Western blot analysis (Fig. 2C, lane 3) revealed a single band at ~64 kDa and SE-HPLC analysis confirmed the presence of a monomeric peak with a purity >99% (Fig. 2D). Of note, the aggregates were eluted with 1 M NaCl suggesting a stronger ionic interaction compared to the monomeric form (Fig. 2C, lane 4).

Another concern in the purification of soluble protein from *E. coli* supernatant is the presence of high levels of endotoxins and HCPs. In-process sampling over the course of the downstream purification showed that the first anion exchange column removed ~97% of the endotoxins and HCPs (Table 1). Washing of the Ni-chelating column with buffer containing Triton-X100 reduced the remaining endotoxin and HCP load by a further 390 and 88-fold, respectively. The stronger association of endotoxin for the AEX on the third column permitted a further reduction of endotoxin levels by another ~9000-fold to 0.2 EU/mg, well within the ICH guidelines specification of <12.5 EU/mg.

Table 1
In-process testing of endotoxin and HCP levels.

Sample	Endotoxins (EU/mg)	HCP (ng/mg)
Pre-column 1 (SM)	24000,000	4,000,000
Column 1 eluate	731,000	85,000
Column 2 eluate	1871	960
Column 3 eluate	0.2	300
Column 5 eluate	<0.2	18
Overall fold reduction	~120 M	~200 K

However, due to the heterogeneity of the HCPs, a portion co-eluted with VB4-845 under the same salt conditions and as a consequence, the AEX only managed to reduce HCPs by an additional threefold but still did not meet the specification of a 100 ng/mg set for the Phase II process.

Host cell protein reduction using ceramic hydroxyapatite – column 4

Typically, at this stage of the process, the final removal of trace impurities such as HCPs is accomplished using orthogonal separation methods. To that end, attempts to capture HCPs on a cation exchange column such as SP-Sepharose or VB4-845 on different hydrophobic resins were unsuccessful suggesting that the remaining impurities and VB4-845 have similar biophysical properties (data not shown). CHT is a mixed mode support comprising of positively charged calcium and negatively charged phosphate groups that bind proteins via carboxyl clusters and cationic exchange, respectively. To test the possibility that VB4-845 and HCPs would bind differentially to the CHT resin, the Q-Sepharose eluate was loaded directly onto a CHT column previously equilibrated with 20 mM sodium phosphate, 200 mM NaCl, buffer, pH 8.0. Analysis of the effluent showed that VB4-845 was in the flow-through while a significant portion of the HCPs bound to the CHT. Further optimization was achieved by decreasing the pH and phosphate concentration (10 mM sodium phosphate, 200 mM NaCl, pH 7.1) resulting in a 94% reduction in HCPs corresponding to a 17-fold reduction (18 ng/mg), well below the Phase II specification.

Concentration step using Q-Sepharose – column 5

At this point, the endotoxin and HCPs levels were well within the release specifications; however, VB4-845 concentration was below the formulation requirement. Therefore, VB4-845 was applied onto a Q-Sepharose column and eluted with 500 mM NaCl to a final concentration of ~9 mg/mL. Note, the use of TFF to concentrate the drug substance at this stage of the process was not suitable as it led to unacceptable levels of aggregate. Overall, the downstream process yielded 0.15 g/L of VB4-845 drug substance corresponding to a 40% recovery and effectively reduced the endotoxin levels by 120 million-fold and the HCPs burden by 200,000-fold.

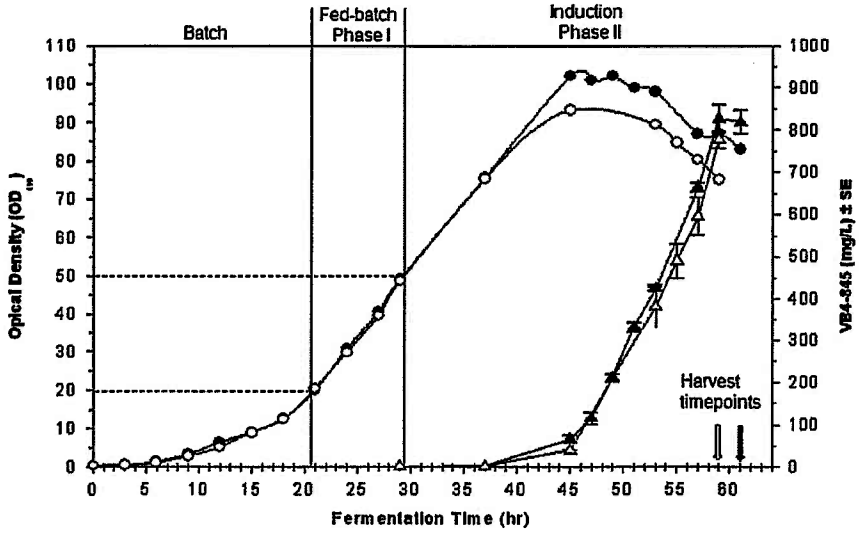

Fig. 3. Growth and expression kinetics for the 15 L (solid symbols) and 1000 L scale (open symbols). Representative examples from three and four independent fermentations at the 15 L and 1000 L scale, respectively are shown. OD_{600} measurements are indicated by circles and VB4-845 supernatant titres by triangles. Arrows indicate the harvest time for the 1000 L and 15 L fermentations.

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Table 2
Summary of VB4-845 downstream recovery at 1000 L scale.

Column	Column recovery (g)[a]	Column yield (%)[b]	Accumulated yield (%)
Q-Sepharose fast flow	63.2	81.0	81.0
Ni^{2+}-charged chelating Sepharose	56.1	88.8	71.9
Q-Sepharose	43.8	78.1	56.1
Ceramic hydroxyapatite	32.3	73.7	41.3
Q-Sepharose[c]	31.3	97.0	40.1

[a] 78.0 g of VB4-845 as estimated by ELISA was loaded onto the first column.
[b] Calculated as a percent of the total protein loaded.
[c] Concentration measured by A_{280}.

Table 3
Determination of drug substance host cell impurities.

Test	Specification	Phase I/II	Phase III
Total DNA (pg/mg)	⩽330	39.8 ± 28.4	27.8 ± 13.8
HCP (ng/mg)	⩽100	18.0 ± 1.5	10.0 ± 1.7
Endotoxins (EU/mg)	⩽25	7.8 ± 1.2	0.3 ± 0.2

Samples were taken from drug substance (formulated API). HCP, significant difference between levels measured in Phase III ($n = 3$) compared to Phase I/II ($n = 3$) ($p = 0.026$, Student's t-test). Endotoxin levels a significant difference ($p = 0.003$) between Phase I/II ($n = 18$) and Phase III ($n = 4$) material as determined by Mann–Whitney rank sum test. Total DNA levels ($p = 0.693$) were not significantly different by Student's t-test.

Clinical production-1000 L scale

At the 1000 L scale, VB4-845 cultures were induced with L-arabinose for 30 h. Growth profiles and expression kinetics at the 1000 L scale are shown in Fig. 3 and are similar to the 15 L scale. The OD_{600} of the culture reached a plateau of ~100 after 45 h where it remained constant for 8 h, and then slowly declined thereafter. VB4-845 was detected after 7 h post-induction and the level continued to increase until harvest. Of note, to minimize membrane fouling during microfiltration and thus maximizing product recovery, the 1000 L scale was harvested 2 h earlier compared to the 15 L scale.

Mass balance of VB4-845 was monitored at all in-process steps by ELISA and by absorbance (A_{280}) on the final purification step. VB4-845 expression in the culture broth upon harvest was estimated by ELISA to be ~0.75 g/L. Of note, acetate levels were determined at end of batch and fed batch phases and every three hours during the induction phase. Acetate accumulation peaked at 0.16 g/L at the end of batch phase, well below levels considered to be inhibitory to cell growth [22,23]. The low acetate accumulation was expected since glycerol was the carbon source, and the amount added to the culture was tightly regulated via the pO_2 loop [24–26].

Due to cost considerations regarding column resins, direct scaling of the columns to purify VB4-845 in a single run was not done. Instead, in order to manage the higher expression levels with the GMM HCD cultivation process, the diafiltered material was purified in batches. Non-purified material can be stored at −20 °C for up to six months without any affect on drug quality (data not shown). For batch processing, dynamic binding capacity (4 g/L) estimated the optimal protein load for the first column (Q-Sepharose) to be 78 g. Representative data from a single 78 g batch showed the percent yield per column to vary between 73% to 97% depending on the column (Table 2). Taken together, this translated into an overall recovery of ~40% similar to the 15 L scale.

Host cell-related impurities were measured at the drug substance stage of the process to confirm the levels were in accordance with the ICH guidelines. All host cell-related impurities were within the permissible specifications; however, endotoxin and HCP levels were significantly lower with the Phase III process compared to the Phase II reference standard (Table 3). In contrast, the amount of Total DNA detected was similar regardless of the process used.

Drug product comparability studies

Biochemical characterization

Comparability studies were conducted to demonstrate the high degree of similarity between VB4-845 reference standard and Phase III product, a regulatory requirement once a manufacturing change has been made.

VB4-845 Phase III drug substance was formulated to 5.2 mg/mL in 20 mM $NaPO_4$, 0.1% polysorbate 80, pH of 8.0 and full release testing completed prior to filling. To create drug product, drug substance was filled at a volume of 8 mL and stored at −20 °C. On visual inspection, the vialed drug product was clear and colorless to slightly yellow, with essentially no visible particulates after thawing. As expected, under reducing conditions, Coomassie staining of VB4-845 Phase III drug product produced by the GMM HCD process showed a single band at ~64 kDa that was indistinguishable from a VB4-845 reference standard prepared using the TB fed-batch process (Fig. 4A, compare lanes 2 and 3 vs. lanes 4 and 5).

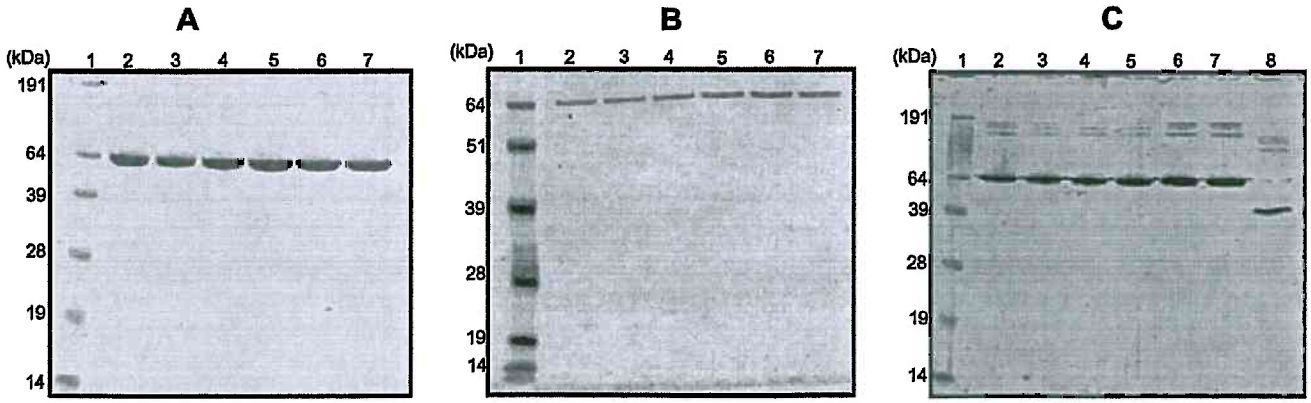

Fig. 4. Biochemical characterization. (A) A total of 1 μg was loaded under non-reducing conditions on an SDS–PAGE gel and Coomassie stained. (B) 50 ng was loaded under reducing conditions and (C) non-reducing conditions on SDS–PAGE gels and immunoblotted with a rabbit anti-PE antibody and silver stained, respectively, as described in materials and methods. Molecular weight standard (lane 1), VB4-845 reference standard (lanes 2 and 3), VB4-845 Phase III drug product (lanes 4 and 5), VB4-845 reference standard and VB4-845 Phase III drug product mixed 1:1 (lanes 6 and 7). BSA, 50 ng was loaded in lane 8 on the silver stained gel.

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The similarity in molecular weight was further demonstrated upon mixing the reference standard with the VB4-845 Phase III drug product (Fig. 4A, lanes 6 and 7) which still resulted in a single band. The identity of VB4-845 band was confirmed by Western blot analysis under reducing conditions (Fig. 4B). In addition, the Western blot analysis also revealed that no low or high molecular weight drug-related fragments were detected with anti-PE antibody or with an antibody specific for the scFv moiety (data not shown). Densitometry analysis of the Coomassie gel showed that the purity of product generated by both processes was in excess of 99% (Fig. 4A). However, under non-reducing conditions, silver stain SDS–PAGE gel of VB4-845 Phase III drug product showed the presence of higher molecular aggregates estimated at 3% by SE-HPLC (Fig. 5). This amount was less than that observed with the reference standard (Fig. 4C, lanes 2 and 3) which yielded ~6% aggregates on average by SE-HPLC, and therefore not significantly different when compared to the Phase III process.

To ensure the structural integrity of the material, CD profiles of both the TB and GMM HCD material were generated. Both lots exhibited similar profiles with a typical negative CD band at ~234 nm indicating no structural differences between products (Fig. 6).

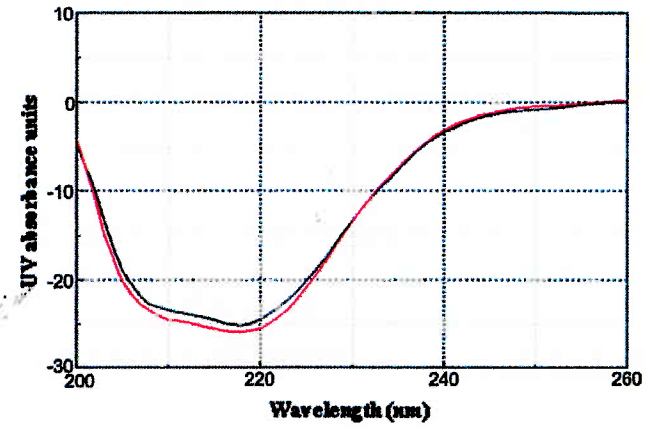

Fig. 6. Structural analysis. Overlay of the CD spectra of VB4-845 reference standard (black) and VB4-845 Phase III drug product (red).

Biological characterization

The biological activity of the VB4-845 Phase III drug product was analyzed by flow cytometry and WST using the EpCAM positive CAL-27 cell line, and compared to the VB4-845 reference standard. Flow cytometry, measuring the cell-surface reactivity of VB4-845 generated by the TB fed-batch and GMM HCD processes, demonstrated that the binding histograms were identical (Fig. 7A). Similarly, representative cytotoxicity curves of material from each of the processes (Fig. 7B) showed overlapping profiles with similar IC_{50}. In contrast, no IC_{50} value was obtained with the EpCAM-negative tumor cell line, COLO-320, demonstrating the specificity of VB4-845.

Stability

To ensure that quality, purity and potency is maintained during storage (−20 °C), drug product is being tested as part of a long term stability program to assess structural integrity and potency. After 12 months, the potency and percent monomer for three separate lots of VB4-845 Phase III drug product was maintained (data not shown).

Discussion

This report describes the development of a Phase III microbial manufacturing process for the production of VB4-845, an immunotoxin under investigation for the treatment of NMIBC. A TB medium fed-batch process was used in the manufacture of VB4-845 for early phase clinical trials; however, this process did not provide sufficient drug to support a pivotal Phase III trial. While further scale-up studies of this process were considered, the cost of goods proved prohibitive thus necessitating the development of a new process.

In an effort to increase VB4-845 titers in the culture medium, two complementary strategies were implemented. The first consisted of cultivating the host in a defined medium (GMM) in order to achieve higher cell density where cultivation conditions were optimized. The second involved optimizing the VB4-845 coding sequence with respect to type, frequency and host codon usage. Taken together, the changes resulted in a 30-fold increase in expression levels in the supernatant at the 15–1000 L scale (from 25 mg/L to 750 mg/L).

To purify VB4-845 product and remove associated impurities in the culture broth, a scalable five-column downstream process (Q-Sepharose, Ni-chelating, Q-Sepharose, CHT and Q-Sepharose) was used. Q-Sepharose as the initial column was effective in capturing the bulk of VB4-845 from the supernatant while successfully removing the majority of the two key process-related impurities, endotoxin and HCP. The high binding affinity of endotoxin for Q-Sepharose permitted the differential elution of VB4-845 at a lower salt concentration [27–29]. Since Ni-chelating was less efficient at reducing endotoxin and HCP levels, Q-Sepharose was the method of choice for primary capture. Nonetheless, Ni-chelating was used as second capture column where a further reduction of endotoxin was achieved with a wash buffer containing Triton-X100. The addition of detergents in the wash buffer, including Triton-X100, has been used successfully with other proteins captured by nickel affinity chromatography [30–32].

Whereas endotoxins bind strongly to the Q-Sepharose column, the bulk of the HCPs flows through. However, a minor,

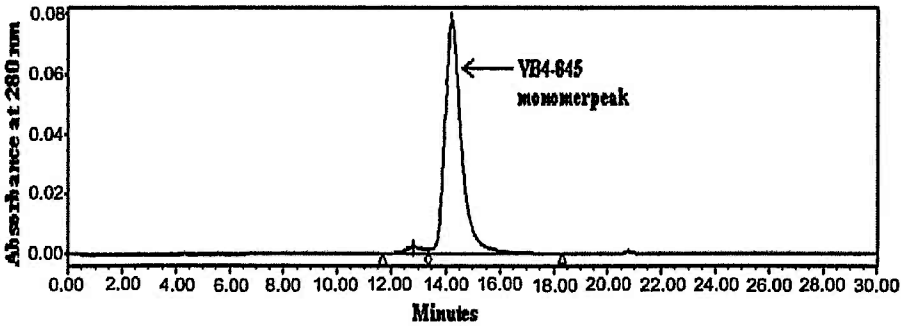

Fig. 5. SE-HPLC analysis of the VB4-845 Phase III drug product produced at the 1000 L scale. Representative chromatographic profile of the drug product analyzed on a BioSep 2000 size exclusion HPLC column at a flow rate of 0.5 mL/min. Arrow indicates the VB4-845 monomer peak.

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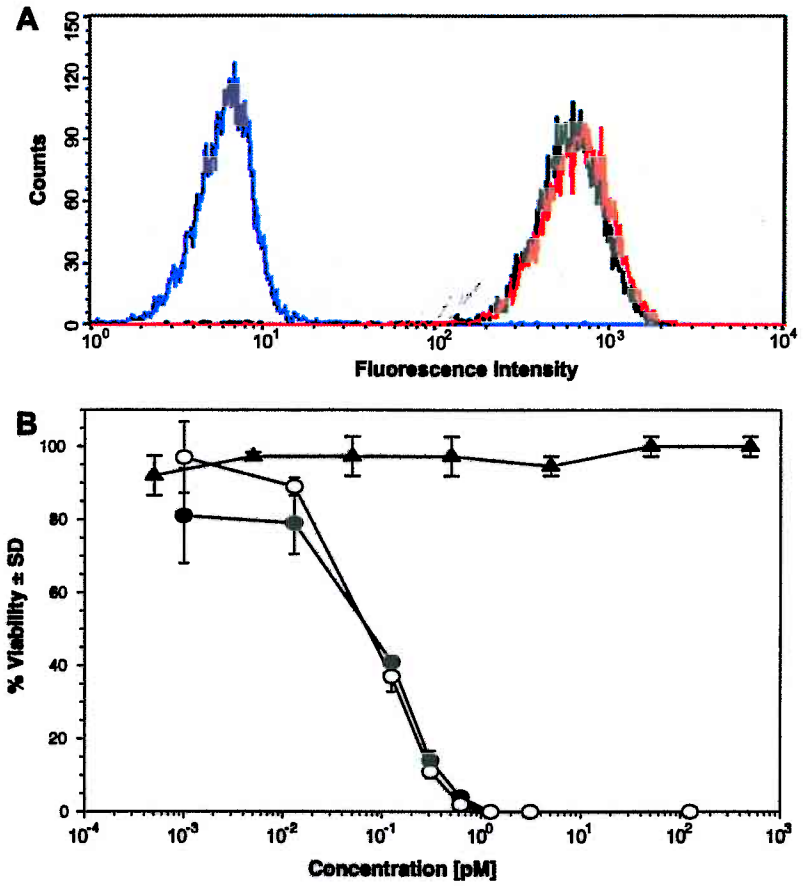

Fig. 7. Biological reactivity. (A) Cell surface binding. VB4-845 Phase III drug product (red) and VB4-845 reference standard (black) binding to EpCAM-positive CAL-27 cells tested at 0.15 μg was detected by flow cytometry. Blue line represents a PBS control. (B) Cytotoxicity. VB4-845 Phase III drug product (solid circle) and VB4-845 reference standard (open circle) were added to CAL-27 cells and in a 96-well plate and viability measured after 3 days using WST-1 reagent. As a negative control, VB4-845 reference standard (solid triangle) was incubated with EpCAM-negative, Colo 320 cells. The results are expressed as % of the untreated control wells. The data is representative of three independent experiments.

but significant HCP fraction did co-elute with VB4-845 after the second Q-Sepharose column. Since the use of a cation exchanger was unfeasible due to the pI of VB4-845, CHT characterized as a mixed-mode resin was assessed. Generally, CHT is used to remove aggregates but more recently its usage has been extended to HCP removal [33]. Typically, CHT is a bind and elute step where product is separated from impurities by salt gradient elution. However, in the present study, the optimal separation was obtained by HCPs binding to the CHT column while the bulk of VB4-845 was in the flow-through.

With respect to product-related impurities, the Ni-chelating column was efficient at removing low molecular weight derivatives of VB4-845 while the second Q-Sepharose column effectively separated aggregates from the monomer. In addition, the low salt concentration not only permitted the separation of monomer from aggregate, but also further reduced endotoxin levels and consequently was preferred to the classical size exclusion approach.

Establishing the conditions at each orthogonal step was based on product quality and gave an overall recovery from the downstream purification of 40%. In spite of a 30-fold increase in VB4-845 expression, the overall process recovery represented only a 10-fold increase in yield over the Phase II process. A higher yield was unachievable as 50% of the product remained in the retentate at the microfiltration step due to cellular debris present in the centrate. Studies are planned to reduce the OD_{600} of the centrate to improve final recovery.

Analysis of purity, potency, biological activity and higher order structure showed that the VB4-845 drug product produced by the GMM HCD and TB Fed-batch processes were comparable. In the case of purity, an even higher level was observed for the VB4-845 from the Phase III process as fewer aggregates were present. In conclusion, the GMM HCD process was effective in generating sufficient VB4-845 drug product for late stage clinical development and commercial production.

Acknowledgments

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