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Production and quality control assessment of a GLP-grade immunotoxin, D2C7-(scdsFv)-PE38KDEL, for a Phase I/II clinical trial

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Abstract

D2C7-(scdsFv)-PE38KDEL (D2C7-IT) is a novel recombinant *Pseudomonas* exotoxin A-based immunotoxin (IT), targeting both wild-type epidermal growth factor receptor (EGFRwt) and mutant EGFR variant III (EGFRvIII) proteins overexpressed in glioblastomas. Initial preclinical testing demonstrated the anti-tumor efficacy of D2C7-IT against orthotopic glioblastoma xenograft models expressing EGFRwt, EGFRvIII, or both EGFRwt and EGFRvIII. A good laboratory practice (GLP) manufacturing process was developed to produce sufficient material for a Phase I/II clinical trial. D2C7-IT was expressed under the control of the *T7* promoter in *Escherichia coli* BLR (λ DE3). D2C7-IT was produced by a 10 L batch fermentation process and was then purified from inclusion bodies using anion exchange, size exclusion, and an endotoxin removal process that achieved a yield of over 300 mg of purified protein. The final vial batch of D2C7-IT for clinical testing was at a concentration of 0.12 ± 0.1 mg/mL, the pH was at 7.4 ± 0.4 , and endotoxin levels were below the detection limit of 10 EU/mL (1.26 EU/mL). The stability of the vial batch of D2C7-IT has been monitored over a period of 42 months through protein concentration, sodium

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Compliance with Ethical Standards

Conflict of interest

Author1 declares that she has no conflict of interest. Author2 declares that he has no conflict of interest. Author3 declares that she has no conflict of interest. Author4 declares that he has no conflict of interest. Author5 declares that he has no conflict of interest. Author6 is inventor on immunotoxin patents, all of which have been assigned to NIH. Author7 owns stock in Istari Oncology and is a consultant to Genetron Health.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent

For this type of study, formal consent is not required.

dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), isoelectric focusing, size exclusion chromatography, cytotoxicity, sterility, and pH measurements. The vialled D2C7-IT is currently being tested in a Phase I/II clinical trial by intratumoral convection-enhanced delivery for 72 h in patients with recurrent glioblastoma (NCT02303678, D2C7 for Adult Patients with Recurrent Malignant Glioma, clinicaltrials.gov).

Keywords

good laboratory practice; recombinant immunotoxin; malignant glioma; epidermal growth factor receptor; mutant EGFR variant III

Introduction

The epidermal growth factor receptor (EGFR), a 170-kDa, transmembrane glycoprotein, is a member of the ErbB/EGFR family of receptor tyrosine kinases (RTKs). The overexpression of EGFR is reported in head and neck cancer (Rubin Grandis et al. 1996), breast cancer (Klijn et al. 1992), ovarian carcinoma (Bartlett et al. 1996), lung cancer (Pavelic et al. 1993), prostate cancer (Fox et al. 1994), and brain tumors (Libermann et al. 1984; Arita et al. 1989). Compared to normal control brain specimens, a 300-fold overexpression of EGFR is observed in glioma (Libermann et al. 1985). Comprehensive molecular analyses have identified mutation, rearrangement, altered splicing, and/or focal amplification of *EGFR* in 57% of glioblastoma patients (Brennan et al. 2013). In the absence of gene amplification, EGFR protein overexpression has also been demonstrated in glioblastoma (Chaffanet et al. 1992).

The *EGFR* gene amplification is often associated with gene rearrangements. The most common rearrangement is the *EGFR variant III* (*EGFRvIII*) mutant, which is present in 67% of glioblastomas with *EGFR* amplification (Frederick et al. 2000). The high prevalence of EGFR fusion and deletion variants in glioblastomas necessitates the development of a therapeutic strategy that will target the different EGFR alterations that exist concurrently in a tumor. An agent targeting multiple variants of EGFR is expected to have a major impact on the survival of glioblastoma patients.

Pseudomonas exotoxin A (PE), secreted by *Pseudomonas aeruginosa*, is a potent toxin that can be chemically or genetically fused to monoclonal antibodies (mAbs) or mAb fragments targeting tumor-specific proteins (Weldon et al. 2011). These mAb-toxin chimeras specifically targeting and eliminating tumor cells are called immunotoxins (ITs).

Recombinant ITs are produced by genetically fusing a 38-kDa truncated mutant form of PE, PE38KDEL, to the single-chain variable-region antibody fragments (scFvs), consisting of the heavy- and light-chain variable regions (VH and VL) (Seetharam et al. 1991). D2C7-(scdsFv)-PE38KDEL (D2C7-IT) is a novel recombinant IT with dual specificity for the EGFRwt and mutant EGFRvIII proteins (Chandramohan et al. 2013). In preclinical studies, the dual-specific immunotoxin D2C7-IT demonstrated a strong anti-tumor response against intracranial glioblastoma xenografts expressing either EGFRwt (prolonged survival by 310%, $P = 0.006$) or both EGFRwt and EGFRvIII (prolonged survival by 166%, $P = 0.001$) (Chandramohan et al. 2013). D2C7-IT (single dose) is currently being evaluated in a Phase I

clinical trial (NCT02303678) to determine the maximum tolerated dose when delivered intratumorally by convection-enhanced delivery for 72 h to recurrent grade III (dose escalation only) and IV (dose escalation and dose expansion) malignant glioma patients and to determine the optimal dose for a subsequent single-arm Phase II trial.

In this report, we describe the production, purification, and stability testing processes of the clinical grade D2C7-IT, which was manufactured under good laboratory practice (GLP) conditions.

Materials and methods

Production of D2C7-IT was performed by the Antibody Engineering and Antibody Therapeutics (AEAT) Program personnel at the Duke University Medical Center AEAT Production Facility. All materials were purchased at the highest purity possible and certificates of analysis from different manufacturers were retained. All equipment was cleaned and tested in accordance with GLP guidelines. All of the AEAT Program personnel were trained on and followed the GLP guidelines during production of the clinical grade D2C7-IT.

D2C7-IT strain construction

Plasmid (pRB199-D2C7-scdsFv-PE38KDEL) expressing D2C7-IT has been previously described (Chandramohan et al. 2013). The pRB199-D2C7-scdsFv-PE38KDEL plasmid was transformed into the expression host *E. coli* BLR (λ DE3) (Novagen-EMD Millipore, Billerica, MA) and positive clones were selected by chloramphenicol resistance. Ten colonies were picked and inoculated into tubes (11CT-20CT) containing 3 ml of Luria-Bertani (LB) broth with 75 μ g/ml chloramphenicol and 12.5 μ g/ml tetracycline. All clones were incubated overnight at 37°C in a rotary shaker at 250 revolutions per minute (rpm). Two hundred microliters of clones 11CT-20CT were seeded into tubes with 3 ml of Turbo Prime-olate media (Athena Environmental Sciences, Inc., Baltimore, MD) with chloramphenicol and tetracycline and incubated at 37°C in a rotary shaker at 250 rpm. After 2 hours of incubation, 3 ml of 40 % glycerol was added to all the tubes and six-1 ml aliquots were stored frozen at –80°C (Accession Cell Bank [ACB] clones). One frozen ACB vial of cells per clone was thawed and 300 μ l of the bacterial stock was added to a tube containing turbo prime-olate media with chloramphenicol and tetracycline and incubated for 6 hours at 37°C in a rotary shaker at 250 rpm. Cultures were induced with 1 mM isopropyl β -D-thiogalactoside (IPTG) overnight at 37°C in a rotary shaker at 250 rpm and inclusion body pellets were analyzed in a NuPage 4-12% Bis-Tris gels (Thermo Fisher Scientific, Waltham, MA) for protein expression.

Production of D2C7-IT master cell bank

One ACB vial of clone 18CT (DsD2C7-PE38-KDEL Clone 18CT BLR[DE3]) was thawed into 25 ml of Animal Product Free Terrific Broth media (Teknova, Hollister, CA) and incubated in a rotary shaker overnight at 250 rpm at 37°C. The following day, 25 ml of the overnight culture of clone 18CT was added to 150 ml of fresh Animal Product Free Terrific

Broth media and incubated in a rotary shaker at 250 rpm at 37°C until the OD₆₀₀ was 0.845. Master cell bank (MCB) vials of D2C7-IT (DsD2C7-PE38-KDEL MCB of clone 18 in *E. coli* BLR[DE3] 3-13-12) were prepared by combining 800 µl of clone 18CT bacterial culture with 200 µl of 100% glycerol per vial.

The following quality control testing of the D2C7-IT MCB was performed by Charles River Biopharmaceutical Services, Malvern, PA; Pennsylvania State University-Gastroenteric Disease Center, University Park, PA; and Genewiz, South Plainfield, NJ:

1. **Bacterial purity:** The purity of the D2C7-IT MCB microbial culture was determined through growth in differential agars, viability testing of microbial suspension, and gene copy number analysis by amplification of specific nucleic acids utilizing fluorescent probes (Charles River Biopharmaceutical Services).
2. **Bacterial identity:** The strain of *E. coli* used for MCB production was verified through pulse field gel electrophoresis (PFGE) (Pennsylvania State University), comparative sequence analysis of genomic DNA encoding Gyrase B, and retention of selectable markers in transformed host cell lines (Charles River Biopharmaceutical Services).
3. **Plasmid identity:** Identity of the transformed pRB199-D2C7-scdsFv-PE38KDEL plasmid was confirmed through the retention of the recombinant construct in transformed *E. coli* cell lines, restriction endonuclease analysis of the plasmid DNA by agarose gel electrophoresis and ethidium bromide staining (Charles River Biopharmaceutical Services), and isolation and sequencing of the pRB199-D2C7-(scdsFv)-PE38KDEL plasmid from the master cell bank by Genewiz (South Plainfield, NJ).
4. **Safety:** The safety of the MCB was established by the testing for Shiga-like toxin/toxin genes (Pennsylvania State University), the induction of bacterial virus from *E. coli* using Mitomycin C, and the ultrastructural evaluation of fluid samples for bacteriophage particles by negative staining (Charles River Biopharmaceutical Services).

D2C7-IT fermentation

One frozen MCB vial was thawed and 0.2 mL of bacterial cells were inoculated into 2.0 mL of SOC media and incubated for 2 hours at 220 rpm and 37°C. The cells were then plated on ten LB-chloramphenicol (75 µg/ml) plates and incubated at 37°C overnight. The ten plates of grown bacteria were inoculated into two 2-L shake flasks, each containing 500 mL of sterile, prepared Animal Product Free Terrific Broth media (Teknova) supplemented with 4.1 mM MgSO₄, 0.5% glucose, and chloramphenicol at 34 µg/mL. The inoculated seed flasks were incubated at 230 rpm and 37°C. When the optical density at 600 nm (OD₆₀₀) of seed culture reached an OD₆₀₀ ≈ 1, both the seed flasks were pooled to yield a 12.5% inoculum for the fermentor.

A 10-L fermentor was prepared for fermentation of the D2C7-IT by batching the fermentor with 7-L of Terrific Broth media, 2% glucose, 0.05% magnesium sulfate, and 34 µg/mL

chloramphenicol. The final volume in the fermentor was 8-L. The fermentor was set to culture parameters as follows: Agitation: cascade; Temperature: 37°C (36.5-37.5°C); pH: 7.0-7.2; Dissolved Oxygen: $\approx$ 70%; Antifoam: as required. The prepared media in the fermentor was inoculated with the prepared D2C7-IT seed culture. The D2C7-IT fermentation culture was sampled hourly for OD₆₀₀ measurement. D2C7-IT expression was induced with 1 mM IPTG for 2 h when the OD₆₀₀ reached 6 ± 1 (Figure 1). Cells were harvested by centrifugation and the paste was stored at -20°C overnight or until lysis.

Inclusion body preparation

Thawed cell paste (30 gms each) was resuspended in 100 mL of 50mM Tris-HCl pH 7.5 buffer and dispersed through homogenization. Next, 0.5 mL of 1M MgSO₄ (5mM final concentration), 6000 units of DNase I, and 300 mg of Lysozyme (10 mgs/gm bacterial pellet) were added to the dispersed cell mixture and incubated at room temperature for 1 hour. Twelve mL of 5M NaCl (500mM final concentration) and 9.6 mL of 25% Triton X-100 buffer (2% final concentration) were added to the bacterial lysate, which was then homogenized and incubated at room temperature for 30 minutes. Finally, 4 mL of 0.5M ethylenediaminetetraacetic acid (EDTA) (16.7 mM final concentration) was added to the bacterial lysate and the final volume was adjusted to 200 mL with 2% Triton-X 100 in TE 50/20 buffer (50mM Tris-HCl pH 7.5 and 20 mM EDTA), dispersed thoroughly by homogenization, and centrifuged at 10,000 rpm at 4°C for 50 min. The bacterial pellets were washed (200 mL total volume) twice with 2% Triton-X 100 in TE 50/20 buffer and three times with TE 50/20 buffer. During each wash step, the bacterial pellet was thoroughly dispersed through homogenization followed by centrifugation, at 10,000 rpm at 4°C for 50 min. The final inclusion body pellets were labeled and stored at -20°C until solubilization.

Inclusion body solubilization and D2C7-IT refolding

Individual inclusion body pellets were dissolved in solubilization buffer (6M Guanidine-HCl) overnight at room temperature on a shaker. The solubilized inclusion body was centrifuged at 16,000 rpm at 4°C for 50 min. The resulting supernatant was collected and the protein concentration was determined by Pierce Coomassie Plus (Bradford) Assay Kit (Thermo Fisher Scientific). The solubilized inclusion body was then diluted to a concentration of 10 mg/mL using an appropriate volume of the solubilization buffer. Solubilized D2C7-IT was then reduced by the addition of dithioerythritol (DTE; Sigma, St. Louis, MO) to 10 mg/mL for the final concentration and incubated for 16 h at room temperature with gentle shaking. Reduced D2C7-IT was slowly diluted 100-fold into refolding buffer (100 mM Tris-HCl pH 8.0, 0.5 M L-arginine-HCl, 0.9 mM oxidized form of glutathione, 2 mM EDTA, 2 ml of Protease Inhibitor/L [Sigma]; pH 10.3) with gentle mixing. The refolding reaction was allowed to proceed for 72 hours at 2-8°C without agitation.

Dialysis, filtration, and purification of D2C7-IT

The refolded protein solution (3-5 L) was dialyzed against 50 liters of dialysis buffer (100 mM Urea, 20 mM Tris-HCl, pH 7.5) overnight at 2-8°C. The dialyzed retentate was filtered through a 0.22 µm bottle top filter for subsequent purification.

A High Resolution (HR16/10) column (GE Healthcare) was packed with Q-Sepharose High Performance (HP) anion exchange resin (GE Healthcare) and had a packed bed volume of 20 mL. The Q Sepharose HP column was equilibrated with ten column volumes (CVs) of Buffer A (20mM Tris-HCl, pH 7.5) until the conductivity of the effluent was within 2 mS/cm of the equilibration buffer. The column was then run at a flow rate of 6 mL/min. The D2C7-IT Q-Sepharose HP Load (4-6 L of dialyzed and filtered protein solution) was applied to the Q-Sepharose HP column, and the flow-through was collected. After washing the Q-Sepharose HP column with five CVs of Buffer A (20 mM Tris-HCl, pH 7.5), the D2C7-IT was eluted (flow rate, 4 mL/min/fraction; total runtime, 60 min) using a linear gradient of Buffer B (20 mM Tris-HCl, pH 7.5, 1M NaCl), with tubes 1-10 collecting fractions with 0 – 10% buffer B, tubes 11-50 collecting fractions with 10% - 50% buffer B, and tubes 51-60 collecting fractions with 50% - 100% buffer B. The main D2C7-IT-containing fractions eluted from Q-Sepharose HP chromatography were pooled based on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis and processed for High Performance Liquid Chromatography-Size Exclusion Chromatography (HPLC-SEC) purification.

Pooled Q-Sepharose D2C7-IT Q-Sepharose fractions were concentrated using a 30 kDa molecular weight cut-off (MWCO) Vivaspin 20 ultrafiltration device (Sartorius, Bohemia, New York). Pooled D2C7-IT fractions were concentrated to approximately 4 to 6 mg/mL and dialyzed (using autoclaved Spectra/Por 25 kDa MWCO dialysis tubing) overnight against filter sterilized (VacuCap filters, Pall Corporation, Port Washington, NY) endotoxin low TSK buffer (70 mM Na₂HPO₄, 62.5 mM NaH₂PO₄, and 100 mM Na₂SO₄, pH 6.7), which was prepared using sterile intravenous injection water. Dialyzed D2C7-IT was then filtered through a 0.2 µm Millex GV filter (EMD Millipore) and run on a TSKgel SuperSW3000 (particle size 4µm, 21.5mm × 30cm; Tosoh Bioscience, King of Prussia, PA) gel filtration column equilibrated with TSK buffer. The column was run at a flow rate of 2 mL/min (total runtime 60 min) and when a stable base line was obtained, a 1 mL D2C7-IT sample was injected and 30-second fractions were collected under sterile conditions. The main D2C7-IT-containing fractions eluted from the TSKgel SuperSW3000 gel filtration column were pooled based on SDS-PAGE analysis, concentrated, and filtered through a 0.2 µm Millex GV filter for endotoxin removal.

A 1 × 10 cm Econo-Column chromatography column (Bio-rad, Hercules, California) was packed with ActiClean Etox resin (Sterogene, Carlsbad, California). The column was equilibrated with 10 CVs of 1N NaOH and incubated over night at 4°C. The column was then rinsed with 20 CVs of sterile intravenous injection water and equilibrated with 5 CVs of TSK buffer (filtered through 0.2 µm Acrodisc Units with Mustang E Membrane for endotoxin removal; Pall Corporation). Concentrated D2C7-IT was passed over the ActiClean Etox column at a flow rate of 1 mL/min for endotoxin removal. The D2C7-IT

eluate from the ActiClean Etox column was then dialyzed overnight at 4°C against Cell Therapy Systems DPBS CTS without Calcium Chloride without Magnesium Chloride (Thermo Fisher Scientific). Purified individual bulk lots of D2C7-IT were transferred to 50 mL conical tubes, labeled, and stored at –80°C.

Preparation of final purified bulk D2C7-IT product

The final purified bulk product was made by combining purified intermediate bulk lots. Individual purified intermediate bulk lots were filtered through a 0.2 µm Millex GV filter and combined into one purified bulk lot. The protein concentration of the bulk lot was assessed by Pierce Coomassie Plus (Bradford) Assay and the volume was adjusted to yield a final concentration of approximately 120 µg/mL using DPBS CTS pH 7.4.

D2C7-IT analytical release testing

The analytical tests performed on the final purified bulk D2C7-IT product for release to Compounding Pharmacy for vialing are described below.

Color, appearance, and clarity by visual inspection

The final purified bulk D2C7-IT product was assessed by visual inspection to determine if the soluble product in the liquid formulation was free from any particulate matter, opaqueness, and/or tint or turbidity of the solution.

Identity by SDS-PAGE

SDS-PAGE (non-reduced and reduced) analysis was performed using NuPAGE Novex 4-12% Bis-Tris pre-cast polyacrylamide gels (Thermo Fisher Scientific) to estimate the molecular weight and identity of the final bulk D2C7-IT product. The final bulk D2C7-IT product ran on NuPAGE gel was visualized by staining with GelCode Blue Stain Reagent (Thermo Fisher Scientific). Molecular weight standards (Precision Plus Protein Dual Color Standards; Bio-Rad) were included on the same gel in order to determine the molecular weight of the purified bulk product through relative electrophoretic mobility comparison with that of the standards.

Protein concentration by pierce coomassie plus (Bradford) assay

Pierce Coomassie Plus (Bradford) Assay was used to determine the protein concentration of the final purified bulk D2C7-IT product. Bovine Serum Albumin (BSA) was used to prepare a known protein concentration reference standard in the same diluent as the final purified bulk D2C7-IT product. Diluted standards and the D2C7-IT sample were added to the Pierce Coomassie Plus reagent, incubated at ambient temperature for 5 – 10 minutes, and the optical density of the samples were measured at 595nm in a spectrophotometer. The protein concentration of the final purified D2C7-IT product was determined using the BSA reference standard curve.

Potency testing by cytotoxicity in NR6M and A431p cell lines

The cytotoxic activity of the final purified bulk product was determined using the CellTiter-Fluor Cell Viability assay reagent (Promega, Madison, WI). The A431p (epidermoid carcinoma cell line expressing EGFRwt) and NR6M (Swiss 3T3 mouse fibroblast cell line transfected with the EGFRvIII) cells were cultured in 1x Zinc Option (ZO) medium (Thermo Fisher Scientific) supplemented with 10% fetal bovine serum. Cells were trypsinized, washed with 1x PBS and counted with a hemocytometer. The cells were then seeded in 96-well plates at 5×10^3 (A431p) and 5×10^4 (NR6M) cells/well in 100 μ L of the growth medium and incubated at 37°C, 5% CO₂ overnight. The final purified bulk D2C7-IT product was diluted in 1x PBS-0.2% BSA and was added to the wells at an initial concentration of 1000 ng/mL, followed by 10-fold serial dilutions. The plates with A431p and NR6M cells were incubated at 37°C and 5% CO₂ for an additional 48 h. After the 48 h incubation period, CellTiter-Fluor Reagent (100 μ L/well) was added and the plates were incubated for 70 mins at 37°C and 5% CO₂. The plates were then read at 400 nm-Excitation/505 nm-Emission. The data were analyzed using GraphPad Prism 4 software. The OD₅₀₅ values were averaged for the cells that were left untreated. One half of this average was considered the 50% growth inhibition value. The concentration of the purified bulk D2C7-IT corresponding to the 50% growth inhibition value was reported as the IC₅₀ value.

Purity by HPLC-SEC

A TSKgel SuperSW3000 gel filtration column connected to a Beckman Coulter Gold high performance liquid chromatography (HPLC) system with pump module 126, detector module 168, and 32 Karat software was utilized to assess the purity of the final bulk D2C7-IT product. The purified bulk D2C7-IT protein (12 μ g) was injected into the SuperSW3000 size exclusion chromatography (SEC) column. The eluate from the column was analyzed through a detector, and the absorbance (at 280 nm) was measured and used to create a chromatogram. From the area of the peak on the chromatogram, the percentages of the bulk D2C7-IT protein, aggregates, and cleavage fragments in the final product were determined.

Purity by isoelectric focusing

The purity of the final bulk D2C7-IT product was determined through isoelectric focusing (IEF). The isoelectric point (pI) of the bulk D2C7-IT was determined using Novex pH 3-10 IEF Protein Gels (Thermo Fisher Scientific), and the IEF Marker 3-10 (Thermo Fisher Scientific) was used as a reference standard.

DNA content (residual *E. coli* DNA)

The purity of the final bulk D2C7-IT product was determined through quantitation of residual *Escherichia coli* (*E. coli*) DNA in the final purified bulk D2C7-IT product (Charles River Laboratories, Malvern, PA). Quantitative fluorescent PCR (QF-PCR) was used to detect the presence and quantity of residual *E. coli* DNA in the final purified bulk D2C7-IT product.

pH

The safety of the final purified bulk product was established through pH measurement. A Accumet AR-15 pH meter (Fisher Scientific) was calibrated using pH 4.0, 7.0, and 10.0 standard buffers. The probe was rinsed and blotted dry before determining the pH of the purified bulk D2C7-IT sample.

Endotoxin testing

A bacterial endotoxin assay was performed on the final purified bulk D2C7-IT product by the Duke University Hospital Radiopharmacy Laboratory. Endotoxin levels in the purified bulk D2C7-IT product were assessed through the Endosafe-PTS (Charles River Laboratories) test system, utilizing an FDA-licensed disposable Endosafe-PTS test cartridge with a handheld spectrophotometer.

Sterility

Sterility testing on the final purified bulk D2C7-IT product was performed by the Duke University Hospital Radiopharmacy Laboratory. The final purified bulk D2C7-IT sample (0.5 mL) was aseptically added to the BBL Thioglycollate tube and incubated at 30-35°C for 14 days. The BBL Trypticase Soy Broth tube was inoculated with 0.5 mL of bulk D2C7-IT sample and incubated at 20-25°C for 14 days. Media was validated for the ability to grow test organisms and was determined to be sterile prior to use. Both the test and negative control tubes were examined for turbidity at specified intervals during the 14-day test period.

Chloramphenicol content

Chloramphenicol content was determined by Toxicology Associates, Inc. (Columbus, OH), according to the standard gas chromatograph mass spectrometry (GC/MS) detection method. Chloramphenicol is a broad spectrum antibiotic that was extracted with a buffer and a solvent, and derivatized with N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane (TCMS) and shot in GC/MS.

IPTG content

Isopropyl β -D-1-thiogalactopyranoside (IPTG) content was determined by Toxicology Associates, Inc. (Columbus, OH), according to the standard GC/MS detection method. IPTG was extracted with a solvent and a buffer, and derivatized with N-Methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) at 70°C for 20 min, and shot in GC/MS.

Vialing of the final purified bulk D2C7-IT product

The final purified bulk D2C7-IT product was sent to the Compounding Pharmacy at Duke University Medical Center for vialing. One mL of the final purified bulk D2C7-IT product, at a concentration of 120 μ g/mL, was aliquoted into a 2 mL amber, serum type 1 glass vial with gray butyl Teflon-faced straight plug stoppers, and sealed with center disc tear-out aluminum. Sealed vials were affixed with a label designating the lot number and storage

conditions, and stored at -70°C to -80°C . The final vial D2C7-IT product was subjected to a product inspection, in which we examined the condition of the straight plug stoppers, the aluminum center disc, and the uniformity of the fill volume. We also checked for any cracks, verified that the product was appropriately labeled, and confirmed that the labels were securely applied. Finally, we ran another round of analytical release testing similar to the testing of the final purified bulk product (with the exception of *E.coli* DNA, chloramphenicol, and IPTG content assessments).

Flow cytometry

Indirect flow cytometry analysis was performed with the D2C7-IT or negative control P588-IT (P588-(scdsFv)-PE38KDEL). Briefly, 1×10^6 cells (murine Swiss 3T3 fibroblast cell line NR6, human epidermoid-carcinoma cell line A431p [expressing EGFRwt], or NR6M [NR6 cell line transfected with human EGFRvIII]) were suspended in 500 μL of phosphate-buffered saline pH 7.4 (PBS) (Invitrogen, Carlsbad, CA) containing 5% FBS (Invitrogen) (5% FBS/PBS). The D2C7-IT or negative control, P588-IT, was added to the cells at a concentration of 5 $\mu\text{g/mL}$ and the samples were incubated for 40 min at 4°C . After washing, the cells were incubated with rabbit anti-Pseudomonas exotoxin A antibody (Sigma, St. Louis, MO) followed by goat anti-rabbit-IgG-FITC antibody (Rockland, Limerick, PA). Stained cells were analyzed on a Becton Dickinson FACSort instrument equipped with CellQuest software (BD Biosciences, San Jose, CA).

Stability testing of the final vial D2C7-IT product

Stability testing of the final vial D2C7-IT product is being conducted under Protocol 2012-001 over a 5-year duration, from November 2012 to November 2017, by the DUMC/AEAT program. Stability testing includes visual inspection, identity by SDS-PAGE, and verification of protein concentration, purity, potency, and pH (as described earlier), and will be tested at 0, 3, 6, 9, 12, 18, 24, 30, 36, 42, 48, 54, and 60-month time points. Sterility testing will be performed annually.

Results

D2C7-IT master cell bank production and validation

The *E. coli* strain BLR(DE3) was transformed with the pRB199-D2C7-(scdsFv)-PE38KDEL plasmid. On the basis of chloramphenicol and tetracycline resistance, ten positive clones labelled 11CT-20CT were selected for the screening of D2C7-IT expression. The culture identified as Clone 18CT demonstrated the highest levels of D2C7-IT expression (data not shown) and was selected as the source of the D2C7-IT ACB (DsD2C7-PE38-KDEL Clone 18CT BLR[DE3]).

From the D2C7-IT ACB, 240 MCB vials of D2C7-IT Lot 212001 (DsD2C7-PE38-KDEL MCB of clone 18 in *E. coli* BLR[DE3] 3-13-12) were prepared and stored frozen at -80°C . Quality control assessment of D2C7-IT MCB Lot 212001 confirmed the identity of the bacteria to be the non-toxic *E. coli* strain BLR(DE3) by comparative sequence analysis of genomic DNA (99.83%), by PFGE (>94% similarity), and through retention of the selection

marker chloramphenicol. A sample of the pRB199-D2C7-(scdsFv)-PE38KDEL MCB (Lot 212001) was sequenced and was demonstrated to have 100% identity to the reference sequence. The sequencing data was used to derive the plasmid map included in Figure 2. Plasmid identity was also confirmed through 100% retention of the pRB199-D2C7-(scdsFv)-PE38KDEL construct in the transformed *E. coli* strain BLR(DE3) and by the generation of predicted fragment sizes upon restriction endonuclease digestion, including *Nde*I (linear): 5151 base pairs (bp); *Nde*I and *Eco*RI: 2 fragments – 3410 and 1883 bp; and *Nde*I and *Hind*III: 2 fragments – 4727 and 800 bp. Purity of the MCB bacterial culture was established due to the absence of contaminating bacterial growth in the differential agar, the ability of MCB to form viable colonies on LB-agar plates with a viability of 1.2×10^8 CFU/mL, and the bacterial copy number pretest of the plasmid digest, which produced positive amplification of the gene of interest with fluorescent probes (306 plasmid copies/genome equivalent). Finally, the safety of the MCB was established by demonstrating the absence of the Shiga-like toxin/toxin gene, the absence of bacterial virus through plaque formation assay, and the absence of particles with bacteriophage morphology through negative staining and transmission electron microscopy examination.

Fermentation and purification of bulk D2C7-IT

To produce purified bulk lots of D2C7-IT, 12 rounds of seed buildup and fermentation (Fermentation Lot Numbers: 212011 – 212022) were performed using vials from the MCB. Each round of seed buildup and fermentation resulted in the generation of a new lot of cell paste, which was processed for inclusion body isolation. Multiple washes of the inclusion body isolation with Triton-X-100 detergent and Tris-EDTA buffer through centrifugation differentially removed a significant amount of contaminating host proteins as demonstrated in Figures 3a and 3b. The final inclusion body yield from each round of fermentation was in the range of 1.7 – 4.3 gms (Table 1). The inclusion body pellets were solubilized in a strong denaturant (6 M Guanidine-HCl), reduced with DTE, and diluted 100-fold in the refolding buffer. Individual batches of the refolded D2C7-IT protein (3 – 5L refolding buffer) from each fermentation lot was dialyzed, filtered, and loaded onto a Q-Sepharose FF anion exchange column. The D2C7-IT protein bound to the Q-Sepharose column was eluted using a differential salt gradient, and 4 ml fractions of the eluate was examined for purity in SDS-PAGE gel. The majority of the D2C7-IT was contained in 3-4 Q-Sepharose eluate fractions, which were pooled for downstream processing. Representative D2C7-IT Q-Sepharose eluate fractions and the final purity of the combined Q-Sepharose fractions are shown in Figures 4a and 4b. The 1.7 gms of the inclusion body obtained from the first batch of D2C7-IT fermentation (Lot 212011) was purified through a total of six individual Q-Sepharose runs yielding a total of 86.3 mg of D2C7-IT. The pooled Q-Sepharose fractions were dialyzed in TSK buffer overnight at 4°C for size exclusion chromatography purification. After overnight dialysis, the protein was filtered and injected into a TSKgel SuperSW3000 gel filtration column equilibrated with TSK buffer, and the eluate corresponding to the major peak of D2C7-IT was analyzed in a SDS-PAGE gel. The fractions that contained only the D2C7-IT monomer (fractions 83-86 in Figure 5) were pooled, concentrated, and loaded on to the ActiClean Etox column for endotoxin removal. The D2C7-IT eluate from the ActiClean Etox column was then dialyzed overnight at 4°C against DPBS CTS. A total of nine batches of bulk lots of D2C7-IT (Lot No. 212032-212040) were purified with a total yield of 307.75

mg, which were then transferred to 50 mL conical tubes, labeled, and stored at -80°C . The D2C7-IT purification process and the protein yields (Fermentation Lot No. 212032 – 212040 and TSK purified Bulk Lot No. 212032-212040) at each step are summarized in Table 1.

Preparation of the final purified bulk D2C7-IT product and analytical release testing

The final purified bulk D2C7-IT product was prepared by filtering and combining three batches of the intermediate bulk product, specifically Lot 212033, Lot 212034, and Lot 212035, for a total volume of 118 mL. The volume was adjusted to 250 mL by adding 132 mL of DPBS CTS, and the protein concentration was initially determined to be 0.36 mg/mL using the Pierce Coomassie Plus assay. However, since the curve was not linear in this initial assessment, the bulk product was further diluted to 500 mL by the addition of 250 mL DPBS CTS. The protein concentration of the diluted pool was determined to be 0.166 mg/mL for a total of 83 mg of protein. The final volume was adjusted to 600 mL by adding 100 mL DPBS-CTS and the final concentration was determined to be 0.123 mg/mL. The final purified bulk D2C7-IT was designated as Lot 212041 and 20-mL vials were frozen and stored at -80°C as the Purified Bulk reference standards. The purified bulk reference standard (D2C7-IT Lot 212041) was subjected to analytical release testing as described in materials and methods, and the results are presented in Figure 6. The purified bulk D2C7-IT Lot 212041 was a colorless clear liquid with no particles at 25°C . The purified bulk D2C7-IT Lot 212041 generated a single band corresponding to 61 kDa and 62.5 kDa in a reduced and non-reduced SDS-PAGE analysis, respectively. The purified bulk D2C7-IT Lot 212041 had a final protein concentration of 0.123 mg/mL. The purified D2C7-IT Lot 212032 was used as a reference standard for the assessment of cytotoxicity (IC_{50}) of the purified bulk D2C7-IT Lot 212041. The cytotoxicity of the reference standard D2C7-IT Lot 212032 and purified bulk D2C7-IT Lot 212041 on mutant EGFRvIII-expressing NR6M cells were 0.310 ng/mL and 0.410 ng/mL and on EGFRwt- expressing A431p cells were 0.038 ng/mL and 0.049 ng/mL, respectively. The purified bulk D2C7-IT Lot 212041 had 98.7% monomers and 1.3% fragments by HPLC-SEC analysis. The isoelectric point of the purified bulk D2C7-IT Lot 212041 was 5.3. The purified bulk D2C7-IT Lot 212041 had a pH of 7.4, DNA content of < 20 pg/mL, endotoxin levels equivalent to 1.09 EU/mL, and exhibited no growth in a sterility assay. The chloramphenicol content of Lot 212041 of the final purified bulk product was 1.2 $\mu\text{g/mL}$. According to the laboratory report from Toxicology Associates Inc., Columbus, OH, the levels of chloramphenicol in biological products needs to be > 25 $\mu\text{g/mL}$ to be toxic in humans. In the clinical study, we have proposed to infuse patients with a maximum of 1,143,504 ng of D2C7-IT, which is the equivalent to 9.53 mL of Lot 212041. Thus, the maximal potential exposure of chloramphenicol to patients in the clinical study will be 11.04 μg , which is below the toxic range of > 25 $\mu\text{g/mL}$. Therefore, the levels of chloramphenicol in Lot 212041 were considered to be non-toxic. Additionally, the level of IPTG in Lot 212041 was undetectable as it was below the limit of sensitivity.

Preparation of the final vialled D2C7-IT product and analytical release testing

Upon clearance of the analytical release testing, the remaining 580 mL of the purified bulk D2C7-IT Lot 212041 was sent to the Compounding Pharmacy at Duke University Medical Center for vialing. The final product contained approximately 1 mL of purified D2C7-IT/

vial at a concentration of 120 µg/mL in DPBS CTS. A total of 560 vials labelled as Lot 211131 were prepared, delivered, and stored at -70°C . The final vial product, D2C7-IT Lot 211131, underwent similar analytical release testing as the final purified bulk product, with the exception of the assessment for DNA, chloramphenicol, and IPTG content, since these tests have already been performed. The analytical release testing results for the final vial product, D2C7-IT Lot 211131, is presented in Figure 7. The final vial D2C7-IT Lot 211131 was a colorless clear liquid with no particles at 25°C . The final vial D2C7-IT Lot 211131 generated a single band corresponding to 61 kDa and 62.5 kDa in a reduced and non-reduced SDS-PAGE analysis, respectively. The final vial D2C7-IT Lot 211131 had a final protein concentration of 0.12 mg/mL. The purified D2C7-IT Lot 212032 was used as reference standard for the assessment of cytotoxicity (IC_{50}) of the final vial D2C7-IT Lot 211131. The cytotoxicity of the reference standard D2C7-IT Lot 212032 and final vial D2C7-IT Lot 211131 on mutant EGFRvIII-expressing NR6M cells were 0.210 ng/mL and 0.120 ng/mL, respectively. The cytotoxicity of the reference standard D2C7-IT Lot 212032 and final vial D2C7-IT Lot 211131 on EGFRwt-expressing A431p cells were 0.031 ng/mL and 0.041 ng/mL, respectively. The final vial D2C7-IT Lot 211131 had 97.7% monomers and 2.1% fragments by HPLC-SEC analysis. The isoelectric point of the final vial D2C7-IT Lot 211131 was 5.3. The final vial D2C7-IT Lot 211131 had a pH of 7.4, endotoxin levels equivalent to 1.26 EU/mL, and exhibited no growth in a sterility assay.

Antigen specificity of the final vial product, D2C7-IT Lot 211131

To determine the target antigen specificity of the final vial product, D2C7-IT Lot 211131, the ability of D2C7-IT to bind to human EGFRwt and EGFRvIII proteins expressed on the cell surface was assessed by indirect flow cytometry (Figure 8). Flow cytometry analysis revealed that the D2C7-IT Lot 211131 bound to both the EGFRwt-expressing A431p cells (Figure 8b) and the EGFRvIII-expressing NR6M cells (Figure 8c). The parental NR6 cells (Figure 8a) and the nonspecific P588-IT were used as negative controls, which confirmed the binding specificity of D2C7-IT Lot 211131 to native EGFRwt and EGFRvIII protein molecules expressed on human tumor and transfected mouse cell lines.

Stability testing of the final vial product, D2C7-IT Lot 211131

Stability testing of the final vial product, D2C7-IT Lot 211131, is being conducted over a 5-year period, from November 2012 to November 2017, by the DUMC/AEAT program. Visual appearance, protein concentration, purity, potency, and pH are being assessed by the methods outlined in the materials and methods section, and will be tested at 0, 3, 6, 9, 12, 18, 24, 30, 36, 42, 48, 54, and 60 month time points. Sterility testing will be performed annually. Specifications for each of the stability tests have been determined and are as follows: (1) the visual appearance specification requires that the results are clear with no foreign matter, (2) the protein concentration by Pierce Coomassie Plus used to determine protein content should specify a range of $0.12 \text{ mg/mL} \pm 0.01 \text{ mg/mL}$, (3) the major bands and the banding pattern reported by SDS-PAGE should conform to the standard reference (reference standard lot 212041), (4) the results reported by IEF gel should conform to the standard reference (reference standard lot 212041), (5) purity results as determined by HPLC-SEC must meet a specification of 95% total protein amounts of monomer, (6) the results of the cytotoxicity assay used to determine the potency of the product must conform

to the standard reference (reference standard Lot 212041), (7) the product must be found sterile, and (8) the acceptable pH range is 7.4 ± 0.4 . The stability tests performed, the assay specifications, and the available test results are summarized in Table 2 and the 42 month stability data is presented in Figure 9. The results indicated that Lot 211131 of the final vial product met the specifications set for the required assays through 42 months.

Discussion

We have successfully manufactured a GLP-grade D2C7-(scdsFv)-PE38KDEL immunotoxin for a Phase I/II clinical trial in patients with recurrent glioblastoma (NCT02303678, D2C7 for Adult Patients with Recurrent Malignant Glioma, clinicaltrials.gov). This is the first study that reports the quality control and stability testing (for a 42-month period since the date of manufacture) of a GLP-grade D2C7-(scdsFv)-PE38KDEL immunotoxin, which is currently being tested in clinical trials.

High levels of D2C7-IT expression in *E. coli* resulted in aggregation of the expressed protein molecules into inclusion bodies. From a total of nine fermentation runs, we generated a total of 26.3gm of inclusion body. Solubilization of the D2C7-IT inclusion body was achieved using a high concentration of Guanidine-HCl, which was then refolded by removal of the solubilization agent through dilution in refolding buffer. Twenty-six grams of the D2C7-IT inclusion body was refolded by dilution in a total of 260L of refolding buffer. Refolded D2C7-IT was dialyzed and then purified through a strong anion-exchange chromatographic step followed by a size exclusion chromatographic step. The total yields of purified D2C7-IT after anion exchange and size exclusion chromatographic steps were 926.9 mg and 356.2 mg, respectively. Because of the use of a bacterial expression system, endotoxin contamination was involved in the D2C7-IT preparation. Thus, preparation required multiple washes with detergent to remove the majority of this contaminant before polishing on secondary chromatography columns and endotoxin removal resins. The total D2C7-IT after endotoxin removal was 341.96 mg, and the final yield of the GLP grade D2C7-IT after dialysis against DPBS CTS was 307.75 mg. As discussed and agreed upon with the Food and Drug Administration (FDA) at our pre-IND meeting, the final purified bulk product was made by combining purified intermediate bulk lots 212033, 212034, and 212035, which were subjected to extensive analytical release testing both pre and post vialing.

A quantitative acceptance criterion, with an upper and lower limit, for the potency assay (cytotoxicity assay) used for release and stability testing of the final purified bulk product (Lot 212041) and the final vial product (Lot 211131) was determined by a statistician. The conclusions are as follows: (1) Final Purified Bulk (Lot 212041) on A431p cells: based upon four available IC₅₀ observations, with a mean=40 and SD=7.39, 68% Prediction Interval: 32.7, 47.4, (2) Final Vial Product (Lot 211131) on A431p cells: based upon 104 IC₅₀ observations, with a mean=40.35 and SD=14.26, 68% prediction interval: 26.1, 54.4, (3) Final Purified Bulk (Lot 212041) on NR6M cells: based upon four available IC₅₀ observations, with a mean=306.3 and SD=114.1, 68% Prediction Interval: 192.2, 420.4, and (4) Final Vial Product (Lot 211131) on NR6M cells: based upon 40 IC₅₀ observations, with a mean=432.9 and SD=161.4, 68% Prediction Interval: 271.5, 594.3. Upon the request from FDA following the pre-IND comments, SDS-PAGE and IEF-Gel analyses were added

to the stability testing protocol of the final vial product (Lot 211131). These additional tests were added to the ongoing stability protocol at the 9-month time point. Therefore, data for these two tests are reported in the stability results summary in Table 2, beginning at 9 months of stability testing. Additionally, upon consultation with the Duke Translational Medicine Institute Regulatory Affairs group, the expiration date of the final vial product (Lot 211131) was extended from 48 months to 60 months after the completion of the 42-month stability testing. Similarly, the expiration date of the final vial product (Lot 211131) will be extended from 60 months to 72 months after the completion of the 66-month stability testing.

Major challenges associated with the production of dsFv-PE38 immunotoxins such as HA22, SS1P, MR1-1, and 55.1 include the expression of V_H and V_L fragments as individual components and mixing the reduced V_H and V_L inclusion body solution at a 1:1 molar ratio prior to refolding (Reiter et al. 1996; Reiter et al. 1996; Reiter et al. 1996; Ochiai et al. 2008). Subsequent to the refolding process, it is critical that the individual V_H and V_L fragments are separated from the correctly refolded dsFv-PE38 immunotoxin fragment, making this a key quality control step during the ion exchange and size exclusion chromatographic purification process (Jiang et al. 2013). This two-step purification process frequently results in low yield because of the inefficient purification of the V_H or V_L fragments from the completely refolded dsFv-PE38 immunotoxin (Jiang et al. 2013). A more recent and improved three-step purification process involving ammonium sulfate precipitation, Phenyl HP hydrophobic interaction, and ion exchange chromatographic purification was described, which resulted in a significant increase in the final yield of the purified dsFv-PE38 immunotoxins MR1-1 and HA22 (Jiang et al. 2013). However, even with the improved three-step purification process, the individual V_H and V_L fragments had to be expressed separately and had to be removed from the final current Good Manufacturing Practice (cGMP)-grade dsFv-PE38 immunotoxin (Jiang et al. 2013). In contrast to the dsFv-PE38 immunotoxins, the D2C7-(scdsFv)-PE38KDEL immunotoxin is expressed as a single chain disulfide stabilized immunotoxin fragment (scdsFv-PE38KDEL) (Chandramohan et al. 2013). Fusing the V_H and V_L fragments via a 15 amino acid linker fragment and increasing the stability of the linked V_H and V_L fragments via a disulfide bond (scdsFv) offers the convenience of a single batch expression while simultaneously avoiding the individual V_H and V_L fragments contamination in the purified D2C7-(scdsFv)-PE38KDEL immunotoxin. The final yield of the GLP-grade D2C7-(scdsFv)-PE38KDEL immunotoxin was approximately 8-10%. We believe that if scaled up to an 80-L fermentation batch, the yield of the D2C7-(scdsFv)-PE38KDEL immunotoxin could be increased to meet the cGMP requirements for a Phase III clinical trial.

A clinical supply of large quantities of immunotoxins such as D2C7-IT is highly desirable (Hassan et al. 2007; Kreitman et al. 2009; Kreitman et al. 2012; Bao et al. 2016; Kreitman et al. 2016). This particular protein typifies the difficulties in supplying single chain disulfide stabilized immunotoxins due to the challenges in producing D2C7-IT at a higher yield and purity under GLP conditions. The main difficulty we encountered during the manufacturing process was the degradation of D2C7-IT when stored at 4°C in the absence of the carrier protein. We had to forego three fermentation batches (Lots 212019, 212020, and 212021) after Q-Sepharose purification due to D2C7-IT degradation at 4°C. We overcame this

problem by storing intermediate purification products at -80°C . Despite these challenges, we were able to produce 307.75 mg of a GLP-grade D2C7-IT from a total of nine, 8L fermentation runs. Most importantly, this material is currently being tested in a Phase I/II clinical trial in patients with recurrent glioblastoma, and we anticipate that it will save many lives.

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References

- Arita N, Hayakawa T, Izumoto S, Taki T, Ohnishi T, Yamamoto H, Bitoh S, Mogami H. Epidermal growth factor receptor in human glioma. *J Neurosurg.* 1989; 70(6):916–919. DOI: 10.3171/jns.1989.70.6.0916 [PubMed: 2715819]
- Bao X, Chandramohan V, Reynolds RP, Norton JN, Wetsel WC, Rodriguiz RM, Aryal DK, McLendon RE, Levin ED, Petry NA, Zalutsky MR, Burnett BK, Kuan CT, Pastan IH, Bigner DD. Preclinical toxicity evaluation of a novel immunotoxin, D2C7-(scdsFv)-PE38KDEL, administered via intracerebral convection-enhanced delivery in rats. *Invest New Drugs.* 2016; 34(2):149–158. DOI: 10.1007/s10637-015-0318-3 [PubMed: 26728879]
- Bartlett JM, Langdon SP, Simpson BJ, Stewart M, Katsaros D, Sismondi P, Love S, Scott WN, Williams AR, Lessells AM, Macleod KG, Smyth JF, Miller WR. The prognostic value of epidermal growth factor receptor mRNA expression in primary ovarian cancer. *Br J Cancer.* 1996; 73(3):301–306. [PubMed: 8562334]
- Brennan CW, Verhaak RG, McKenna A, Campos B, Nounshmehr H, Salama SR, Zheng S, Chakravarty D, Sanborn JZ, Berman SH, Beroukhi R, Bernard B, Wu CJ, Genovese G, Shmulevich I, Barnholtz-Sloan J, Zou L, Vegesna R, Shukla SA, Ciriello G, Yung WK, Zhang W, Sougnez C, Mikkelsen T, Aldape K, Bigner DD, Van Meir EG, Prados M, Sloan A, Black KL, Eschbacher J, Finocchiaro G, Friedman W, Andrews DW, Guha A, Iacocca M, O'Neill BP, Foltz G, Myers J, Weisenberger DJ, Penny R, Kucherlapati R, Perou CM, Hayes DN, Gibbs R, Marra M, Mills GB, Lander E, Spellman P, Wilson R, Sander C, Weinstein J, Meyerson M, Gabriel S, Laird PW, Haussler D, Getz G, Chin L, Network TR. The somatic genomic landscape of glioblastoma. *Cell.* 2013; 155(2):462–477. DOI: 10.1016/j.cell.2013.09.034 [PubMed: 24120142]
- Chaffanet M, Chauvin C, Laine M, Berger F, Chedin M, Rost N, Nissou MF, Benabid AL. EGF receptor amplification and expression in human brain tumours. *Eur J Cancer.* 1992; 28(1):11–17. [PubMed: 1567659]
- Chandramohan V, Bao X, Keir ST, Pegram CN, Szafranski SE, Piao H, Wikstrand CJ, McLendon RE, Kuan CT, Pastan IH, Bigner DD. Construction of an immunotoxin, D2C7-(scdsFv)-PE38KDEL, targeting EGFRwt and EGFRvIII for brain tumor therapy. *Clin Cancer Res.* 2013; 19(17):4717–4727. DOI: 10.1158/1078-0432.CCR-12-3891 [PubMed: 23857604]
- Fox SB, Persad RA, Coleman N, Day CA, Silcocks PB, Collins CC. Prognostic value of c-erbB-2 and epidermal growth factor receptor in stage A1 (T1a) prostatic adenocarcinoma. *Br J Urol.* 1994; 74(2):214–220. [PubMed: 7921940]
- Frederick L, Wang XY, Eley G, James CD. Diversity and frequency of epidermal growth factor receptor mutations in human glioblastomas. *Cancer Res.* 2000; 60(5):1383–1387. [PubMed: 10728703]

- Hassan R, Bullock S, Premkumar A, Kreitman RJ, Kindler H, Willingham MC, Pastan I. Phase I study of SS1P, a recombinant anti-mesothelin immunotoxin given as a bolus I. V. infusion to patients with mesothelin-expressing mesothelioma, ovarian, and pancreatic cancers. *Clin Cancer Res.* 2007; 13(17):5144–5149. [PubMed: 17785569]
- Jiang H, Xie Y, Burnette A, Roach J, Giardina SL, Hecht TT, Creekmore SP, Mitra G, Zhu J. Purification of clinical-grade disulfide stabilized antibody fragment variable--*Pseudomonas* exotoxin conjugate (dsFv-PE38) expressed in *Escherichia coli*. *Appl Microbiol Biotechnol.* 2013; 97(2):621–632. DOI: 10.1007/s00253-012-4319-2 [PubMed: 22890777]
- Klijn JG, Berns PM, Schmitz PI, Foekens JA. The clinical significance of epidermal growth factor receptor (EGF-R) in human breast cancer: a review on 5232 patients. *Endocr Rev.* 1992; 13(1):3–17. [PubMed: 1313356]
- Kreitman RJ, Stetler-Stevenson M, Jaffe ES, Conlon KC, Steinberg SM, Wilson W, Waldmann TA, Pastan I. Complete Remissions of Adult T-cell Leukemia with Anti-CD25 Recombinant Immunotoxin LMB-2 and Chemotherapy to Block Immunogenicity. *Clin Cancer Res.* 2016; 22(2): 310–318. DOI: 10.1158/1078-0432.CCR-15-1412 [PubMed: 26350263]
- Kreitman RJ, Stetler-Stevenson M, Margulies I, Noel P, Fitzgerald DJ, Wilson WH, Pastan I. Phase II trial of recombinant immunotoxin RFB4(dsFv)-PE38 (BL22) in patients with hairy cell leukemia. *J Clin Oncol.* 2009; 27(18):2983–2990. DOI: 10.1200/JCO.2008.20.2630 [PubMed: 19414673]
- Kreitman RJ, Tallman MS, Robak T, Coutre S, Wilson WH, Stetler-Stevenson M, Fitzgerald DJ, Lechleider R, Pastan I. Phase I trial of anti-CD22 recombinant immunotoxin moxetumomab pasudotox (CAT-8015 or HA22) in patients with hairy cell leukemia. *J Clin Oncol.* 2012; 30(15): 1822–1828. DOI: 10.1200/JCO.2011.38.1756 [PubMed: 22355053]
- Libermann TA, Nusbaum HR, Razon N, Kris R, Lax I, Soreq H, Whittle N, Waterfield MD, Ullrich A, Schlessinger J. Amplification, enhanced expression and possible rearrangement of EGF receptor gene in primary human brain tumours of glial origin. *Nature.* 1985; 313(5998):144–147. [PubMed: 2981413]
- Libermann TA, Razon N, Bartal AD, Yarden Y, Schlessinger J, Soreq H. Expression of epidermal growth factor receptors in human brain tumors. *Cancer Res.* 1984; 44(2):753–760. [PubMed: 6318976]
- Ochiai H, Archer GE, Herndon JE 2nd, Kuan CT, Mitchell DA, Bigner DD, Pastan IH, Sampson JH. EGFRvIII-targeted immunotoxin induces antitumor immunity that is inhibited in the absence of CD4+ and CD8+ T cells. *Cancer Immunol Immunother.* 2008; 57(1):115–121. DOI: 10.1007/s00262-007-0363-7 [PubMed: 17634939]
- Pavelic K, Banjac Z, Pavelic J, Spaventi S. Evidence for a role of EGF receptor in the progression of human lung carcinoma. *Anticancer Res.* 1993; 13(4):1133–1137. [PubMed: 8394672]
- Reiter Y, Brinkmann U, Lee B, Pastan I. Engineering antibody Fv fragments for cancer detection and therapy: disulfide-stabilized Fv fragments. *Nat Biotechnol.* 1996; 14(10):1239–1245. DOI: 10.1038/nbt1096-1239 [PubMed: 9631086]
- Reiter Y, Pastan I. Antibody engineering of recombinant Fv immunotoxins for improved targeting of cancer: disulfide-stabilized Fv immunotoxins. *Clin Cancer Res.* 1996; 2(2):245–252. [PubMed: 9816166]
- Reiter Y, Wright AF, Tonge DW, Pastan I. Recombinant single-chain and disulfide-stabilized Fv-immunotoxins that cause complete regression of a human colon cancer xenograft in nude mice. *Int J Cancer.* 1996; 67(1):113–123. DOI: 10.1002/(SICI)1097-0215(19960703)67:1<113::AID-IJC19>3.0.CO;2-F [PubMed: 8690511]
- Rubin Grandis J, Melhem MF, Barnes EL, Tweardy DJ. Quantitative immunohistochemical analysis of transforming growth factor- α and epidermal growth factor receptor in patients with squamous cell carcinoma of the head and neck. *Cancer.* 1996; 78(6):1284–1292. DOI: 10.1002/(SICI)1097-0142(19960915)78:6<1284::AID-CNCR17>3.0.CO;2-X [PubMed: 8826952]
- Seetharam S, Chaudhary VK, FitzGerald D, Pastan I. Increased cytotoxic activity of *Pseudomonas* exotoxin and two chimeric toxins ending in KDEL. *J Biol Chem.* 1991; 266(26):17376–17381. [PubMed: 1910044]
- Weldon JE, Pastan I. A guide to taming a toxin--recombinant immunotoxins constructed from *Pseudomonas* exotoxin A for the treatment of cancer. *FEBS J.* 2011; 278(23):4683–4700. DOI: 10.1111/j.1742-4658.2011.08182.x [PubMed: 21585657]

Fig. 1. D2C7-IT fermentation profile. A representative 8L D2C7-IT fermentation profile is shown. The X-axis represents fermentation time in minutes and the Y-axis represents OD₆₀₀ values.

Fig. 2.
pRB199-D2C7-(scdsFv)-PE38KDEL plasmid map with key features.

Fig. 3.

Representative coomassie-stained SDS-PAGE gel of crude D2C7-IT bacterial lysate and purified D2C7-IT inclusion body. **a** *E. coli* BLR (λ DE3) cells transformed with pRB199-D2C7-(scdsFv)-PE38KDEL were grown in fermentor until OD_{600} reached around 6–8 and the D2C7-IT expression was induced with 1 mM IPTG for 2 h. Samples were harvested at the end of the induction period and different volumes (5 and 15 µl) of crude bacterial lysate were analyzed in a SDS-PAGE gel. M: molecular weight standard. **b** 25 µl of the D2C7-IT inclusion body was analyzed in a SDS-PAGE gel during the final TE 50/20 buffer wash. M: molecular weight standard and IB: inclusion body.

Fig. 4. Representative chromatographic profile and coomassie-stained SDS-PAGE gel of D2C7-IT Q-Sepharose fractions. **a** Chromatographic profile of D2C7-IT eluate from the Q-Sepharose FF column. 30 μ l of the D2C7-IT eluate fractions 25-42 from the Q-Sepharose FF column was analyzed in a SDS-PAGE gel. M: molecular weight standard. **b** 20 μ l of the pooled D2C7-IT Q-Sepharose fractions 29-32 was analyzed in a SDS-PAGE gel. M: molecular weight standard and MQ: merged Q-Sepharose fractions 29-32.

5) Chromatographic profile and coomassie-stained SDS-PAGE gel of D2C7-IT TSK fractions

Fig. 5.

Representative chromatographic profile and coomassie-stained SDS-PAGE gel of D2C7-IT TSK SEC fractions. A chromatographic profile of D2C7-IT eluate from the TSKgel SuperSW3000 column. A D2C7-IT sample was injected into the TSK column and 30-second fractions (2 mL/min, total runtime 60 min) were collected and 30 μ L of the D2C7-IT eluate fractions 81-88 from the TSK SEC column was analyzed in a SDS-PAGE gel. M: molecular weight standard.

6) Purified bulk D2C7-IT Lot 212041 analytical release testing summary

CERTIFICATE OF ANALYSIS		
Product Name: D2C7-IT Purified Bulk Lot Number: 212041 Production Date: 11/5/2012 Lot Size: 20 vials Container Size/ Fill volume: 2 mL/ 1mL Storage Temperature: -80°C Description: D2C7-IT (scdsFv-PE38KDEL) is a dual specific single-chain disulfide-stabilized variable domain immunotoxin (containing Pseudomonas exotoxin) that targets both the epidermal growth factor receptor (EGFR) wild-type and tumor-specific variant form EGFRvIII. Colorless liquid in a solution of sterile PBS, pH 7.4.		
Test Description	Assay Specification	Results
Product Inspection Color, Appearance and Clarity	Colorless, No Foreign matter, clear to slightly opalescent solution at 25°C	Colorless, clear liquid with no particles @25°C
Identity SDS-PAGE, Reduced & Non-reduced, Coomassie Blue Stain	Report Major Bands, Conforms to Standard	Reduced: 1 band @61 kDa Non-reduced: 1 band @62.5 kDa Conforms to Std
Content Protein Concentration by Pierce Coomassie Plus Assay	0.12 ± 0.01 mg/mL	0.123 mg/mL
Potency Cytotoxicity in NR6M & A431p cell lines	Conforms to Standard Lot 212032	IC ₅₀ values 212041 = 410 pg/mL @ NR6M 212041 = 49 pg/mL @ A431p Ref Std = 310 pg/mL @ NR6M Ref Std = 38 pg/mL @ A431p Conforms to Std
Purity HPLC-SEC	≥ 95% Total Protein, amounts of monomer, aggregates, & fragments Conforms to Standard	98.7% Purity, 98.7% monomer 0% aggregates, 1.3% fragments pI = 5.3
Gel IEF		
Safety DNA Content (E. coli)	Report Results	< 20 pg DNA/mL
Endotoxin (LAL)	≤ 10 EU/ mL	1.09 EU/ mL
Sterility	No growth	No Growth
pH	7.4 ± 0.4	7.4
Chloramphenicol Content	Report Results	1.2 µg/mL
Isopropyl-β-D-1-thiogalactopyranoside Content	Report Results	< 50 ng/mL Limit of sensitivity. Not detected
Results Confirmed and Approved by:
Date: 11-04-2013
Project Scientist		Date: 11-04-2013
Date: 11/5/13
Project Scientist		Date: 11/5/13
Date: 11/10/13
Director of EAT Program		Date: 11/10/13

Quality Assurance Oversight		

Fig. 6.

Purified bulk D2C7-IT Lot 212041 analytical release testing summary. The purified bulk reference standard, D2C7-IT Lot 212041 was subjected to analytical release testing including product inspection, identity, content, potency, purity, and safety.

7) Final vial D2C7-IT Lot 211131 analytical release testing summary

CERTIFICATE OF ANALYSIS

Product Name: D2C7-IT Final Vial Product Lot Number: 211131 Production Date: 11/7/2012		
Lot Size: 560 vials Container Size/ Fill volume: 2 mL/ 1mL Storage Temperature: -80°C		
Description: D2C7-IT (scd5Fv-PE38KDEL) is a dual specific single-chain disulfide-stabilized variable domain immunotoxin (containing Pseudomonas exotoxin) that targets both the epidermal growth factor receptor (EGFR) wild-type and tumor-specific variant form EGFRvIII. Colorless liquid in a solution of sterile PBS, pH 7.4.		
Test Description	Assay Specification	Results
Product Inspection Color, Appearance and Clarity	Colorless, No Foreign matter, clear to slightly opalescent solution at 25°C	Colorless, clear liquid. Free from particles @25°C
Identity SDS-PAGE, Reduced & Non-reduced, Coomassie Blue Stain	Report Major Bands, Conforms to Standard	Reduced: 1 band @61 kDa Non-reduced: 1 band @62.5 kDa Conforms to Std
Content Protein Concentration by Pierce Coomassie Plus Assay	0.12 ± 0.01 mg/mL	0.12 mg/mL
Potency Cytotoxicity in NR6M & A431p cell lines	Conforms to Standard	IC ₅₀ Values 211131 = 120 pg/mL @ NR6M 211131 = 41 pg/mL @ A431p Ref Std = 210 pg/mL @ NR6M Ref Std = 31 pg/mL @ A431p Conforms to Std
Purity HPLC-SEC	≥ 95% Total Protein, amounts of monomer, aggregates, & fragments Conforms to Standard	97.7% Purity, 97.7% monomer 0% aggregates, 2.1% fragments
Gel IEF		pI = 5.3
Safety Endotoxin (LAL)	≤ 10 EU/ mL	1.26 EU/ mL
Sterility	No growth	No Growth
pH	7.4 ± 0.4	7.4

Results Confirmed by /Date: _____ Date: 9-18-2013

Project Scientist _____ Date: 09-18-2013

Project Scientist _____ Date: _____

Approvals/ Date: _____ Date: 9/19/13

Director AEAT Program _____ Date: 9/20/13

Quality Assurance Oversight _____ Date: _____

Fig. 7.

Final vial D2C7-IT Lot 211131 analytical release testing summary. The final vial product, D2C7-IT Lot 211131, was subjected to analytical release testing including product inspection, identity, content, potency, purity, and safety.

Fig. 8.

Flow cytometric analysis to determine the specificity of D2C7-IT Lot 211131. **a** Parental NR6 cells were used as control. Indirect flow cytometry analysis demonstrated the specificity of D2C7-IT Lot 211131 against **b** A431p cells expressing EGFRwt or **c** NR6M cells transfected with human EGFRvIII. Cells were stained with D2C7-IT Lot 211131 (black open peaks) or a non-specific scFv derived immunotoxin (P588-IT) control (filled grey peaks).

a) Protein concentration of D2C7-IT, Lot 211131

b) IEF gel of D2C7-IT, Lot 211131

IEF marker 3-10.	pI
Cytochrome C	10.7
Ribonuclease A	9.5
Lectin	8.3,8.0,7.8
Myoglobin	7.4, 6.9
Carbonic anhydrase	6.0
Beta-Lactoglobulin	5.3, 5.2
Trypsin Inhibitor	4.5
Glucose oxidase	4.2
Amyloglucosidase	3.5

c) SDS-PAGE analysis of D2C7-IT, Lot 211131

D2C7-IT 42 month stability gel			
		Band No.	Band Label Mol. Wt. (kDa)
Bio-Rad Precision Std	Lane 1	1	250.0
		2	150.0
		3	100.0
		4	75.0
		5	50.0
		6	37.0
		7	25.0
		8	20.0
		9	15.0
		10	10.0
Lot 211131 non-reduced 3.6ug	Lane 2	1	61.8
Lot 211131 non-reduced 1.8ug	Lane 3	1	60.9
Lot 211131 reduced 3.6ug	Lane 4	1	66.3
Lot 211131 reduced 1.8ug	Lane 5	1	67.0
Lot 212041 non-reduced 3.6ug	Lane 6	1	61.7
Lot 212041 non-reduced 1.8ug	Lane 7	1	61.2
Lot 212041 reduced 3.6ug	Lane 8	1	66.6
Lot 212041 reduced 1.8ug	Lane 9	1	66.9
Bio-Rad Precision Std	Lane 10	1	250.0
		2	150.0
		3	100.0
		4	75.0
		5	50.0
		6	37.0
		7	25.0
		8	20.0
		9	15.0
		10	10.0

d) HPLC-SEC analysis of D2C7-IT, Lot 211131

Sample ID: D2C7-IT 42 month stability test
 FileName: D:\32Karat\Projects\Default\Data\D2C7-IT 42 month stability test.dat
 Method: D:\32Karat\Projects\Default\Method\Super TSK 3000 UV only.met Run
 Time 6/8/2016 3:08:26 PM
 User Name: System

Det 168-280nm
Results

Pk #	Retention Time	Area	Area Percent	Height	Height Percent	Width
1	14.200	3475310	97.104	77762	96.442	2.72
2	16.250	103662	2.896	2869	3.558	0.97
Totals		3578972	100.000	80631	100.000	

e) A431pp18

■ 0.2% HSA-Saline
 ▲ Bulk D2C7-IT Lot 212041 IC₅₀ 61 pg/ml
 ▼ Viald D2C7-IT Lot 211131 IC₅₀ 50 pg/ml

f) NR6Mp63

■ 0.2% HSA-Saline
 ▲ Bulk D2C7-IT Lot 212041 IC₅₀ 300 pg/ml
 ▼ Viald D2C7-IT Lot 211131 IC₅₀ 230 pg/ml

Fig. 9.

Forty two month stability data including **a** protein concentration, **b** IEF gel, **c** SDS-PAGE analysis, **d** HPLC-SEC analysis, and Potency-Cytotoxicity of D2C7-IT Lot 212041 (▲) and D2C7-IT Lot 211131 (▼) against **e** A431p and **f** NR6M cells.

Table 1

D2C7-IT purification process yield summary

Fermentation	Lot 212011	Lot 212012		Lot 212013
Bacterial Culture	8L	8L		8L
Total Inclusion Body	1.7g	1.8g	2.4g	2.7g
Refolding Volume	17L	18L	24L	27L
Pooled Q/S Fractions	86.3mg	76.6mg	79.1mg	116.5mg
Dialysis and Filtration	75.9mg	64.9mg	64.9mg	105.1mg
Pooled TSK Fractions	28.5mg	24.1mg	27.9mg	39.2mg
Endotoxin Column	28.4mg	23.9mg	21.3mg	40.9mg
Dialysis vs PBS	24.25mg	17.63mg	18.87mg	37.44mg
Purified Bulk Number	Lot 212032	Lot 212033	Lot 212034	Lot 212035
Fermentation	Lot 212014	Lot 212015	Lot 212016	
Bacterial Culture	8L	8L	8L	
Total Inclusion Body	2.4g	2.8g	4.3g	
Refolding Volume	24L	28L	40L	
Pooled Q/S Fractions	80.9mg	102.6mg	126.1mg	
Dialysis and Filtration	70.25mg	88.7mg	100.5mg	
Pooled TSK Fractions	30.7mg	50.4mg	56.3mg	
Endotoxin Column	30.7mg	49.56mg	50.9mg	
Dialysis vs PBS	29.81mg	42.60mg	51.69mg	
Purified Bulk Number	Lot 212036	Lot 212037	Lot 212038	
Fermentation	Lot 212017	Lot 212018	Lot 212022	
Bacterial Culture	8L	8L	8L	
Total Inclusion Body	3.8g	1.7g	2.7g	
Refolding Volume	38L	17L	27L	
Pooled Q/S Fractions	144mg		114.8mg	
Dialysis and Filtration	114.3mg		86.75mg	
Pooled TSK Fractions	56.7mg		42.4mg	
Endotoxin Column	51.1mg		45.2mg	
Dialysis vs PBS	48.13mg		37.33mg	
Purified Bulk Number	Lot 212039		Lot 212040	

Table 2

Stability Results for D2C7-IT, Lot 211131, Time 0 through 5 Years Production Date: 11/7/2012

Test	Specification	Time Point										
		0 Month 11/2012	3 Months 02-03/2013	6 Months 05-06/2013	9 Months 08-09/2013	12-14 Months 12/2013-02/14	18 Months 05-06/2014	24 Months 11-12/2014	30 Months 05-06/2015	36 Months 01/2016	42 Months 05-06/2016	48 Months 11-12/2016
Appearance	Colorless, Clear, No particles or precipitation	Colorless, clear solution	Colorless, clear liquid. Free from particles	Colorless, clear solution with no particles	Colorless, clear liquid with no particles	Colorless, clear liquid with no particles	Colorless, clear liquid with no particles	Colorless, Clear, No particles or precipitation	Colorless, Clear, No particles or precipitation	Colorless, Clear, No particles or precipitation		
Content Protein Conc. by Pierce Coomassie Plus Assay	0.12 ± 0.01 mg/mL	0.12 mg/mL	NA		0.117 mg/mL	0.123 mg/mL	0.121 mg/mL	0.112 mg/mL	0.118 mg/mL	0.125 mg/mL	0.122 mg/mL	
SDS-PAGE, Reduced & Non-reduced, Coomassie Blue Stain	Report Major Bands, Conforms to Standard *	Non-reduced: 1 band @62.5 kDa Conforms to Std	Non-reduced: 1 band @62 kDa Conforms to Std	Non-reduced: 1 band @61.5 kDa Conforms to Std	Reduced: 1 band @61.5 kDa Non-reduced: 1 band @63.3 kDa Conforms to Std	Reduced: 1 band @63.1 kDa Non-reduced: 1 band @62.9 kDa Conforms to Std	Reduced: 1 band @63.2 kDa Non-reduced: 1 band @60.2 kDa Conforms to Std	Reduced: 1 band @66.2 kDa Non-reduced: 1 band @62.55 kDa Conforms to Std	Reduced: 1 band @63.4 kDa Non-reduced: 1 band @60.55 kDa Conforms to Std	Reduced: 1 band @66.65 kDa Non-reduced: 1 band @61.35 kDa Conforms to Std		
Gel IEF	Conforms to Standard *	5.3	NA	NA	5.3	5.3	5.3	5.3	5.3	5.3		
Purity HPLC-SEC	95% monomer	97.7% Purity 97.7% monomer 0% aggregates 2.1% fragments	NA	NA	99.8% Purity 98.8% monomer 0% aggregates 1.1% fragments	100% Purity 100% monomer 0% aggregates 0% fragments	97.4% Purity 97.4% monomer 0.95% aggregates 1.67% fragments	98.213% Purity 98.213% monomer 0% aggregates 1.787% fragments	98.150% Purity 98.150% monomer 1.850% aggregates 0% fragments	96.442% Purity 96.442% monomer 0% aggregates 3.558% fragments		
Potency Cytotoxicity in NR6M & A431p cell lines	Conforms to Reference Standard ** Lot 212041 (Relative Activity%)	IC ₅₀ values 211131 = 120 pg/mL @ NR6M (175%) Ref Std = 210 pg/mL @ NR6M (100%) 211131 = 41 pg/mL @ A431p (76%) Ref Std = 31 pg/mL @ A431p (100%) Conforms to Std	IC ₅₀ values 211131 = 400 pg/mL @ NR6M 211131 = 42 pg/mL @ A431p Conforms to Std	IC ₅₀ values 211131 = 410 pg/mL @ NR6M 211131 = 41 pg/mL @ A431p Conforms to Std	IC ₅₀ values 211131 = 400 pg/mL @ NR6M (100%) Ref Std = 400 pg/mL @ NR6M (100%) 211131 = 39 pg/mL @ A431p (105%) Ref Std = 41 pg/mL @ A431p (100%) Conforms to Std	IC ₅₀ values 211131 = 205 pg/mL @ NR6M (100%) Ref Std = 205 pg/mL @ NR6M (100%) 211131 = 36 pg/mL @ A431p (108%) Ref Std = 39 pg/mL @ A431p (100%) Conforms to Std	IC ₅₀ values 211131 = 290 pg/mL @ NR6M (100%) Ref Std = 290 pg/mL @ NR6M (100%) 211131 = 48 pg/mL @ A431p (125%) Ref Std = 60 pg/mL @ A431p (100%) Conforms to Std	IC ₅₀ values 211131 = 300 pg/mL @ NR6M (77%) Ref Std = 230 pg/mL @ NR6M (100%) 211131 = 21 pg/mL @ A431p (100%) Ref Std = 21 pg/mL @ A431p (100%) Conforms to Std	IC ₅₀ values 211131 = 460 pg/mL @ NR6M (100%) Ref Std = 460 pg/mL @ NR6M (100%) 211131 = 23 pg/mL @ A431p (122%) Ref Std = 28 pg/mL @ A431p (100%) Conforms to Std	IC ₅₀ values 211131 = 230 pg/mL @ NR6M (100%) Ref Std = 300 pg/mL @ NR6M (100%) 211131 = 50 pg/mL @ A431p (122%) Ref Std = 61 pg/mL @ A431p (100%) Conforms to Std		
Sterility	No Growth	No Growth	Not Required	Not Required	No Growth	Not Required	No Growth	Not Required	No Growth	Not Required		
pH	7.4 ± 0.4	7.4	NA	NA	7.4	7.4	7.39	7.2	7.18	7.23		

*, “Conforms to Standard”; there are no new bands as compared to reference standard, and the relative percentage of each species remains constant.

**, The revised acceptance criteria values for future DS (lot 211131) and DP (lot 212041) stability and intermediate bulk lot stability on A431p and NR6M cells are as follows:

1.	NR6M cells: DS (Reference Standard) 306.3±114.1 pg/ml [192.2-420.4 pg/ml]; DP 432.9±161.4 pg/ml [271.5-594.3 pg/ml].
2.	A431p cells: DS (Reference Standard) 40±7.39 pg/ml [32.6-47.4 pg/ml]; DP 40.35±14.26 pg/ml [26.1-54.6 pg/ml]