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Process development and production of cGMP grade Melan-A for cancer vaccine clinical trials

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ABSTRACT

Melan-A is a cancer testis antigen commonly found in melanoma, and has been shown to stimulate the body's immune response against cancerous cells. We have developed and executed a process utilizing current good manufacturing practices (cGMP) to produce the 6 times-His tagged protein in C41DE3 *Escherichia coli* for use in Phase I clinical trials. Approximately 11 g of purified Melan-A were produced from a 20 L fed-batch fermentation. Purification was achieved through a three column process utilizing immobilized metal affinity, anion exchange, and cation exchange chromatography with a buffer system optimized for low-solubility, high LPS binding capacity proteins. The host cell proteins, residual DNA, and endotoxin concentration were well below limits for a prescribed dose with a final purity level of 91%.

Introduction

Melan-A, also known as MART-1, has been used as a diagnostic marker [1] for melanoma as well as an immunotherapeutic agent [2]. This tumor associated antigen (TAA)[2] contains 118 amino acids, with a 21 amino acids predicted transmembrane domain and a 92 amino acid solvent exposed domain [3]. Melan-A was originally cloned by Coulie et al. [4] and was independently cloned by Kawakami et al. [5] who termed it MART-1 (Melanoma Antigen Recognized by T-cells) [6]. The protein is expressed only in melanocytes, the retina, and most melanoma cancers. While it is expressed in virtually all metastatic melanomas, some primary, cutaneous melanomas have stained Melan-A negative [7].

Melan-A is a member of the MAGE gene family [6], which includes many TAAs that are recognized by cytotoxic T lymphocytes (CTL). Once recognized, CTLs lyse the cancerous cell [8]. NY-ESO-1 [9] and SSX-2 [10] are two related TAAs which provoke similar reactions from CTLs and have had some success as cancer vaccines [11,12]. Interestingly, it has been demonstrated that Human Leukocyte Antigen (HLA) phenotype strongly influences the efficacy of Melan-A vaccination due to the ability of the HLA molecule to present the antigen [13]. Other TAAs appear to be antigenic when displayed by multiple HLA phenotypes [14,15].

Melan-A peptides have been delivered through viral vectors and directly as peptide solutions for use as cancer vaccine antigens. Virally, Melan-A has been expressed using lentiviral [15], adenoviral [16], and poxviral vector [17,18] systems. The studies' results strongly supported the use of Melan-A as an immunotherapy. This is especially well supported by the phase I/II clinical trials using inactivated vaccinia virus. Individuals have also been vaccinated with Melan-A peptides intravenously as part of clinical trials. In order to elicit a stronger CTL response, adjuvants [19] and combination therapies have been used. There has been significant evidence that masking the CTLA-4 protein on helper T-cells can significantly increase the immune response to vaccine antigens [20–22]. As with any selective agent, resistance to CTLs has been observed [24] and is further evidence that combination therapies are required for these vaccine treatments.

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[2] *Abbreviations used:* TAA, tumor associated antigen; MART-1, melanoma antigen recognized by T-cells; CTL, cytotoxic T lymphocytes; HLA, human leukocyte antigen; cGMP, current good manufacturing process; ICH, international conference on harmonisation; FDA, food and drug administration; TGA, therapeutic goods administration; ICH, international conference on harmonization; CSE, control standard endotoxin; MVD, maximum valid dilution; CID, collision induced dissociation; emPAI, exponentially modified protein abundance index; IDT, integrated DNA technologies; MCB, master cell bank; DO, dissolved oxygen; LPBF, low protein binding filter; IMAC, immobilized metal affinity chromatography; CV, column volumes; AXC & CXC, anion & cation exchange chromatography; PID, proportional–integral–differential; TFF, tangential flow filtration; HIC, hydrophobic interaction column; IB, inclusion bodies.

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We have developed a process to produce His-tagged current Good Manufacturing Process (cGMP) grade Melan-A at the pilot-scale for clinical trials. The process utilizes a 20 L fed-batch fermentation and a three column purification scheme to remove contaminating host proteins and endotoxin. Together with the protein's initial success in limited trials [25–27], we have filled the gap between laboratory and industry scale to provide cGMP grade Melan-A for use in vaccine clinical trials.

Materials and methods

Materials

All materials used were obtained at the highest purity level possible. All equipment was cleaned and tested in accordance with cGMP protocols detailed in the harmonized International Conference on Harmonization (ICH) quality guidelines [28]. Production staff followed strict cGMP training and operating procedures during production of the biopharmaceutical material. Injection grade water was used for all solutions (Hyclone Inc.; Logan, UT). All column chromatography purification steps were performed using an Akta Purifier FPLC system (GE Healthcare, Piscataway, NJ) equipped with either a BPG 200/500 or 100/500 column (GE Healthcare) controlled by Unicorn software version 4.12 (GE Healthcare). Our production technicians packed all chromatography columns. The columns were then checked for symmetry and plate count as directed by the manufacturer. Protein and endotoxin concentrations were measured using Bradford and Limulus Amebocyte Lysate assays respectively, as previously described [29].

Buffer composition

Lysis buffer: 50 mM Tris Base, 100 mM NaCl, 1 mM $MgSO_4$, 1 mM β-mercaptoethanol, 2.5×10^{-3}% (v/v) "Turbo" DNase (Ambion, Inc.; Austin, TX) at pH 8.0. *Solubilization buffer:* 2% m/v deoxycholate (sodium salt), 1% v/v Triton-114, 8 M urea, 50 mM phosphate, 200 mM NaCl, 100 mM KCl, 10 mM imidazole, 2.5 mM β-mercaptoethanol at pH 7.5. *Urea buffer:* 4 M urea, 50 mM phosphate, 2.5 mM β-mercaptoethanol at pH 7.5. *Imidazole buffer:* Formulated as urea buffer with 500 mM imidazole. *Carbonate buffer:* 4 M urea, 10 mM Carbonate, 1 mM 2-Mercaptoethanol at pH 10.5 Carbonate elution buffer: Formulated as carbonate buffer with 1 M sodium chloride at pH 10.5 Final Bulk Buffer: 4 M urea, 50 mM phosphate, 145 mM NaCl, 50 mM glycine at pH 6.5. All buffers cleared endotoxin testing before use.

Description of facility

The manufacturing facility is approximately 1100 sq. ft. and consists of five (5) process suites: Bioreactor, Cell Disruption, Downstream Purification, Buffer Prep & Wash, and Storage. In addition there is a common hallway and a gowning room. Interlocking pass-throughs are positioned between the Downstream Purification Room and Cell Disruption as well as between the Downstream Purification Room and Buffer Prep & Wash Room. Adjacent to the manufacturing suite is the mechanical room housing an oil-free air compressor for process and instrument air, HVAC system with dedicated air conditioners, on-demand dry steam humidifier, dedicated purified water-USP water system and a dedicated recirculating refrigerated chiller. The facility has a dedicated boiler for plant (dirty) steam. The GMP manufacturing facility suites are classified as ISO level 8 with the exception of the downstream purification room which has an ISO classification level 7.

Methods

Data analysis

The standard curve, spike recovery, and sample dilutions for all quantitative assays were analyzed for linearity, accuracy, and intra-assay precision. For linearity, the mean corrected value versus expected concentration was plotted and a best fit line using linear regression analysis was generated. For endotoxin assay, $\log_{10}$ of the mean onset time versus the $\log_{10}$ of the expected endotoxin concentration was plotted. The accepted correlation coefficient and the residual sum of squares were $\geqslant 0.98$ and $\geqslant 0.97$, respectively. Accuracy was determined by Eq. (1). The accepted accuracy of the lowest standard was ±20% of its expected concentration and all other standards had an accepted accuracy of ±10%. The precision was calculated using the coefficient of variation. The accepted CV for the standard was $\leqslant 20\%$ for the lowest concentration and $\leqslant 10\%$ for all other samples. Spike recovery was calculated by Eq. (2). The accepted spike recovery was within 70–130% of the expected value.

$$\text{Accuracy } (\%) = ((\text{Calculated Mean Conc.} - \text{Expected Conc.})/(\text{Expected Conc.})) \times 100 \quad (1)$$

$$\text{Spike Recovery } (\%) = ((\text{Spiked Sample} - \text{Sample})/\text{Spike}) \times 100. \quad (2)$$

Assay validation

Preparation and execution of assay validation was the responsibility of the Facility Manager and assigned personnel. Approval of the qualification protocol and completed qualification report was the responsibility of Quality Assurance. All designated test instruments and standards used in assay validation and in the exercise of the assay were calibrated as NIST traceable where applicable. References used for assay validation include FDA (Food and Drug Administration)-Code of Federal Regulations (21 CFR Part 211): Subpart I-Laboratory Controls, TGA (Therapeutic Goods Administration) – Australian Code of Good Manufacturing Practice for Medicinal Products; Chapter 6 – Quality Control, ICH (International Conference on Harmonization) Q2A – Validation of Analytical Procedures, and ICH (International Conference on Harmonization) Q2A – Validation of Analytical Procedures: Methodology.

Picogreen dsDNA quantification assay

PicoGreen dsDNA quantitation assay was developed from Molecular Probes PicoGreen® dsDNA Quantitation Kit (Life Technologies; Carlsbad, CA). 8 μg/mL of working solution of dsDNA was diluted in 1 times TE working solution (10 mM Tris–HCl, 1 mM EDTA, pH 7.5). Eight dsDNA standards were prepared from 2000 mg/mL to 0 ng/mL. 90 μL of each dsDNA standard and experimental sample was loaded into the corresponding microplate well, in triplicate. Two dilutions of selected experimental samples were spiked with 9 μL of 8000 ng/mL dsDNA standard. 90 μL of 1 times PicoGreen® dsDNA quantitation reagent was transfer into each well containing blank, standard, or sample. The fluorescence intensity of the samples at 515 nm (ex.485 nm) was measured on a Tecan Genios Microplate Reader (Tecan; San Jose, CA).

Endotoxin quantification assay

Endotoxin quantification assay was adapted from Endosafe® Endochrome-K™ (Charles River; Wilmington, MA) reagent protocol. First, Endochrome-K™ was rehydrated in 3.2–3.4 mL of LAL reagent water. Endosafe® Control Standard Endotoxin (CSE) was reconstituted in manufacturer specified volume using LAL reagent water to a final concentration of 50 EU/mL. Standards were prepared in depyrogenated glass test tubes and vortexed for 30 s. If