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Production and characterisation of recombinant human chaperonin 10 for treatment of inflammatory disease

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ABSTRACT

Human chaperonin 10 (Cpn10) and chaperonin 60 (Cpn60) fulfil an essential role in mitochondrial protein folding. Cpn10 is located in the mitochondrial matrix; however, it has also been detected in extra-mitochondrial compartments where it has demonstrated anti-inflammatory and immunoregulatory activity, suggesting a potential therapeutic value for the treatment of inflammatory and autoimmune disorders that corresponds with its recently proposed role as a resolution-associated molecular patterns (RAMPs) molecule. Ala-Cpn10, a recombinant, minimally modified Cpn10 synthesised in *Escherichia coli*, is formulated for use in clinical trials and pre-clinical studies with intravenous or subcutaneous administration. Herein, we report the development of a bioprocess for the production of ~115 g of recombinant human Ala-Cpn10 from a 100 L *E. coli* fermentation with >99% purity (SDS-PAGE), <0.6 EU mg^{-1} endotoxin, <18 pg mg^{-1} DNA and 3.2% molecular variants. This bioprocess was achieved through a careful optimisation of the gene construct, the promoter system and the fermentation process. Recombinant Ala-Cpn10 produced in this process is active as a molecular chaperone indicating correct tertiary and quaternary structure and the stability profile indicates no significant changes with storage as a liquid for at least 3 years at 2–8 °C. The results of validated characterisation assays demonstrate that purified Ala-Cpn10 produced using the optimised process reported here is suitable for its intended purpose as an investigational drug product, and this material is currently being tested in a phase II study for efficacy, safety and impact on biomarkers in subjects with mildly active Systemic Lupus Erythematosus (SLE) under US IND 116156.

1. Introduction

Chaperonin 10 (Cpn10) is a member of the stress or heat shock family of proteins. This group of proteins is among the most highly conserved in nature, with respect to both structure and function. Human Cpn10 has 100% homology with bovine, porcine and rabbit Cpn10 while rat Cpn10 differs from human by one amino acid [1]. Localised in various intracellular compartments, heat shock proteins (HSPs) have a well-defined role in protein assembly, translocation and degradation. Cpn60 together with its co-chaperone Cpn10, reside primarily within mitochondria where they fulfil an essential role in mitochondrial protein folding [2]. Stress proteins are up-regulated in response to hypoxia, hypoglycaemia, ionising radiation and heat-shock [3,4] and released from cells through as yet undefined mechanisms. Once in the extracellular environment, a number of stress proteins have demonstrated autocrine, paracrine and endocrine immunological activity [5]. Cpn10 has demonstrated anti-inflammatory and immunoregulatory activity and has been proposed as a member of the resolution-associated molecular patterns (RAMPs) family of molecules [6]. As a RAMP, Cpn10 is hypothesised to regulate the acute immune response with therapeutic potential to restore

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homeostasis in a dysregulated setting such as chronic inflammation and autoimmune disease.

Cpn10 members are dome-shaped, heptameric rings comprised of identical 10 kDa subunits [7,8]. The test material described herein is Cpn10 produced in bacteria as a recombinant protein. An N-terminus comparison of native human Cpn10 with recombinant Cpn10 reveals the substitution of two hydrogen (H) groups for a methyl (CH_3) and an amino (NH_3^+) group in recombinant Cpn10 (Ala-Cpn10).

In vitro, recombinant Cpn10 inhibited TNF-α and IL-6 secretion from human peripheral blood mononuclear cells (PBMC) stimulated with bacterial lipopolysaccharide (LPS) [9]. Data from animal models of inflammation show that Cpn10 pre-treatment of mice significantly reduced serum concentrations of TNF-α and regulated upon activation normal T cell expressed and secreted (RANTES) while increasing serum IL-10 following sub-lethal LPS administration [9]. Observations of potential immunomodulatory activity in animal models of human disease, including adjuvant arthritis [10], experimental autoimmune encephalitis [11], allogeneic skin grafting [12], Graft-versus-host disease [9] and systemic lupus erythematosus [13] provide a rationale for clinical trials of Cpn10 safety and efficacy in humans.

Nine clinical studies have been completed investigating recombinant Cpn10 administration in various populations. In the clinical studies conducted to date there appear to be no hepatic, haematologic or other obvious effects of concern. The proof of concept studies in subjects with rheumatoid arthritis (RA) and psoriasis (PS), showed modest immunomodulatory effects and a reduction in the signs and symptoms of disease from pre-treatment levels with intravenous (i.v.) Cpn10 dosing. Cpn10 reduced production of TNF-α, IL-1β and IL-6 in response to LPS stimulation of human PBMC from healthy volunteers and patients with autoimmune disease [multiple sclerosis (MS), psoriasis (PS), rheumatoid arthritis (RA)] [9,14,15]. Combined with the toxicokinetic profile generated thus far, these data confirm the safety and tolerability of Cpn10 in patients and support further investigation in subjects with systemic lupus erythematosus or other autoimmune diseases.

Currently the standard of care for subjects with mild-to-moderate manifestations of SLE includes small molecule compounds such as non-steroidal anti-inflammatory drugs, antimalarial drugs such as hydroxychloroquine and corticosteroids such as prednisone. Patients with active disease and organ threatening lupus are usually treated with immunosuppressants (either methotrexate, leflunomide or azathioprine). Recently approved biological agents (belimumab) target specific cytokines, such as BLyS [B lymphocyte stimulator, also known as BAFF (B-cell activating factor)] a cytokine essential for the survival of B cells, although questions remain regarding the durability of the clincial response [16]. Thus opportunities still exist for the development of new, safe and effective treatments for SLE, including other biological therapeutics for this autoimmune disease.

The production of biologics (such as proteins) in an *Escherichia coli* system incurs the risk of contamination with molecules known as pathogen-associated molecular patterns (PAMPs), the most well-known example being LPS. PAMPs describe the evolutionarily conserved microbial components that are recognised by the eukaryotic host through binding to pattern-recognition receptors such as CD14 or the Toll-like receptors (TLRs) [17]. Endotoxins are pro-inflammatory and thus their presence in biologics may be toxic and result in artefactual effects on eukaryotic cells. It is therefore critical that recombinant protein preparations have minimal impurities such as endotoxin, DNA and other microorganism-derived components.

Regulatory bodies stipulate that the manufacturer of a biologic should assess and characterise both process-related impurities (i.e. host cell proteins, endotoxin, DNA, etc.), and the levels of product-related substances (i.e. molecular variants of the native product) (EMEA, CPMP/ICH/365/96). The product specification sets out an acceptance criteria for impurities and product-related substances within numerical limits, and includes some detail of specific tests and analytical procedures used. Characterisation of the product is performed in the early stages of bioprocess development, and following subsequent changes to the bioprocess. Apart from meeting specifications for process-related impurities, an important facet of bioprocess optimisation is minimising levels of product variant.

In compliance with regulatory requirements and in order to facilitate the safe and effective testing of Cpn10 administration in patients with autoimmune disease, it was essential that a rigorous purification methodology be devised to remove process-related impurities from Cpn10.

This study describes the bioprocess development, production and subsequent characterisation of recombinant human Cpn10 produced in *E. coli*, the first chaperone protein to enter clinical trials for treatment of disease indications associated with inflammation.

2. Materials and methods

2.1. E. coli *host strains*

XL1-Blue (*endA1 gyrA96* (nal^R) *thi-1 recA1 relA1 lac glnV44 hsdR1* F′[::Tn10 *proAB*$^+$ *lacI*^{q} Δ(*lacZ*)M15]) (Stratagene, CA, USA). BR067 (*glnV44 (AS) rfbC1 endA1 spoT1 thi-1 hsdR17 creC510 ΔompT)* is a proprietary derivative of *E. coli* strain MM294 (ATCC #33625) developed at Hospira, Adelaide, Australia.

2.2. *Cloning and expression of human Cpn10 in* E. coli

Initial cloning and expression of Cpn10 was as described in Somodevilla–Torres et al. [18]. Ala-Cpn10 pPL550, a heat-inducible expression plasmid encoding human Cpn10 with an additional N-terminal alanine was transformed into XL1-Blue and Cpn10 expression examined in complex media. A master cell bank (MCB) of the XL1-Blue production clone was established at Acyte Pty Ltd., Sydney, Australia which was subsequently expanded at Hospira, Adelaide, Australia. Synthetic genes with the appropriate flanking restriction enzyme cleavage sites were obtained from GENEART AG, Regensberg, Germany.

Cpn10 expression from Hospira's host strain BR067 and lac promoter-based expression vectors was examined following growth and induction in defined media. A final production strain for manufacture of clinical trial material was selected on the basis of high level Cpn10 expression and concomitant low level expression of product variants. A MCB was established from a single selected clone and a working cell bank (WCB) generated by expansion of a single MCB vial. Both cell banks were characterised to ensure suitability for use in manufacture of clinical grade material.

2.3. *GMP fermentation: XL1-Blue cell line*

A MCB of XL1-Blue with the Ala-Cpn10 pPL550 plasmid was 'pre-cultured' overnight in Soya Peptone Broth with no antibiotic supplementation at 30 °C. An inoculum culture was subsequently prepared for the 150 L bioreactor maintaining the above media and growth temperature parameters. An aliquot of this inoculum was dispensed into a 150 L bioreactor in a minimal media supplemented with 1.5 g L^{-1} vegetable peptone and 1.0 g L^{-1} yeast extract and no antibiotic supplementation. The bioreactor cultivation did not require nutrient feeding and the temperature was maintained throughout the growth phase at 30 ± 2 °C. The pH was maintained at 7.0 ± 0.3 by the addition of an ammonia solution. Induction of Cpn10 was achieved by a temperature shift to 42 °C when the culture reached an optical density at 600 nm ($OD_{600\,nm}$) of 10 and