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From laboratory to Phase I/II cancer trials with recombinant biotherapeutics

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ABSTRACT

Many promising recombinant cancer medicines are generated by academic research and increasing the number of these products that are translated into the clinic will increase the pipeline of new therapies. Recombinant proteins for use in Phase I/II cancer trials must be produced to standards of Good Manufacturing Practice (GMP) in compliance with EU law. This can be a major obstacle for translating experimental products to clinical reality especially when there is no established process or prior experience with GMP. Here, we illustrate the principals of GMP with a step-by-step guide and we show that GMP can be achieved on a relatively small scale in the researchers own institution. The process is exemplified with an antibody-based therapeutic expressed in the yeast *Pichia pastoris*. The purified product has been used safely in patients and the principles are applicable to any recombinant protein required for Phase I/II cancer trials.

1. Introduction

Biological medicinal products such as vaccines, cytokines and antibodies have potential to provide a wide range of new cancer treatments. Antibodies, for example, have resulted in notable success[1] and exciting recombinant antibody-based treatments are becoming increasingly available.[2,3] To evaluate the potential of new recombinant therapies they should be rapidly tested in Phase I/II trials as Investigational Medicinal Products (IMPs).

Approval for a medicinal product intended for use in EU countries is obtained by applying to the European Agency for the Evaluation of Medicinal Products (EMEA; www.emea.eu.int) observing guidelines on the evaluation of anti-cancer medicinal products in man.[4] An essential part of the procedure is that products are manufactured to standards of Good Manufacturing Practice (GMP) in compliance with the EU Clinical Trials Directive.[5,6] The Directive became implemented in national law in May 2004. Although the aim of the Directive is to improve the standards of clinical trials in the Member States without holding back discovery, GMP manufacture of biologics can be a major obstacle for clinical researchers. One option is to finance production by accredited GMP manufactures but it can be more beneficial and cost-effective to develop a GMP production facility in the researchers own institution. Once established, the facility can be used for a variety of subsequent products. Here, we describe how GMP is achieved and we

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set this in context with the pathway of a therapeutic, from molecular design to application for clinical trials authorisation.

The biotherapeutic used to exemplify the process is MFECP1, a fusion protein of an anti-carcinoembryonic antigen (CEA) single chain Fv antibody (scFv) and the enzyme carboxypeptidase G2 (CP).[7,8] MFECP1 is a complex multifunctional molecule, expressed in the yeast *Pichia pastoris* and currently in a Phase I/II clinical trial of Antibody Directed Enzyme Prodrug Therapy (ADEPT).

2. Materials and methods

2.1. *Pathway from bench to bedside*

The developmental pathway from concept to clinical trials authorisation is shown in Fig. 1, with references to relevant guidelines and regulatory documents.[4–6,9–26] The first stage, i.e. fundamental research is generally completed in the laboratory and would normally include *in vitro* and/or *in vivo* models of efficacy. This stage is exemplified with MFECP1 in a

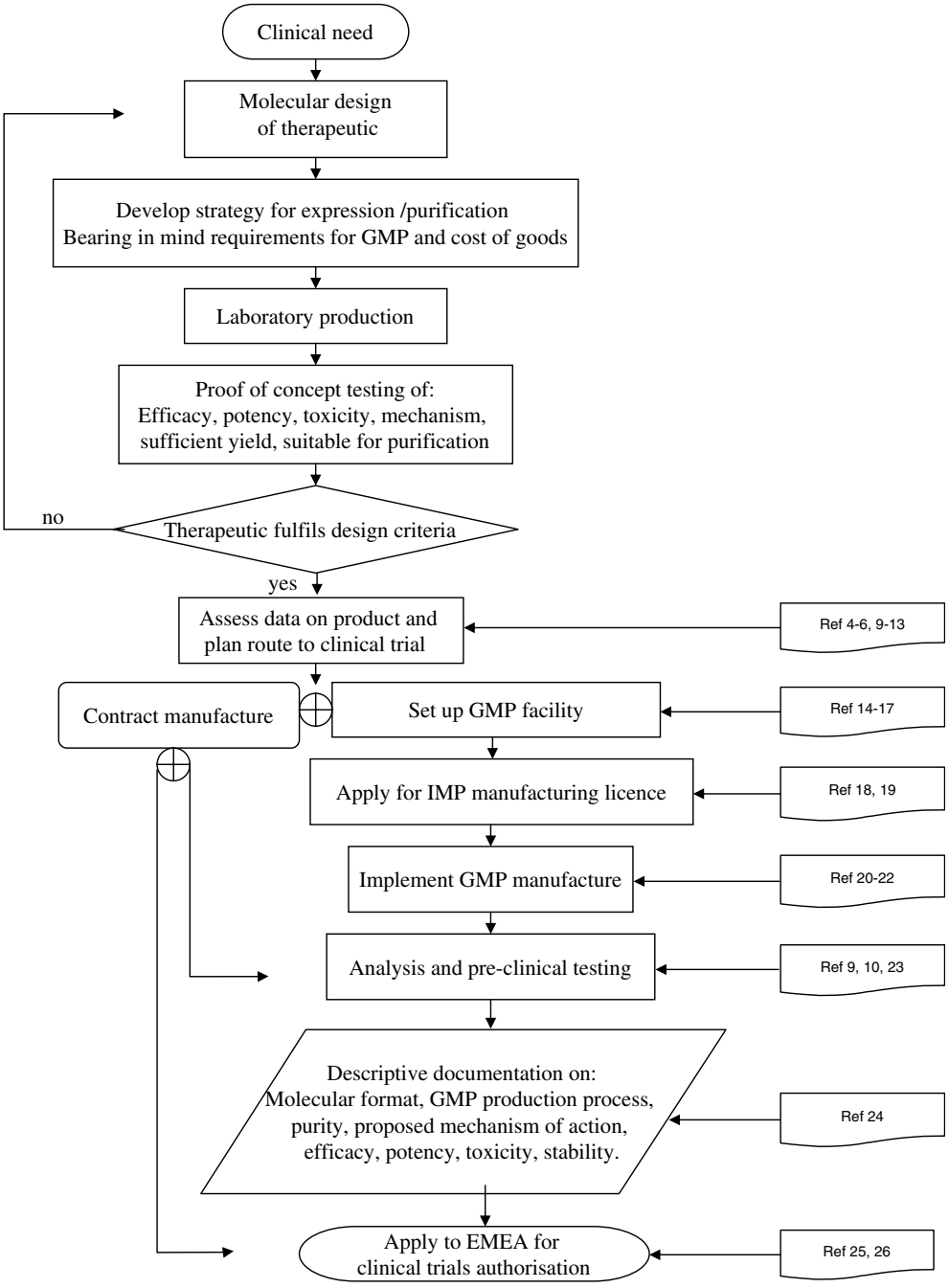

Fig. 1 – Developmental pathway for a recombinant biological. The flow scheme of the major quality assurance activities and the MFECP1 GMP-manufacturing process are indicated. All manufacturing steps, from production of the site master file to the final release by a QP, must be quality assured.

research publication showing preclinical proof of concept.[7] If the new therapeutic agent meets design criteria, and a decision is made to proceed to a clinical trial, the route for GMP manufacture must be established. Companies providing specialist advice and practical support on GMP issues are available to assist with this stage (e.g. www.edenbiodesign.com). The feasibility of GMP manufacture is assessed based on the research data, quantity and quality of product required and the yield and stability of product obtained from the laboratory process. When designing the production process it is important to consider all the product information that will be required to make an application for clinical trials authorisation (Fig. 1).[24]

2.2. GMP

The first step in establishing a GMP process is to take advice from the governing body of the European Economic Area (EEA) member state. Details of these agencies may be obtained via the Medicines Authorities in the European Union and Norway, Iceland, Liechtenstein (http://heads.medagencies.org). When a new application is submitted and a GMP inspection is deemed necessary it is the competent authority of the member state where the site is located that carries out the inspection. For example, to manufacture an IMP in the UK, a licence is required from Medicines and Healthcare Products Regulatory Agency (MHRA). We use a UK example but the principles are applicable to all the EEA because, although practice differs from state to state, there are essentially three main requirements for GMP: a quality system, appropriate environment and trained personnel. Table 1 provides definitions of the regulatory terms used.[14,27,28]

2.2.1. *Quality system*

A quality system is an essential component of compliance and comprises the documents and systematic processes that ensure that quality is evident in all stages of the manufacturing process.[14] For MFECP1 a Quality Policy document was created to define the quality system. This document details all Standard Operating Procedures (SOPs) used in the production process. SOPs are controlled documents with a unique number and version number. SOPs are signed and dated by the preparer and a qualified reviewer.[15] Each element and stage in the manufacturing procedure (processing, product testing, control of the production environment, equipment) must be described by its own SOP.[15] In case an incidental process deviation is required this must be investigated and fully documented and has to be declared on a 'Non-Conformance Procedure' SOP. Permanent amendments or changes to SOPs can be made by using a 'Change Control Procedure' SOP. The layout, approval, issuing, distribution, review and archiving of SOPs is controlled by a superseding SOP the 'Document Control Procedure'. The SOPs relevant to the production process for MFECP1 are available from the authors upon request.

All equipment used within the quality system must be calibrated and maintained in accordance with good laboratory practice (GLP).[16] After establishing a quality system, it should be maintained by regular audits to review and improve the system. Any change to the quality system must be carried out in a controlled way. Change control actions should be fully documented to ensure that all required testing is completed prior to a change and that staff are retrained and documentation supporting the change is maintained.

A product specification must be defined for the IMP and individual batches tested for compliance with the set specification prior to release. This assures identity, reproducibility, purity, biological function and safety. The batch review

Table 1 – Definitions of regulatory terms

Term	Definition
Quality	The degree of fitness for purpose[27]
Quality Assurance (QA)	All activities and functions concerned with the attainment of quality[14]
Good Manufacturing Practice (GMP)	The part of QA that ensures that products are consistently produced and controlled to the quality standards appropriate to their intended use and as required by the marketing authorisation or product specification[14]
Quality Control (QC)	The part of GMP concerned with sampling specifications, testing, documentation and release procedures that ensure that the necessary and relevant tests are carried out and that no materials are released for use until their quality has been judged to be satisfactory[14]
Quality Management	Description of the responsibility for the attainment of the quality objectives undertaken by senior management which requires the participation and commitment by staff at all levels within an organisation[14]
Quality System	Defines and documents a series of systematic processes that are followed by all those working within an organisation. These processes are designed to ensure that quality is evident in every part of the organisation[14]
Standard Operating Procedure (SOP)	Controlled documents that are used to ensure that all activities that affect the quality of the product are controlled[14]
Specification	Describes in detail the requirement with which the products used or obtained during manufacture have to conform in order to enable quality evaluation[27]
Good Laboratory Practice (GLP)	Defines a system of management controls for laboratories and research organisations to ensure the consistency and reliability of data in compliance with international standards[27]
Qualified Person (QP)	Person whose primary legal responsibility is to certify and release batches of medicinal product for use[28]

process is controlled by an SOP, which defines the roles of the batch reviewers. A technical review of each batch is completed by the Quality Control Manager and overall batch approval by the Qualified Person (QP).[20,28]

2.2.2. Environment

The environment component comprises the production facility, equipment and materials. The manufacturing process must take place in a dedicated clean room, which provides a controlled environment that minimises product contamination. For example, the clean room established for MFECP1 was a Class C environment, which means the entrance air is filtered by Class J terminal High Efficiency Particle Arrestance (HEPA) filters. For critical processes a class 100 laminar flow hood (Labcaire VLF6) was used to provide a class A environment. A positive air pressure cascade was created to minimise the ingress of material to the facility. Non-shedding garments were worn in the clean room to minimise contamination from human shedding (a major source of contamination). The pressure differentials for the clean room were logged routinely. Environmental monitoring was achieved using micro-monitoring plates and reviewed as part of the batch release process. The recommended limits for microbial contamination were taken from Annex 1.[21] The clean room is validated annually to ensure that the specification is continuously met. Maximum permitted number of particles/m^3 is derived from Annex 1.[21]

2.2.3. Personnel and training

The roles of personnel must be clearly designated. In compliance with the Directive, the key roles of Production Manager, Quality Control Manager and Qualified Person for Manufacture of MFECP1 are held by different people and their names are listed on the IMP Manufacturing Licence. All personnel involved in Manufacture are trained in GMP, Quality Systems and the regulatory requirements for the conduct of clinical trials.[22] A training record file must be kept for all personnel and all training recorded including external and internal training against SOPs.

2.2.4. Site master file

A site master file must be submitted prior to an IMP licensing inspection. The site master file contains information on the production facility, the product, the quality management system and personnel, including training records.[18] Manufacturing process control is part of a quality system and is usually summarised in a flow chart outlining the interlinkage of all relevant process steps. All stages in the flow chart are controlled by SOPs and materials entering the process are documented as to type, grade and supplier. A certificate of analysis is obtained for all incoming materials. Each batch of recombinant protein produced is given a unique batch number on a batch processing record in which all raw material batch numbers were logged. All crucial stages of the process are signed by manufacturing personnel on the batch processing record. Any deviations during the production process are logged. If further processing takes place in a different department, a Technical Agreement must be generated to clearly define the requirements for both parties.

2.3. The manufacturing process exemplified with MFECP1

The MFECP1 production strain was constructed by transformation of the MFECP1/pPIC001 plasmid[7] into *P. pastoris* X33 strain (Invitrogen Ltd., Paisley, UK). The master seed bank was prepared from a single colony of *P. pastoris* X33::pPIC001 and expanded into a working seed bank as previously described.[29] MFECP1 was produced using our standard protocols for fermentation.[29] The resultant protein is secreted into growth media. There is an engineered C-terminal hexa-histidine tag[7] to facilitate capture and concentration using immobilised metal affinity chromatography (IMAC).[30] Following IMAC, MFECP1 was filtered on a 0.2 μm filtration unit (Nalgene Fisher Scientific Ltd., Leicestershire, UK), concentrated 10-fold and dialysed against five volume changes of phosphate buffered saline (PBS) using a Labscale™ tangential flow filtration unit (Millipore Ltd., Watford, UK) fitted with a Pellicon XL 50 Biomax 30 (50 kDa cut-off) ultrafiltration device. The dialysed product was further purified by size exclusion chromatography using an Akta-Purifier Fast Performance Liquid Chromatography (FPLC; GE Healthcare, Berkshire, UK) fitted with a Superdex 200 column (GE Healthcare). Endotoxins were removed by successive applications to an endotoxin removal gel (Pierce Perbio Science Ltd., Northumberland, UK) until the sample contained less than 2.5 EU of endotoxin/ml as tested with a Limulus Amebocyte Lysate (LAL) test (Pyrogent® Plus 64 Gel Clot Assay, Lonza Biologics plc, Slough, UK). Finally, the product was filter sterilised (0.2 μm) and dispensed into glass vials (GE Healthcare) in the Hospital Pharmacy class A environment with a class B background. The vials were stored at −80 °C in a dedicated freezer.

2.4. Final product analysis

MFECP1 enzyme activity was quantified as previously reported by measuring a change in absorbance at 320 nm due to CPG2 cleavage of methotrexate.[31] All measurements were performed in triplicate. The interaction of MFECP1 with CEA was determined by enzyme-linked immunosorbent assay (ELISA) at room temperature using 96 well plates coated with 1 μg/ml N-A1 recombinant CEA domains,[32] tests were performed in triplicate. Analytical size exclusion chromatography was performed on Superdex 200 column (GE Healthcare). An OD_{280} trace was collected and the elution point (ml) of each batch recorded. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and Western blot analysis were performed essentially as previously described[32] using rabbit polyclonal antibodies to MFE-23 and CPG2 and mouse monoclonal antibody to the hexa-histidine tag. Total glycosylation was measured with a glycoprotein carbohydrate estimation kit (Pierce) according to the manufacturer's instructions. More detailed analysis was performed using the DSA-FACE method.[33]

Sterility and endotoxin analysis were performed by accredited contract laboratories using tests validated in compliance with the European Pharmacopeia 2000. Specifications set were: sterility, negative (14 day incubation) on thioglycollate and tryptone soya media; LAL, maximum 2.5 EU per ml. Samples prior to final filtration and aliquoting were also tested for bioburden. The recommendation for bioburden is

not more than 10 colony forming units per 100 ml prior to fill (for details: www.emea.europa.eu). Contamination with materials used in the production process, such as copper, methanol and imidazole, were also tested by contract laboratories. Contamination with *P. pastoris* host cell components was tested with a Technology F140 kit (Cygnus Technologies, Inc. Southport, NC, USA) according to the manufacturer's instructions.

3. Results

3.1. Purification process

A typical size exclusion FPLC elution profile of material purified by expanded bed IMAC and subsequent concentration/dialysis is shown in Fig. 2a. This profile shows that the partially purified material contained two major protein peaks (OD 280, solid line) and that the enzymatically active protein (+ symbol) elutes in the second of these. Fractions in the shaded area were pooled for further purification by application to the endotoxin removal column. The efficiency of purification is illustrated by removal of *P. pastoris* host cell products throughout the process. Results are shown in Table 2 and demonstrate substantial increases in purity in particular after size exclusion chromatography where the majority of contaminant was found in the high molecular weight fractions. Later stages resulted in even further increases in purity but these were not quantifiable because *P. pastoris* host cell contamination was below the level of detection after application to the endotoxin removal column and subsequent steps of filter sterilisation and vialing (Table 2).

Table 2 – Elimination of P. pastoris host cell contamination during purification

Stage	*P. pastoris* contamination (ng/ml)
Fermentation supernatant	5928
40 mM imidazole wash step	3367
200 mM imidazole eluent	1400
Protein fraction after concentration	589
Protein fraction after dialysis	537
After size exclusion chromatography	12
Final product: after passage through endotoxin gel and filter sterilisation	Not detectable

Table 3 – Final yield and contaminants in final product

Compound	Average, standard deviation ($n = 40$)
Protein yield	19.7 mg/L supernatant, ±7.04
Protein specific activity	134 U/mg, ±16.8
Copper	3.02 μM/L, ±1.06
Glycosylation	7.53%, ±1.82
Methanol	<10 ppm
Imidazole	<5 ppm
P. pastoris host cell	<0.0005%
Endotoxin	<1.0 EU

3.2. Final product

The final product was stored in 1 ml and 10 ml aliquots for analysis and for patient use. Individual vials were defrosted and tested prior to product release and during the clinical trial. The results from 40 independent fermentations are shown in Table 3 and illustrate that all batches were enzymatically active. Furthermore, all batches were reactive with target antigen when tested by ELISA (results not shown) and gave comparable profiles on FPLC. A typical FPLC result is shown in Fig. 2b. Release specification was set to ensure that the MFECP1 eluted at the same volume on a validated column and that the trace did not differ significantly from a reference batch. Specification for SDS–PAGE and Western blot analysis was that the MFECP1 band should be reactive with antibodies to MFE-23, CPG2 and the hexa-histidine tag, all batches met these criteria (results not shown). Consistency of total percentage glycosylation was measured in comparison to a reference batch and to previous batches. Release criteria met by all

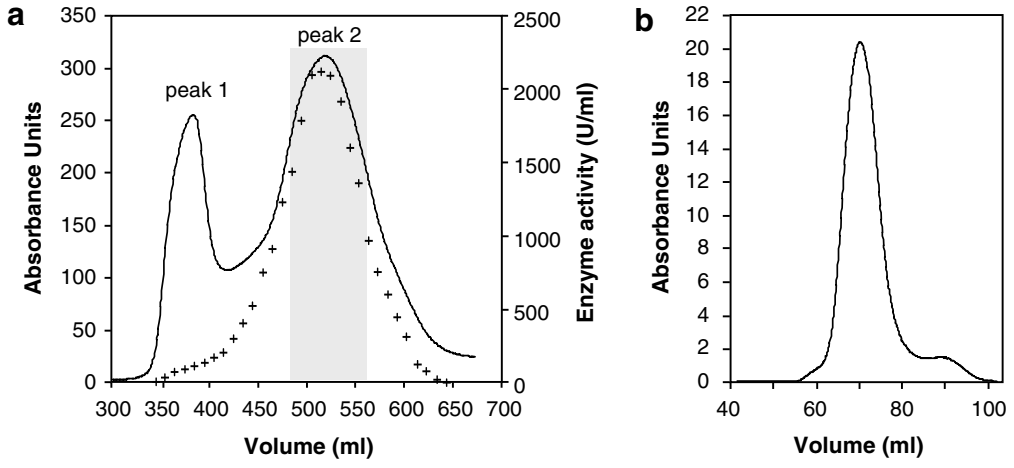

Fig. 2 – Typical size exclusion profiles (Superdex 200) of MFECP1 during purification and analysis. (a) Profile of dialysed product (solid line). Fractions showing CPG2 enzyme activity are indicated (++++). Shaded area shows fractions pooled to form final product. (b) Elution profile of the final product.

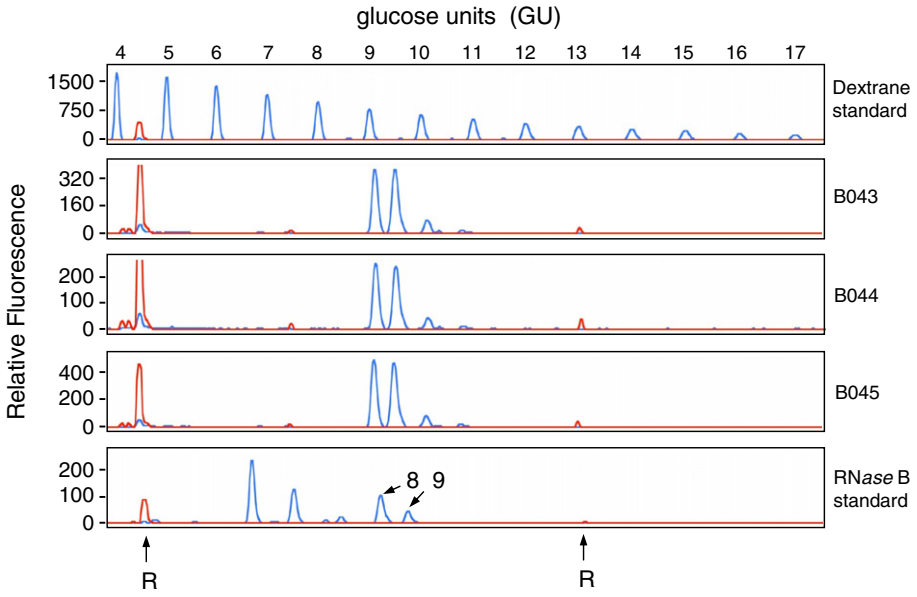

Fig. 3 – DSA-FACE glycosylation profiles. MFECP1 batch comparison of N-glycosylation, as revealed by PNGaseF mediated release of N-glycans. The peaks show relative fluorescence intensity corresponding to eluting N-glycans. Profile 1, molecular weight standard (malto-oligosaccharides or dextrane standard) and the corresponding number of glucose units is indicated; profile 2, MFECP1 batch B043; profile 3, MFECP1 batch B044; profile 4, MFECP1 batch B045; profile 5, RNase B standard. Symbols used: arrows, peaks corresponding to Man_8- and $Man_9GlcNAc_2$; R, peaks corresponding to the rhodamine-labelled internal standards for alignment of individual glycosylation profiles.

batches were that each sample was within 2 standard deviations of the mean of all samples tested in the same assay. The results for all 40 samples are shown in Table 3. All batches were also within set specification for endotoxin copper, methanol and imidazole contamination (Table 3).

3.3. *Consistency of glycosylation*

DSA-FACE analysis of 18 batches of MFECP1 produced over a 1 year period showed a predominant occupation of the N-glycosylation sites by $Man_8GlcNAc_2$ and $Man_9GlcNAc_2$ structures. The average percentage of the glycoforms $Man_8GlcNAc_2$, $Man_9GlcNAc_2$ and Man10GlcNAc2 within these batches is: 41.2 (±4.5)%, 48.8 (±1.8)% and 10.0 (2.8)%, respectively (Fig. 3).

3.4. *Storage stability*

Results obtained by repeating the enzyme activity, ELISA, FPLC, SDS–PAGE and Western blot analysis on aliquots of MFECP1 stored at −80 °C demonstrated that the product was within specification for over 2 years.

3.5. *Clinical trials*

The trial results indicate that MFECP1 is safe to use in man and that the molecular design criteria of CEA binding *in vivo* and rapid elimination from normal tissues[7] are successfully achieved in patients.[8]

4. Discussion

We present a system for production of recombinant proteins in compliance with the EU Clinical Trials Directive. The process is described and exemplified using MFECP1, an antibody-enzyme fusion protein produced in *P. pastoris*. Results from analysis of 40 individual batches are given and all these were within set specification for product release. This *P. pastoris* system was chosen because of its many advantages for production of clinical material. *P. pastoris* grows in chemically defined media without requirement for animal products, cloning is relatively simple and there is potential to produce up to gram quantities of soluble functional mammalian proteins,[34,35] including whole antibodies.[36] Furthermore, *P. pastoris* circumvents safety concerns related to adventitious agents, such as viruses and prions, which can be associated with mammalian expression systems. *P. pastoris* expressed proteins are in clinical development e.g.,[34,37,38] demonstrating feasibility and safety of using these products in man.

P. pastoris performs post-translational modifications such as glycosylation and strains which add human glycoforms are available.[34,36,39] For ADEPT, we exploited the *P. pastoris* high-mannose type N-glycosylation to clear surplus MFECP1 from blood via the mannose receptor.[7,40,41] This resulted in favourable tumour:normal tissue ratios of enzyme, which are required for cancer therapy and safe administration of prodrug to patients.[7,8] To test for batch-to-batch consistency of glycosylation we employed the DSA-FACE method.[33] Our results showed that the oligosaccharide chain length in 18 batches of MFECP1 analysed was the same as reported in initial studies using mass spectrometry.[40]

Our results focus on final product analysis, but it is important to note that this is not sufficient for quality assurance. Regular testing to confirm control and reproducibility of process are also vital for good practice and parameters such as *P. pastoris* biomass development, consistency of feed, the

bioburden and yields of intermediate products should be trended and monitored for variation.

Pre-clinical toxicity testing is a critically important aspect of GMP but is beyond the scope of the current manuscript, largely because the requirements and relevant species for early safety prediction in man will differ according to the type of molecule to be tested. Guidelines are available from the EMEA.[9,10] In some instances, for small molecules, rodent toxicology has been used to predict a safe starting dose and dose limiting toxicity in man.[42] However, biologics are complex, often multi-functional molecules and many have species-specific modes of action for which it is difficult to adequately model.[43] Recombinant biologics aimed specifically at human molecular targets may not show similar activity in other species. Antibody-based molecules, for instance, can have very potent and unpredictable effects[44–47] and require careful consideration in respect to mechanism of action and species of choice for appropriate pre-clinical testing.[48] Adverse effects may be difficult to predict and the need to determine starting doses for clinical trials on the basis of predictions of 'Minimum Anticipated Biological Effect Level' (MABEL) as well as 'No Observed Adverse Effect Level' (NOAEL) has been recently highlighted following enquiries into the phase 1 trial of the anti-CD28 monoclonal antibody TGN1412.[47] Useful information addressing issues of species relevance and primate use for pre-clinical testing has emerged from a recent international workshop.[48]

The aim of the work presented in this paper is to demonstrate feasibility of GMP production for new recombinant anti-cancer therapies in Compliance with EU Clinical Trials Directive. Whilst it is not possible to provide all the information required for such an endeavour, we have aimed to provide insight into GMP and outlined key areas with working examples.

Conflict of interest statement

There is no conflict of interest. The authors confirm that there are no financial or personal relationships with other people or organisations that could inappropriately influence (bias) their work.

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