PRODUCTION OF AN ORPHAN DRUG FOR PHASE I CLINICAL TRIAL: RECOMBINANT HUMAN BMP4

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Introduction

BMP4 belongs to the superfamily of Transforming Growth Factor β (TGF-β), and regulates the early stages of the embryonic development and many processes of the adult tissues homeostasis.
It is a homodimer of two subunits (116 residues for subunit) with three disulfide bridges (two intra-monomer and one inter–monomers). Each monomer has two glycosylation sites.
BMP4 is secreted as mature protein, but it is synthesized as 408 residues molecule: residues 1-19 (signal peptide), residues 20-292 (propeptide). The post-translational modification is regulated by the Furin, which cleaves the proteins along the motif -R-X-R/K-R-, and also -R-X-X-R- (Molloy at al., 1992). Cui Y et al (2001) demonstrated that a sequential cleavage is necessary to have the active mature BMP4.
Recent studies have pointed out a possible use of BMP4 as a putative therapeutic agent against tumors and particularly against the human glioblastomas (Piccirillo et al., 2006). In 2015, the European Medicinal Agency (EMA) has granted Stemgen the status of Orphan Drug for the use of BMP4 in the treatment of glioblastoma and a phase I clinical study, sponsored by Stemgen itself, is going to begin in Europe.
The whole manufacturing process of BMP4 has been developed at IBI-Lorenzini, from the R&D department to the Biotechnology and Fill&Finish Departments, where the production of the API and the finished product are currently been made.

Process Development (Upstream)

The upstream development started from the gene transfection and the clone selection.
The parameters optimized were: temperature, dissolved oxygen; % CO_2; pH; rate of the stirrer (for flasks); speed rate and rock angle (for waves).
Indeed the most critical parameter was the selection of the best culture media and the feeds.

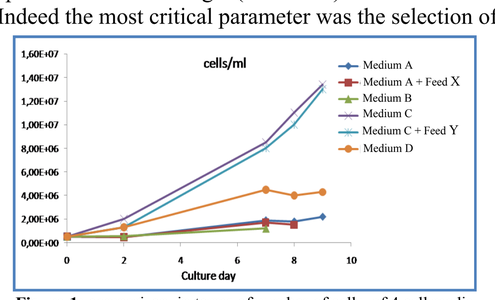


Figure 1: comparison, in terms of number of cells, of 4 cell media and 2 feeds. Medium C is clearly those where cells growth well.

The main responses were: number of cells (fig. 1); vitality; productivity (fig. 2); quality of the protein (fig. 3).

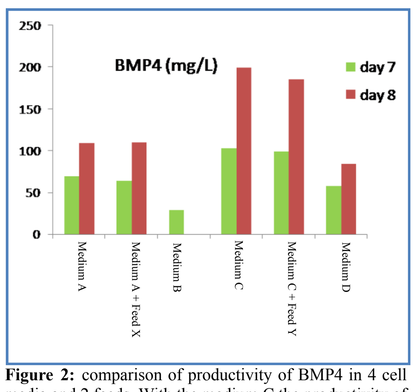


Figure 2: comparison of productivity of BMP4 in 4 cell media and 2 feeds. With the medium C the productivity of BMP4 is higher. However the ratio between the productivity of medium C and A, at the day 8, is about 2.0, while the ratio of number of cells (fig. 1) is about 6.0.

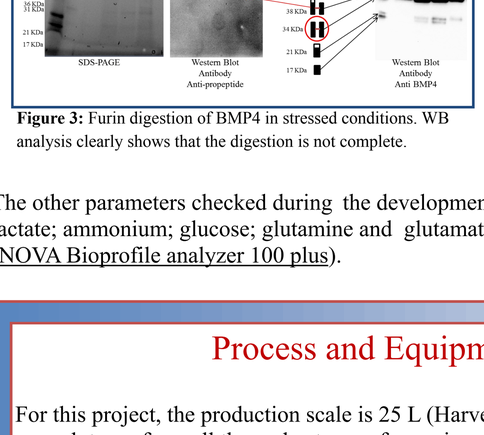


Figure 3: Furin digestion of BMP4 in stressed conditions. WB analysis clearly shows that the digestion is not complete.

The other parameters checked during the development, were:
lactate; ammonium; glucose; glutamine and glutamate concentration (NOVA Bioprofile analyzer 100 plus).

Process Development (Downstream)

The downstream development has been carried out in parallel with the upstream, mainly supporting its development.
In the early stage it has been collected data about some general properties of BMP4, for example: pI (fig. 4); hydrophobicity of the protein; stability at different pH and at different salt concentration (fig. 5).

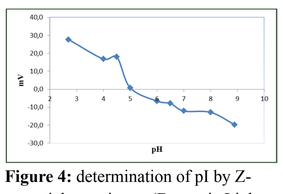


Figure 4: determination of pI by Z-potential experiment (Dynamic Light Scatter, Zetasizer nano series, Malvern)

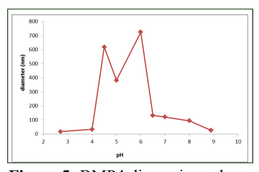
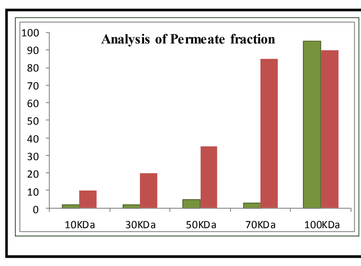
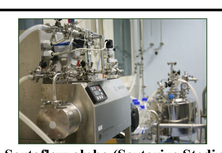


Figure 5: BMP4 dimensions changes at different pH (Dynamic Light Scatter, Zetasizer nano series, Malvern)





Sartoflow alpha (Sartorius Stedim)

Figure 6: % BMP4 (green) and % of other proteins recovered (red) in the permeate fraction at different MWCO

In parallel, several filters have been tested for the clarification step (removal of cells and cells debris), as well as some filters for a possible dial-filtration (fig. 6).

AKTA Explorer

The optimization involved: resin selection; buffers composition (pH, conductivity); volumetric flow rate; binding capacity; residence time; columns dimension (AKTA Explorer).

These parameters have been optimize by DoE experiments (**Minitab 17**): the responses are yield and purity of the protein (fig. 7).

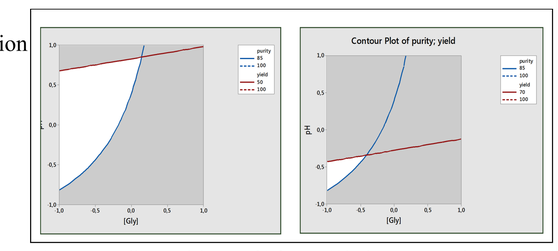


Figure 7: Full Factorial DoE of 2 factors (Glycine concentration and pH), 2 levels and 2 responses (yield and purity). White area shows where the factors contemporary fulfill both the responses target. Maintaining the lowest purity level at 85%, in the left panel it has been chosen a lowest yield level of 50%, while in the right panel a lowest level of 70%.

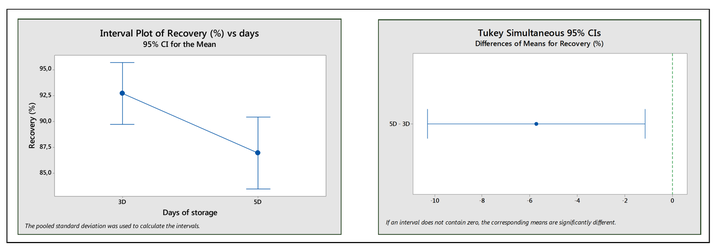
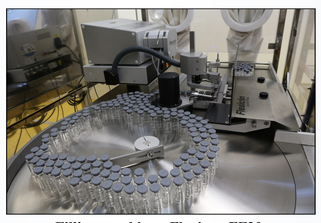


Figure 8: ANOVA regression of the percentage of recovery of BMP4 concentrated after a storage at RT for 3 day and 5 days. The analysis of variance shows that there is a significant difference between the means with a confidence interval of 95%, so the null hypothesis (the means are equal) was rejected .

Statistical software was also useful to operate studies of stability and to check all the critical parameters of the process (fig. 8)

BMP4 specifications (HCP content, % of aggregates; RP-HPLC purity; residual DNA, viral clearance) were in accordance with the current ICH guidelines.
The process has been further scaled-up (AKTA Pilot).
In parallel other critical process parameters have been investigated by a risk assessment approach: life-time of the columns; cleaning in place and holding time of the equipment.

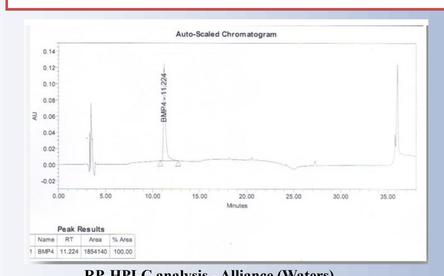
Freeze-dryer - Criofarma C80-7S



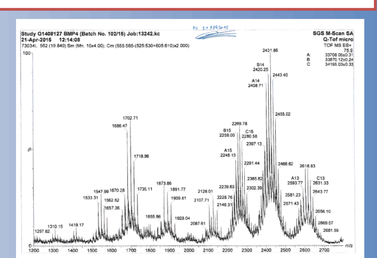
Filling machine - Flexicon FF20

Protein Characterization

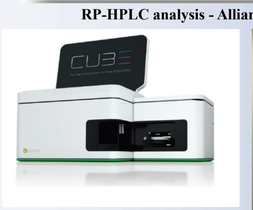
In parallel to the upstream and downstream development, it has been performed a characterization of the protein, as required for the phase I clinical study. The most analytical methods have been developed and qualified in our laboratories.



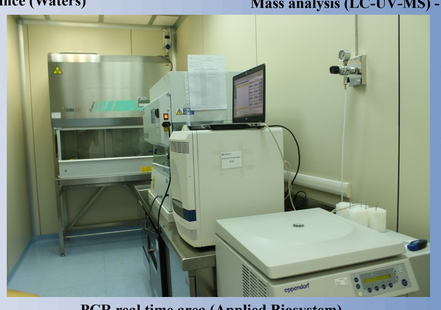
RP-HPLC analysis - Alliance (Waters)



Mass analysis (LC-UV-MS) - by SGS



Flow cytometer CyFlow Cube 8 (Partec)



PCR real time area (Applied Biosystem)

Blitz (Fortebio PALL) Quantitation and kinetics analysis

Process and Equipment (see Flow-Chart)

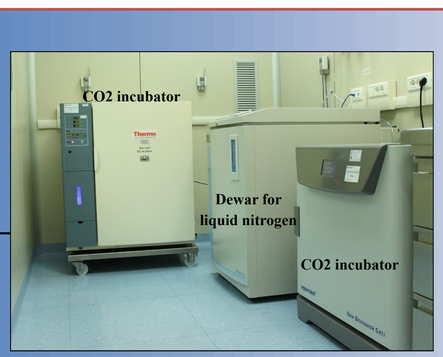
For this project, the production scale is 25 L (Harvest), similar to a traditional pilot scale, and it is enough to perform all the early stages of experimental trials, from pre-clinical to phase I.

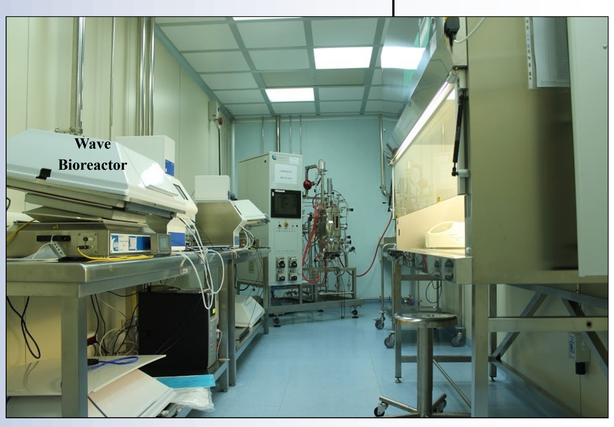
Upstream

The upstream starts from one vial of a WCB/MCB . The cell banks are stored in a vapour phase of liquid N_2.
Expansion in flask: CO2 incubator with a orbital Shaker.
Expansion in single-use cell bag: Wave Bioreactor 20/50 (GE Healthcare).
During the cell culture expansions, media formulation changes. The viability of the cells are the main parameter which must be controlled.

Downstream

There are two capture steps (mixed-mode and cation exchange) and one polishing step (anion exchange, removal of HCP and aggregates). Between the capture steps, there is a viral inactivation and, at the end of BMP4 purification, a nano-filtration is performed (viral clearance).





AKTA Pilot

BPG and Axichrom columns

Stainless steel tanks for buffers preparation

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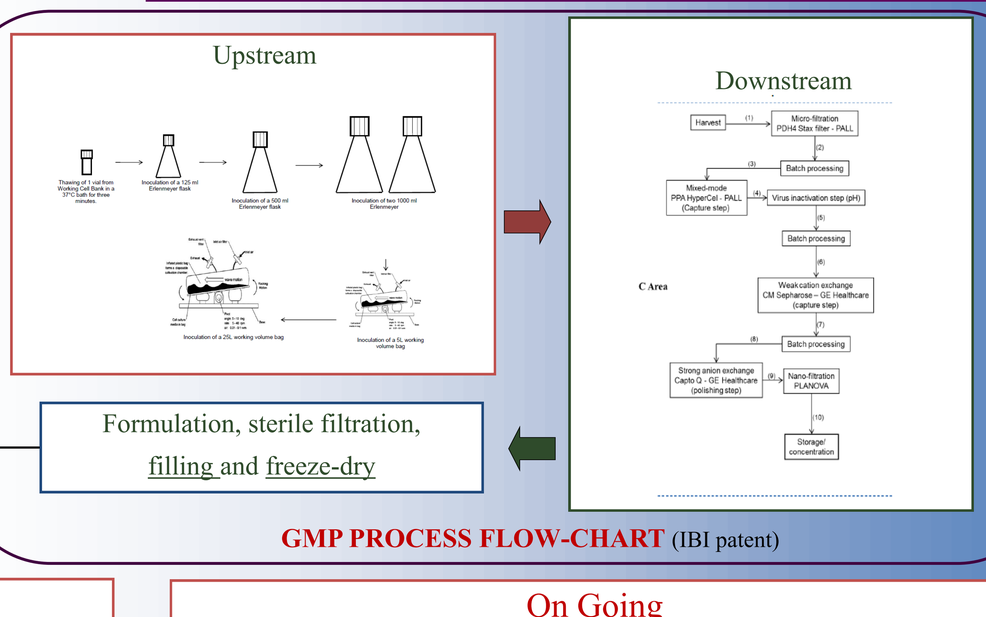

GMP PROCESS FLOW-CHART (IBI patent)

Process Controls

The key question is: **Is the process in control?**
The answer is not easy and needs a control plan of the process.

Upstream controls

1. aminoacid composition of the harvest;
2. analysis of the main metabolites;
3. number of cells (million/ml) (fig.9).

Downstream control

Analysis of the intermediates of purification:

1. recovery;
2. purity;
3. percentage of aggregates.

Other key parameteres of the process are constantly monitored and analyzed by a statistical method (fig. 10).

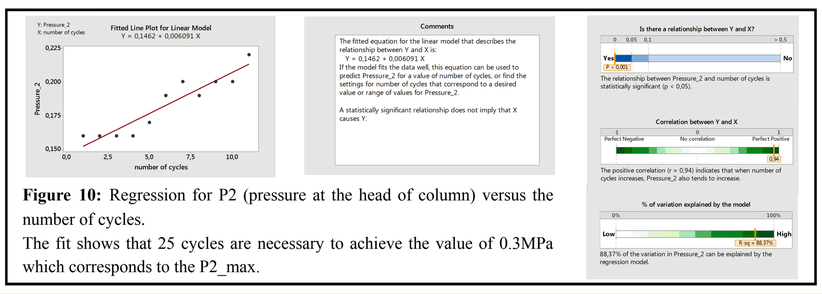

Figure 10: Regression for P2 (pressure at the head of column) versus the number of cycles.
The fit shows that 25 cycles are necessary to achieve the value of 0.3MPa which corresponds to the P2_max.

On Going

The phase I clinical study of an Orphan Drug is quite fast because of the few number of patients who should be treated. This is the reason why it is very important to be ready for the phase II.

Working plan: 1.scale-up of the process; 2. improvement of the process .

Scale-up: our target is to achieve 200L of harvest for upstream (Wave Bioreactor 200).
We are going to introduce an AKTAProcess for the chromatographic steps.
We will introduce some mixers with 3D single-use bags of 1000L (working volume 100-1000L) provided of pH and conductivity probes (NO CLEANING VALIDATION).

Improvement: in parallel the process will be reviewed and improved trying to increase the productivity, and to better understand the process, according to the approach of **Quality by Design**.

References

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Y. Cui, R. Hackenmiller, L. Berg, F. Jean, T. Nakayama, G. Thomas, and J. L. Christian; Genes & Dev. 2001. 15: 2797-2802

S. G. M. Piccirillo, B. A. Reynolds, N. Zanetti, G. Lamorte, E. Binda, G. Broggi, H. Brem, A. Olivi, F. Dimeco & L. Vescovi, Nature, 2006, ***444****, 761-765*