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New Expression Method and Characterization of Recombinant Human Granulocyte Colony Stimulating Factor in a Stable Protein Formulation

Ralitza Boubeva[a], Christian Reichert[a], René Handrick[b], Claudia Müller[a], Jürgen Hannemann[b], and Gerrit Borchard*[a]

Abstract: Human recombinant granulocyte colony stimulating factor (rhG-CSF) is widely used in hematology and oncology for the treatment of neutropenia, for the restoration of neutrophil production after bone marrow transplantation, for myelodysplastic syndromes, and aplastic anemia. The *E. coli* expression system is commonly used for fast recombinant production of rhG-CSF at a large scale. We have applied a novel autoinduction method for the batch expression of rhG-CSF to study whether this new system would increase cell mass and target-protein yield compared to conventional *E. coli* cell culture and induction with isopropyl β-D-thiogalactopyranoside (IPTG). We could demonstrate 3-fold higher culture densities and a 5-fold higher protein yield compared to IPTG induction without the need to monitor cell growth in a shortened 24 h expression procedure. rhG-CSF expressed in autoinduction media was successfully extracted from *E. coli* inclusion bodies and refolded by dialysis. After size exclusion chromatography (SEC) purification, rhG-CSF showed similar conformation, biological activity and aggregation profile compared to the commercially available biosimilar TEVAgrastim® (TEVA Pharma AG). Expression by autoinduction is suggested as a cost- and time-effective method for rhG-CSF production.

Keywords: Autoinduction media · Protein expression · Protein refolding · Protein stability · rhG-CSF

Introduction

Colony-stimulating factors are hematopoietic proteins that stimulate the proliferation and differentiation of hematopoietic cells from pluripotent stem cells. Granulocyte colony stimulating factor (G-CSF), in particular, enhances the proliferation of specific myeloid precursor cells into granulocytes.[1] Currently human G-CSF is widely used in hematology and oncology for the treatment of neutropenia, for the restoration of neutrophil production after bone marrow transplantation, for myelodysplastic syndromes, aplastic anemia as well as in the treatment of immunodeficiency and in prevention of infectious complications.[2]

There are three marketed recombinant forms of human G-CSF (rhG-CSF), which were approved chronologically as filgrastim (Amgen Inc., 1991), lenograstim (Chugai Pharmaceutical Co., 1993) and pegfilgrastim (Amgen Inc., 2007).[3] Among the three, lenograstim is the only version of rhG-CSF produced in a mammalian (Chinese hamster ovary, CHO) cell line and presents therefore the glycosylated pattern of the natural human G-CSF. Even though it has been demonstrated that O-glycosylation confers a higher stability to rhG-CSF by protecting the sulfhydryl group of a free Cys17 residue, it does not influence the biological activity of the therapeutic protein.[4] Thus, the majority of patients are presently treated with filgrastim or its PEGylated version, pegfilgrastim. For both, the protein is recombinantly expressed in *Escherichia coli* (*E. coli*) after insertion of the human G-CSF gene into the bacteria and has the same amino acid sequence as human G-CSF plus an N-terminal methionine residue needed for expression. Since the ≈19kDa protein is expressed in bacteria, the typical O-glycosylation obtained in mammalian cells as well as the solubility that the latter confers to G-CSF are lost. The resulting non-soluble rhG-CSF is produced in *E. coli* as inclusion bodies (IBs) and needs to be subjected to oxidative refolding in order to restore biological activity of the protein.[5] The refolding of rhG-CSF after expression in *E. coli* is a crucial step for obtaining filgrastim on a large scale and constant efforts are aiming at improving refolding yields.[6] Besides the hurdle of downstream refolding, biosynthesis in *E. coli* has the advantage of fast and industrial-scale expression of rhG-CSF and remains among the preferred methods for producing therapeutic proteins. Successful production of rhG-CSF at different scales for non-therapeutic use has been also reported in the COS cell line,[7] *Pichia pastoris*,[8] *Saccharomyces cerevisiae*,[9] transgenic animals,[10] or plant cultures.[11]

The aim of the present work was to improve the expression yield of rhG-CSF in *E. coli* for small batch production by developing a process that would be easily scalable for clinical rhG-CSF manufacture. We have applied a recently developed autoinduction method for the batch expression of rhG-CSF to study whether this new system would increase cell mass and target-protein yield compared to conventional *E. coli* cell culture and induction with isopropyl β-D-thiogalactopyranoside (IPTG).[12] The second goal of this study was to compare the conformation, activity and stability of rhG-CSF produced in autoinduction medium to the commercially available filgrastim (TEVAgrastim®, TEVA Pharma AG).

*Correspondence: Prof. G. Borchard[a]
Tel.: +41 22 379 69 45
Fax: +41 22 379 65 67
E-mail: Gerrit.Borchard@unige.ch
[a]School of Pharmaceutical Sciences
University of Geneva, University of Lausanne
30 Quai Ernest-Ansermet, CH-1211 Geneva 4
[b]University of Applied Sciences
35 Hubertus-Liebrecht Straße, D-88400 Biberach, Germany

Experimental

All chemicals were purchased from Applichem (Germany), unless otherwise indicated.

Cloning

cDNA of non-glycosylated mature 174 amino acid splice variant of human G-CSF (CSF3, Genbank accession NP_757373; aa31-aa204 of the isoform B precursor) was synthesized at GeneArt® Gene Synthesis (Life Technologies Corp.) into a pMA shuttle vector. The synthetic construct was designed with an additional N'-terminal methionine, with adaptation of codon usage for expression in pET23-system (Merck4Biosciences)[12b] and with both 5' NdeI and a 3' XhoI restriction sites for further subcloning into the expression vector pET23a(+). pET 23a(+) carrying the cDNA for rhG-CSF was then used to transform BL21(DE3)pLysS *E. Coli* (Merck4Biosciences).

Expression

Transformed BL21(DE3)pLysS *E. Coli* cells were resuspended in 2 × 5 ml Luria-Bertani (LB) medium (Difco Laboratories, USA) supplemented with 100 µg/ml ampicillin. The pre-cultures were incubated at 29 °C, 200 rpm till OD_{600} = 0.8. 1L of selective autoinduction medium containing 10 g Tryptone (Difco Laboratories, USA), 5 g yeast extract (Difco Laboratories, USA), 137 mM NaCl, 0.5 M $(NH_4)_2SO_4$, 1 M KH_2PO_4, 1 M Na_2HPO_4, 25 g glycerol, 2.5 g D-glucose, 10 g α-lactose[12a] was inoculated with the first rhG-CSF pre-culture at 29 °C, 170 rpm for 24 h. In parallel, 1 L of selective LB medium was inoculated with the second rhG-CSF pre-culture and left at 29 °C, 180 rpm till OD_{600} = 0.8. Then, rhG-CSF expression was induced with 2 M IPTG for 16 h at 29 °C, 170 rpm. Both cell cultures were centrifuged at 4 °C, 3500 × g for 20 min and cell pellets were stored at –20 °C.

IB Isolation and Solubilization

Cell pellets were resuspended in 30 ml Lysis buffer (30 mM Tris pH 8.5, 1 mM ethylene diamine tetra acetic acid (EDTA), 0.1% Triton X-100) supplemented with benzonase nuclease 25 U/ml (Merck4Biosciences) and 1 mM phenylmethylsulfonyl fluoride (PMSF). Cells were disrupted by three cycles of homogenization using a French press. Cell debris and IBs were sedimented by centrifugation at 4 °C, 7000 × g for 40 min. The supernatant was discarded and the IBs were resuspended and washed in 30 ml Lysis buffer supplemented with 1 mM PMSF for 30 min at 4 °C. IBs were sedimented by centrifugation at 4 °C, 7000 × g for 20 min. The supernatant was discarded and IBs were resuspended and washed in 30 ml Lysis buffer supplemented with 0.2% deoxycholic acid for 30 min at 4 °C. IBs were sedimented by centrifugation at 4 °C, 7000 × g for 20 min. The supernatant was discarded and the IBs were resuspended and washed twice for 30 min in 30 ml 30 mM Tris pH 8.5 in endotoxin-free water. IBs free of cell debris, DNA/RNA and endotoxins were finally sedimented by centrifugation at 4 °C, 7000 × g for 20 min and solubilized in 10 ml 30 mM Tris pH 8.5, 1 mM EDTA, 8 M urea and 0.1 M 2-hydroxy-1-ethanethiol overnight at 4 °C under 50 rpm agitation.

rhG-CSF Refolding

rhG-CSF extracted in 8 M urea was refolded by stepwise dialysis against 2 M urea buffer (30 mM Tris pH 8.5, 2 M urea, 2.5 mM reduced glutathione, 0.8 mM oxidized glutathione) at 4 °C, 50 rpm through a 6–8 kDa MWCO Spectra/Por® membrane (Spectrum Laboratories).

Size Exclusion Chromatography (SEC) and Formulation

After dialysis rhG-CSF was concentrated to 2 ml with Corning Spin-X UF concentrator 5 kDa MWCO (Corning Inc.) at 4 °C, 4000 × g. The concentrated protein was then filtered and loaded onto a Superdex 75 column (GE Healthcare) equilibrated with 30 mM Tris pH 8.5, 2 M urea buffer. The protein was eluted at room temperature at a flow-rate of 0.5 ml/min using the ÅKTA FPLC system (GE Healthcare). Pooled fractions containing refolded rhG-CSF were submitted to three cycles of sequential concentration (4 °C, 10 000 × g) and dilution with formulation buffer (10 mM acetate buffer pH 4.2, sorbitol 5% m/w, Tween 0.003% v/v) through a 5 kDa MWCO membrane.

Bioactivity Studies on rhG-CSF Produced in Autoinduction Medium vs. TEVAgrastim®

Proliferation Test

Cells of the mouse myeloid line NFS-60 (CLS, Germany) were cultured in suspension in RPMI 1640 GlutaMax® (Life Technologies) supplemented with 10% fetal calf serum (Life Technologies), 1 mM sodium pyruvate, 50 µg/ml streptomycin, 50 U/ml penicillin (Life technologies), 10 ng/ml IL-3 (Sigma-Aldrich) and maintained at 37 °C/5% CO_2. The cells were allowed to reach an exponential growth phase with the density of ≈10^6/ml and the media was exchanged to supplemented RPMI 1640 GlutaMax® with no IL-3. Then the cells were transferred to a 96-well plate (20 000 cells/well) and the test rhG-CSF preparations were added to the wells; each concentration was tested on six wells. After 48 h of incubation, 10 µl of the WST-1 reagent (Roche Applied Science) were added to each well and the incubation was continued for 3 h. The color change reflecting the number of viable cells was detected at 450 nm *vs.* 690 nm on a BioTek ELISA reader (BioTek).

Activation of the Jak/STAT-3 Signaling Pathway

Mouse myeloid NFS-60 cells were cultured in supplemented RPMI 1640 GlutaMax® with 10 ng/ml of test rhG-CSF preparations and IL-3, respectively. Six million cells of each culture were then sedimented at 450 × g for 5 min at 4 °C and the supernatant was discarded. 100 µl of cold RIPA buffer (Sigma-Aldrich) was added to the cell pellet, vortexed and incubated on ice for 10 min. The cell lysate was centrifuged at 8000 × g at 4 °C and the supernatant was used for immunoblotting.

50 µg of total protein from the cell lysate was boiled at 95 °C for 5 min and separated on a 10% SDS gel for 45 min/200 V. The 0.2 µm pore nitrocellulose membrane (Sigma-Aldrich) and the extra thick blot paper (BioRad) were equilibrated in water and transfer buffer (25 mM Tris, 0.2 M glycine, 10% methanol, 0.05% SDS), respectively, for 20 min. The protein transfer was carried out at 18 V, at room temperature for 90 min in a Trans-Blot Semi-Dry Transfer Cell (BioRad). The nitrocellulose membrane was blocked for 60 min in 20 mM Tris, 137 mM NaCl, 5% skimmed milk and 0.05% Tween 20. The primary antibody solution (1:20 000 rabbit anti-phosphoTyr$_{705}$-STAT-3 or 1:2000 rabbit anti STAT-3 (Merck-Millipore) in blocking buffer) was incubated overnight at 4 °C. The secondary antibody solution (1:4000 goat anti-rabbit HRP-conjugated antibody (Merck-Millipore) in blocking buffer) was incubated 60 min at room temperature. Chemiluminescence Detection Kit (Applichem) and FUJI medical X-ray film (FUJIFILM Corp.) were used to reveal the western blot after 30 s of exposure.

Physico-chemical Studies on rhG-CSF Produced in Autoinduction Medium vs. TEVAgrastim®

Conformation-Sensitive SDS-PAGE

3 µg of rhG-CSF samples and TEVAgrastim® were loaded on a SDS-free acrylamide gel. Electrophoresis was run for 2 h at room temperature at 125 V on a Mini-PROTEAN® electrophoresys system (BioRad Laboratories). A SilverQuest Silver Staining Kit (Life Technologies) was used for protein visualization.

Circular Dichroism Spectra

Circular dichroism (CD) spectra were recorded at 4 °C on a Jasco J-815 spec-

trophotometer (Jasco Inc.) equipped with a water-cooled Peltier unit in 0.05 cm Hellma quartz cuvette model 110-QS. CD spectra measurements were taken for 0.15 mg/ml rhG-CSF at a scan rate of 100 nm/min with a data pitch of 0.5 nm and a band width of 1 nm. Each spectrum represents the mean of two scans from 250 nm to 200 nm. Buffers were used as blanks and their spectrum was subtracted from the recorded spectra of the samples. The secondary structure content was estimated by deconvolution procedures using Spectra Manager (Jasco Inc.). The CD unit used is the mean molar ellipticity (θ) expressed in (deg. $cm^2.dmol^{-1}$) where rhG-CSF and TEVAgrastim® contained 175 residues.

Melting Temperature Studies

To define the midpoint of thermal denaturation (T_M) for rhG-CSF and for TEVAgrastim® spectra were collected at 220 nm within the temperature range of 4–90 °C on J-815 spectrophotometer (Jasco Inc.) equipped with a water-cooled Peltier unit in 0.05 cm Hellma quartz cuvette model 110-QS. Data were collected from 0.15 mg/ml rhG-CSF samples. The rate of heating was fixed at 1 °C/min with data pitch of 1 °C and a delay time of 1 sec. T_M was calculated using Spectra Manager (Jasco Inc.).

Turbidity Studies

The turbidity of rhG-CSF and TEVAgrastim® was evaluated by measuring the absorbance of both formulations at 350 nm on a Cary 50 UV-Vis spectrophotometer (Varian Inc.) in triplicate.

Fluorescence Studies

The extrinsic fluorescence of rhG-CSF and TEVAgrastim® was measured with a Fluoromax-4 spectrofluorometer (Horiba Jobin Yvon Inc.) using 1 mM Nile Red (Acros Organics). Both protein formulations were at a concentration of 0.15 mg/ml in water. Fluorescence spectra were recorded at 25 °C in a 1 cm pathlength quartz cuvette (Hellma). Settings for the measurements were: λ_{ex} = 541 nm, excitation slit 2 nm and emission slit 5 nm, integration time 1 s. Each measurement was performed in triplicate.

Results

Transformed *E. coli* BL21 (DE3)pLysS harboring pET23(a)-rhG-CSF were grown in 1 L shake flask batch cultures. The expression of the protein was conducted in parallel in LB medium with induction by IPTG as a standard procedure and in autoinduction medium as described by Studier.[12a] rhG-CSF produced by both methods was expressed in the form of IBs, which were then washed and solubilized as outlined in the Experimental section. Proteins from both expression media were then solubilized in 8 M urea, refolded and purified by SEC under identical conditions. The expression in autoinduction media allowed over three-fold higher culture density and over five-fold higher protein mass recovery per 1 L of media (Table 1). The specific yield (rhG-CSF recovered from IBs per gram biomass) was improved by more than 1.7-fold.

Table 1. Summary of the efficiency of rhG-CSF production and recovery after expression in 1L LB upon IPTG induction vs. 1L autoinduction media

Expression method	1% glucose / 2M IPTG /48 h	Autoinduction media /24 h
Wet cell mass	2.6 g	7.1 g
IBs' mass	39.4 mg	185.7 mg
rhG-CSF recovered from IBs	37.6 mg	180.3 mg
Refolded rhG-CSF	14.6 mg	75.7 mg
Efficiency of refolding[a]	39%	42%

[a]The efficiency of refolding is calculated as the ratio between the protein recovered from IBs and the amount of refolded protein.

Refolded and purified rhG-CSF from autoinduction media was formulated as TEVAgrastim® and its conformation was compared to the marketed rhG-CSF from TEVA Pharma AG. CD spectra and conformational-sensitive SDS-PAGE indicated similar α-helical content and conformation between the formulated rhG-CSF from autoinduction media and the marketed TEVAgrastim® (Fig. 1).

The biological activity of rhG-CSF expressed in autoinduction media was assayed by two *in vitro* approaches. The first method measures the specific activity of the protein formulation through the proliferation of murine myeloid NFS-60 cells in presence of the studied and reference rhG-CSF. The bioactivity of rhG-CSF (0.85 ng/ml *i.e.* 0.85×10^6 IU/mg) expressed in autoinduction media was comparable to the activity of TEVAgrastim® (0.80 ng/ml *i.e.* 0.8×10^6 IU/mg) (Fig. 2A). The second assay is based on the activation of the Jak/STAT-3 pathway and the phosphorylation of STAT-3 in murine myeloid NFS-60 cells in presence of active rhG-CSF. STAT-3 phosphorylation was similar when NFS-60 cells were cultured with rhG-CSF expressed in autoinduction media and with TEVAgrastim® (Fig. 2B). The aggregation level of rhG-CSF expressed in autoinduction media was investigated at 25 °C by absorbance at 350 nm and fluorescence spectroscopy, and compared to TEVAgrastim®. The extrinsic fluorescence emission of rhG-CSF measured in presence of Nile Red was 5.7% lower than the extrinsic fluorescence emission of TEVAgrastim® (Fig. 3A). The turbidity of rhG-CSF expressed in autoinduction media remained comparable to the one of TEVAgrastim® (Fig. 3B). The stability of rhG-CSF expressed in autoinduction media *vs.* TEVAgrastim® was also mapped by circular dichroism under temperature stress. The thermal stability of both protein formulations was identical (Fig. 4).

Discussion and Conclusions

E. coli is the most widely used system for the production of recombinant human G-CSF at small and industrial scale[12b] because of its simple nutrient requirement,

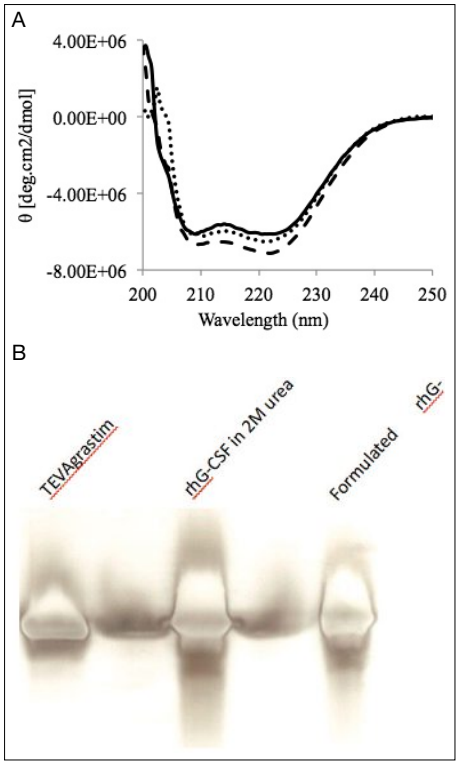

Fig. 1. Conformational analysis of refolded and formulated rhG-CSF after expression in autoinduction medium. A. CD spectrum of refolded rhG-CSF (dotted line), formulated rhG-CSF (full line) and TEVAgrastim® (dashed line) at 0.15 mg/ml in water. Data are plotted in terms of molar ellipticity [θ]. For each protein an average of two measurements is shown. B. Conformation-sensitive poly-acrylamide electrophoresis on 15% sodium dodecyl sulfate-free gel for formulated rhG-CSF expressed in autoinduction media and TEVAgrastim®. Visualization was achieved by silver-staining.

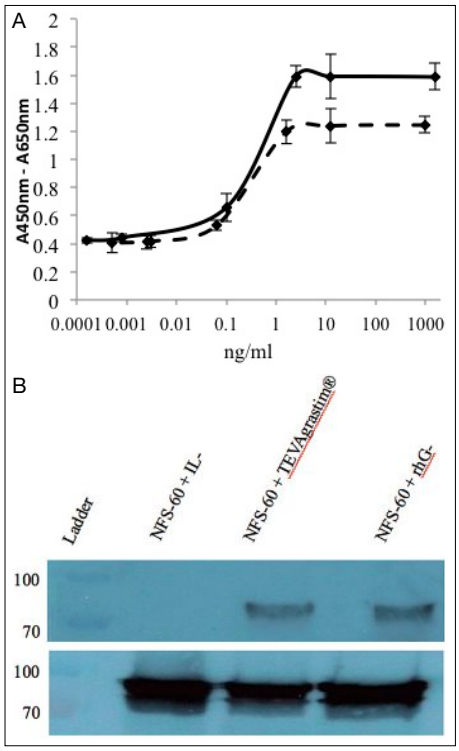

Fig. 2. Activity of rhG-CSF expressed in autoinduction medium. A. WST-1 cell proliferation assay on murine myeloid NFS-60 cells for formulated rhG-CSF and TEVAgrastim®. B. STAT-3 phosphorylation by formulated rhG-CSF and TEVAgrastim® detected by Western Blot with anti-phosphoTyr$_{705}$-STAT-3 antibody (on the top). STAT-3 expression detected with anti-STAT-3 antibody (on the bottom). IL-3 was used as negative control for STAT-3 phosphorylation.

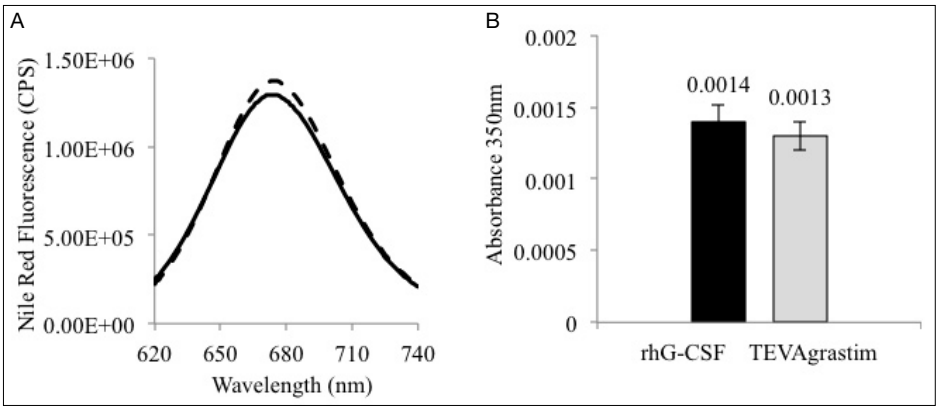

Fig. 3. Detection of protein aggregates in formulated rhG-CSF expressed in autoinduction medium. A. Comparison of fluorescence spectra of formulated rhG-CSF expressed in autoinduction medium (full line) and TEVAgrastim® (dashed line). Both proteins were measured at 25 °C in water at a concentration of 0.15 mg/ml. For each protein an average of three measurements is shown. The fluorescence emission spectrum was measured in presence of 1mM Nile Red. B. Turbidity (absorbance at 350 nm) of rhG-CSF expressed in autoinduction medium and of TEVAgrastim® at 0.15 mg/ml in water.

high growth rate and its well known molecular genetics and physiology.[13] Four main strategies may be adopted to enhance the recombinant protein production, namely, choice of culture medium, mode of cultivation, strain development and expression system control.[13] In this work we aimed at developing a simple, inexpensive and routine production of rhG-CSF at small, laboratory scale by flask batch culture and by the widely used *E. coli* BL21(DE3)pLysS strain and pET expression system for rhG-CSF expression.[6b,14] Thus, we have compared the use of two different expression system controls, a conventional IPTG induction system common for the recombinant protein expression in pET systems and a recently described autoinduction control, which has not been investigated yet for the up-stream processing of rhG-CSF.[12a,15]

The autoinduction medium provides for 'autoinduction' of protein expression without the need to add inducers such as IPTG during mid-log phase of the cultures. The method is based on a buffered medium that contains a mixture of carbon sources, including lactose. Bacteria initially use glucose; when the glucose is exhausted, lactose can enter the cell and induce expression of the T7 polymerase from the DE3 lambda lysogen. The medium provides high culture densities and high yields of proteins, usually in inclusion bodies and may be used for the expression of recombinant proteins that are ultimately driven by the *lac* promoter such as recombinant proteins encoded into the pET expression system. The expression of rhG-CSF in autoinduction medium allowed over three-fold higher cell densities and over five-fold higher protein yield per 1L of culture medium compared to expression under IPTG induction. Moreover, the recombinant protein production was faster, due to the spontaneous induction by lactose, without monitoring cell density and without induction with IPTG. The overall costs of expression were also reduced by omitting the addition of IPTG.

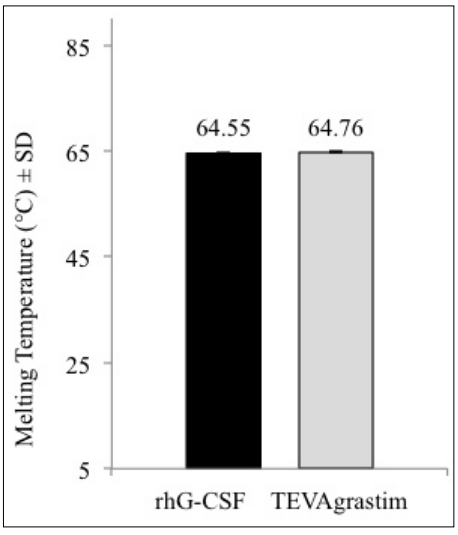

Fig. 4. Thermal stability of formulated rhG-CSF after expression in autoinduction medium and TEVAgrastim®. The midpoint of the thermal denaturation curve (T_M) was defined for samples containing 0.15 mg/ml of protein in water with Jasco Manager (Jasco, Japan) on melting temperature curves registered at 220 nm, within the temperature range of 5–90 °C. Standard deviations (SD) are shown as vertical bars and represent the average of two independent experiments.

The second goal of the present work was to compare the conformation, activity and physical stability of rhG-CSF expressed in autoinduction medium to the commercially available rhG-CSF, TEVAgrastim®. rhG-CSF expressed in autoinduction medium demonstrated similar specific activity and Jak/STAT-3 activation as the control and thus remained in the range of 80–120% of the TEVAgrastim® activity, thus remaining in the biological experiment reproductivity range.

The comparable migration profile of the rhG-CSF formulation to the TEVAgrastim® control on a conformational-sensitive gel suggested that rhG-CSF expressed in autoinduction medium did not present oligomeric or aggregated forms neither partially unfolded nor modified conformations in comparison to the control. The α-helical content (47%) was slightly lower than the α-helical content calculated for the control (50%), but remained comparable to reported experimental values (50% α-helices in rhG-CSF)[16] and their variation might be due to the fact that complete spectra, including the far UV region from 200 nm to 180 nm, could not be measured because of traces of formulation buffer within both samples.

The level of aggregation of the rhG-CSF formulation was investigated by two complementary techniques. The UV absorbance at 350 nm was used to measure the turbidity of the pharmaceutical preparation and the extrinsic fluorescence in presence of Nile Red dye was employed to evaluate the presence of aggregates and/or partial protein denaturation.[17] In both

assays rhG-CSF expressed in autoinduction medium showed similar levels of aggregation (turbidity) or even lower than TEVAgrastim® (Nile Red Fluorescence). Moreover, the thermal unfolding temperature of formulated rhG-CSF obtained by autoinduction appeared to be similar to the one of TEVAgrastim®.

In conclusion, various assays analyzing the conformation, activity and aggregate levels indicate that rhG-CSF from autoinduction medium has a similar profile to the marketed filgrastim (TEVA). rhG-CSF expression by autoinduction in *E. coli* is suggested as a simple, cost- and time-effective alternative to rhG-CSF production and can be further investigated for combination with various downstream rhG-CSF protocols[6a,6c,18] and for an up-scaled hG-CSF recombinant production.

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