ISSN 1682-296X (Print)
ISSN 1682-2978 (Online)

Bio Technology

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Biotechnology 14 (4): 200-205, 2015
ISSN 1682-296X

SHORT COMMUNICATION OPEN ACCESS

DOI: 10.3923/biotech.2015.200.205

Site-Specifically Modified Recombinant Human Granulocyte Colony-Stimulating Factor with Polyethylene Glycol

See-Hyoung Park
Department of Transdisciplinary Studies, Program in Nano Science and Technology, Seoul National University Graduate School of Convergence Science and Technology, Suwon, 443-270, South Korea

ARTICLE INFO
Article History:
Received: April 06, 2015
Accepted: May 26, 2015

Corresponding Author:
See-Hyoung Park
Department of Transdisciplinary Studies, Program in Nano Science and Technology,
Seoul National University Graduate School of Convergence Science and Technology, Suwon, 443-270,
South Korea

ABSTRACT
To prepare PEGylated recombinant human granulocyte colony-stimulating factor (rHuG-CSF) with enhanced pharmacokinetic properties, we prepared a cysteine-substituted mutant of rHuG-CSF (mrHuG-CSF). For site-specific PEGylation of mrHuG-CSF, Threonine 134 residue that is one of the glycosylation sites and thought not to be critical in structure and function of rHuG-CSF was substituted by Cysteine. Also, Cysteine 17 residue at N-terminus that does not make di-sulfide bonds and is partially buried was substituted by Serine. This mrHuG-CSF was then site-specifically conjugated with 5 or 20 kDa of polyethylene glycol-maleimide and the correct molecular weight of the conjugates was confirmed by MALDI-TOF mass spectrometry analysis. Compared with unmodified rHuG-CSF, both PEGylated mrHuG-CSF showed similar biological activities *in vitro* and the plasma half-life of the 20 kDa PEGylated mrHuG-CSF was about 5-folds increased. Taken together, site-specific PEGylation of mrHuG-CSF may increase their therapeutic potency in humans.

Key words: Granulocyte colony-stimulating factor, polyethylene glycol, pharmacokinetics

INTRODUCTION

Granulocyte Colony-Stimulating Factor (G-CSF) is used to care cancer patients, who are treated by chemotherapy or radiotherapy in order to alleviate the decrease of white blood cell levels by non-specific cytotoxic therapeutics (Baldwin *et al*., 1996; Chaiken and Williams, 1996). The G-CSF is one of the growth factors involving in proliferation, differentiation and functional activation of blood cells (Demetri and Griffin, 1991). Currently, recombinant human G-CSF (rHuG-CSF) has been produced in genetically engineered *Escherichia coli* (*E. coli*) for treating cancer patients suffering from chemotherapy-induced neutropenia clinically (Vemula *et al*., 2015). The rHuG-CSF from *E. coli* is composed of 175 amino acids with an additional Methionine (at position-1) at N-terminal. Also, rHuG-CSF from *E. coli* has a free Cysteine (at position 17) and 2 intra-molecular disulfide bonds (Cys36-Cys42 and Cys64-Cys74) (Zink *et al*., 1994; Mott and Campbell, 1995; Wells and de Vos, 1996). Like other *E. coli* derived recombinant proteins, rHuG-CSF from *E. coli* needs an oxidative folding step in order to regain its biological activity from inclusion body (Fahnert *et al*., 2004). In addition, rHuG-CSF has to be administered to patients with subcutaneous injection of large quantity of rHuG-CSF (5-10 g kg^{-1}) frequently (Baldwin *et al*., 1996). Furthermore, like other protein therapeutics, rHuG-CSF needs strict storage condition and specific formulation method (Lauffenburger *et al*., 1998). So, people have been trying to develop the improved method that still keep the biological function of the protein drugs, but have the enhanced pharmacological properties by studying protein reengineering technology.

A modification (PEGylation) of protein drug with Polyethylene Glycol (PEG) is one of the most efficient ways to increase the pharmacological properties of them by increasing their molecular weight and size, which leads into improved plasma half-lives and proteolytic-stability and a

diminished immunogenicity and kidney/hepatic uptake (Pai *et al*., 1991; Chaffee *et al*., 1992; Yabe *et al*., 1999). However, so far almost of the PEGylation of a protein drugs are using amine-PEGylation method, which is reported to be often nonspecific and conjugated at all of the free amine group of lysine residues in the protein (Fontana *et al*., 2008). This unexpected modification causes the protein drugs to be less active biologically than the unmodified protein drugs since the lysine may be in or near the active-site of the protein. Thus, site-specific PEGylation of the Cys substituted protein for the free thiol reaction has been used for the attachment of PEG to protein drug. Substitution of a free Cys residue should be into a amino acid residue that is not making a disulfide bond and thought to be not critical in proteins drug's conformational structure and biological function. A free Cys residue can provide an attachment point for the covalent chemical bond between the free thiol of the Cys-substituted protein drugs and the PEG-maleimide molecule. This chemical modification is very specific since other Cys residues in protein drugs are taking a part in disulfide bonds during the oxidative re-folding steps and so cannot be assessed and reacted by cysteine-reactive PEG-maleimide molecules (Benhar *et al*., 1994; Kuan *et al*., 1994; Tsutsumi *et al*., 2000). Thus, site-specific PEGylation by using free thiol chemistry can have a protein drug be selectively modified by PEG-maleimide molecules at the chosen residues and lower the possible problems of the final PEGylated protein drugs' heterogeneity caused by amine-PEGylation.

In this study, we chose a different approach to site-specific PEGylation. To keep the receptor-binding and molecular conformation, we prepared a mutant of rHuG-CSF (mrHuG-CSF) with one cysteine in one of the glycosylation sites that was modified with PEG-maleimide. The PEGylated mrHuG-CSF had comparable *in vitro* specific activity against cells, but stability and plasma half-life were greatly improved.

MATERIALS AND METHODS

Materials: Methoxy-PEG-maleimide (PEG-maleimide, molecular weight: 5 or 20 kDa) was obtained from Shearwater Polymers (Huntsville, AL). Other reagents were from standard sources.

Bacterial strains and plasmid: *Escherichia coli* pWAGF, which was derived from the ara host strain *E. coli* MC1061, was used to produce the recombinant human G-CSF and as template for oligodeoxynucleotide-directed mutagenesis. The plasmid pGW2.0 in which the structural gene of rHuG-CSF was cloned under the control of the L-arabinose promoter, encodes rHuG-CSF (J. Microbiology Biotechnology, 2000, 10, 3, 321-326).

Mutagenesis of rHuG-CSF: In order to substitute a free Cys residue, site-directed mutagenesis of rHuG-CSF was done by the mutagenesis kit from Stratagene (La Jolla, CA) with some modifications. Mutations in the plasmid were confirmed by DNA sequencing. Synthetic oligonucleotide primers were used to amplify the supercoiled double-stranded DNA template plasmid. Primers complementary to opposite strands of the vector were extended using Pfu Turbo polymerase (Stratagene, La Jolla, CA) for 18 cycles. The resulting PCR product was digested with DpnI to eliminate parental DNA and transformed into DH5 *E. coli*.

Expression of mrHuG-CSF: The components of Cys-substituted mrHuG-CSF were produced from *E. coli* MC1601 containing the corresponding expression plasmids (pGW2.0 or mutant pGW2.0) as described (Choi *et al*., 2000). Cys-substituted mrHuG-CSF was cultured in 5 mL of LB media supplemented with Ampicillin for 12 h that was transferred to 500 mL LB media for main culturing. When the Optical Density (OD) of the culture broth reached to 0.5, l% (w/v) of arabinose was added to the culture media to induce the expression of mrHuG-CSF.

Oxidative refolding of mrHu G-CSF: Strains of the overexpressed cysteine-induced mutant G-CSF were centrifuged to be suspended in Tris 20 and 150 mM NaCl and the suspended strains were disrupted using a sonicator. The disrupted strains were centrifuged and washed with 2% (w/v) sodium deoxycholate to recover inclusion bodies. The inclusion bodies of the cysteine-induced mutants were dissolved in 7 M urea, 20 mM Tris and 100 mM NaCl at pH 8.8 and 10-fold diluted in 3.3 M urea, 5 mM Tris, 2 mM reduced glutathione and 0.2 M oxidized glutathione at pH 8.8. The refolding step was performed with agitation at 4°C for 18 h.

PEGylation of mrHuG-CSF: A procedure for preparation of the PEGylated mrHuG-CSF is as follows. mrHuG-CSF with a free thiol group (at Thr134) in PBS was allowed to react with 5-10 folds molar excess of 5 or 20 kDa PEG-maleimide (PEG 5 or 20K) at 4°C for 12 h. The reaction mixture was adjusted to pH 4.0 with 10% HCl solution and directly loaded on a SP-Sepharose column that was previously equilibrated with equilibrium buffer (20 mM monobasic sodium phosphate, pH 4.8). The column was washed with 50 volumes of equilibrium buffer for removing uncoupled PEG 5 or 20K and then was eluted with elution buffer (20 mM monobasic sodium phosphate, 200 mM NaCl, pH 4.8) to roughly separate PEGylated mrHuG-CSF from unmodified mutant rHuG-CSF. After concentrating the appropriate fractions by Vivaspin 6 (Vivaproduct, Littleton, MA), the crude PEGylated mrHuG-CSF was separated on Superdex-75 or -200 that was equilibrated and eluted with proper buffer (20 mM monobasic sodium phosphate, 150 mM NaCl, pH 4.8). The obtained PEGylated mrHuG-CSF was extremely pure, according to SDS-PAGE analysis and iodine analysis.

Pharmacokinetic study: Normal Sprague Dawly male rat (6-7 weeks old; about 200 g, 5 per each group), was injected subcutaneously with 100 μg of rHuG-CSF, 20 kDa

PEG-mrHuG-CSF, or Neulasta (as control). Blood samples (0.3 mL) were collected from the eye at 0.5, 1, 2, 4, 8, 12, 16, 20, 24, 30, 36, 48, 60, 72, 96 and 120 h after the injection. Blood samples were allowed to clot, then centrifuged and stored frozen until analysis. For a quantity of plasma G-CSF, samples were analyzed by means of an enzyme linked immunological and biological binding assay. In brief, after appropriate dilution with a suitable buffer solution, samples are bound to a microtiter plate coated with mouse monoclonal antibodies against the human G-CSF and after incubation and washing, the concentration of G-CSF are detected by incubation with a peroxidase-conjugated anti-G-CSF polyclonal antibody and peroxidase substrate solution coupled to horseradish peroxidase.

RESULTS

Preparation and characterization of PEGylated mrHuG-CSF: To prevent loss of the receptor binding and biological functions of rHuG-CSF that are necessary for its specific activity, we prepared a mutant form of rHuG-CSF with one Cys instead of the Thr134 residue that is one of the glycosylation site. The free Cys residue was used for site-specific PEGylation using free thiol chemistry. As shown in Fig. 1a, the PEGylated mrHuG-CSF molecules ran as a single band on SDS-PAGE, when eluted from a Superdex-75 size exclusion column as a single peak. We used naïve PAGE gel for analysis of the purified PEGylated mrHuG-CSF since, there is problem such as smear band shape when we have used reduced SDS-PAGE gel analysis. However, still naïve PAGE analysis resulted in the higher molecular weight location than we expected (Fig. 1a). Thus, we tried to adapt MALDI-TOF mass spectrometry analysis for measuring the exact and correct molecular weight of the finally purified PEGylated mrHuG-CSF. As shown in Fig. 1b and c, we could check that our PEGylated mrHuG-CSF has the correct mass spectrum distribution. The specific *in vitro* bioactivity of PEGylated mrHuG-CSF was similar to that of native rhG-CSF. The yield of highly purified PEGylated mrHuG-CSF prepared from inclusion bodies was 7.0%, which is the almost same as that of the native rhG-CSF (7.3%). Thus, we purified mrHuG-CSF with a free thiol group that was site-specifically modified with PEG 5 or 20K-maleimide by the formation of a thio-ether bond. After purification, both types of PEGylated mrHuG-CSF had similar biological activities that were the same as the unmodified native rhG-CSF.

Pharmacokinetics of PEGylated mrHuG-CSF: Rats were injected subcutaneously with a single dose of 100 µg of rHuG-CSF, 20K PEG-mrHuG-CSF and Neulasta. Neulasta was used for the one of controls. Neulasta is a trade name of a 20 kDa PEGylated rHuG-CSF developed by Amgen, which has been prepared using N-terminus PEGylation method. It has been known that Neulasta has a half-life of 15-80 h in human

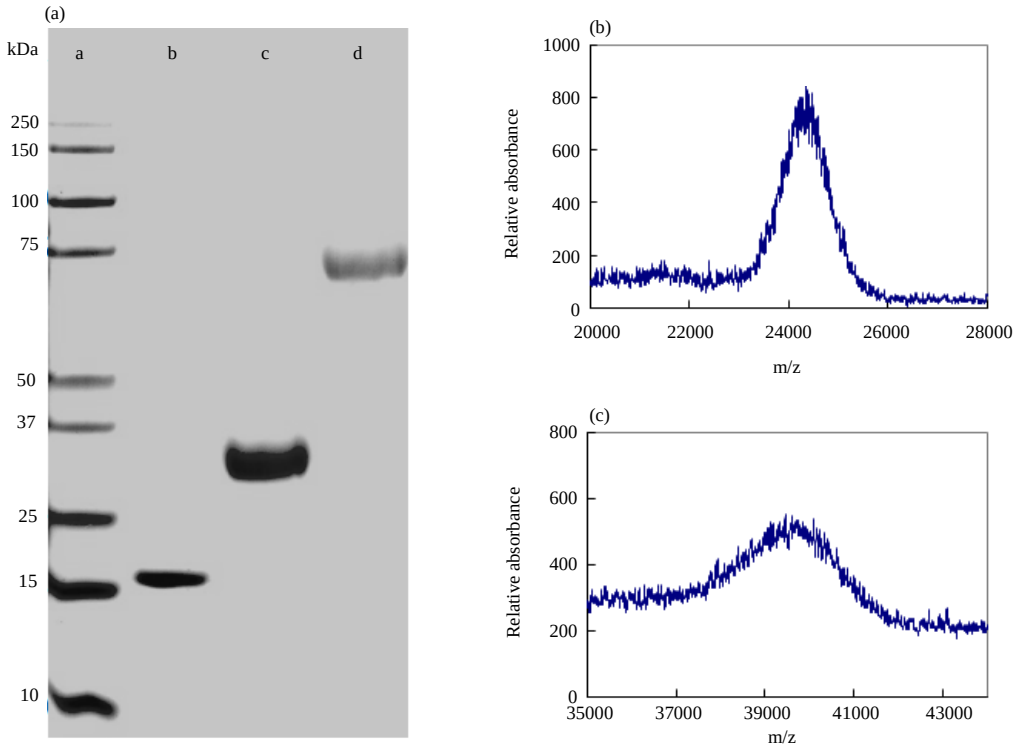

Fig. 1(a-c): Properties of 20K PEG-mrHuG-CSF, (a) Naïve-PAGE analysis of 20K PEG-mrHuG-CSF, a: Molecular weight standards from top to bottom, b: rHuG-CSF, c: 5K PEG-mrHuG-CSF, d: 20K PEG-mrHuG-CSF), (b) MALDI-TOF mass spectra of 5K PEG-mrHuG-CSF and (c) MALDI-TOF mass spectra of 205K PEG-mrHuG-CSF

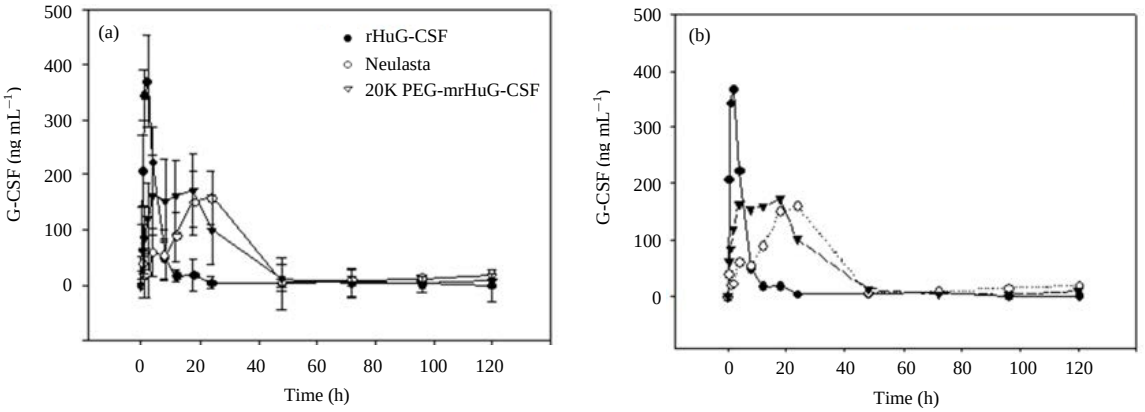

Fig. 2(a-b): Pharmacokinetic study of 20K PEG-mrHuG-CSF in rat. Normal Sprague Dawly male rat (5 per group) were injected subcutaneously with 100 µg of rHuG-CSF, Neulasta and 20K PEG-mrHuG-CSF, receptively. Blood samples were drawn at different times. The level of G-CSF was measured by ELISA method by anti-human G-CSF antibody, (a) Mean serum concentration-time profile with standard error and (b) Mean serum concentration-time profile without standard

Table 1: Pharmacokinetic parameters

Parameter	rHug-CSF	Neulasta	20K PEG-meHuG-CSF
C_{max} (ng mL^{-1})	368	159	173
T_{max} (h)	2	24	18
$T_{1/2}$ (h)	5	36	28

that is much longer than the unmodified rHuG-CSF. Thus, Neulasta served as a kind of positive control for our pharmacokinetic study. Blood was drawn at different times after the injection and was assayed for rHuG-CSF levels. Pharmacokinetic parameters such as the elimination half life ($T_{1/2}$) (Table 1), the maximum serum concentration (C_{max}), the time to reach the maximum serum concentration (T_{max}) were calculated using, Sigma Plot fitting analysis software. As shown in Fig. 2a and b, the 20K PEG-mrHuG-CSF showed 5 times higher plasma half life than that of rHuG-CSF *in vivo* suggesting that of 20K PEG-mrHuG-CSF can be circulated in blood stream of rat for a longer period than the parent rHuG-CSF, thereby improving the therapeutic efficacy. However, the serum concentration profiles of both Neulasta and 20K PEGylated mrHuG-CSF showed the similar elimination curves (Fig. 2a and b). The plasma half-life of Neulasta and 20K PEGylated mrHuG-CSF was 36 and 28 h that was increased about 5-fold and that of PEG20K-LMB-2 about 8-fold. The 20K PEG-mrHuG-CSF reached its peak plasma concentration at 18 h after injection which was much longer (about 9 times) than the rHuG-CSF. Furthermore, C_{max} of 20K PEG-mrHuG-CSF as the indicator of the long acting drugs, was about 2 times lower than that of rHuG-CSF. Thus, 20K PEGmrHuG-CSF has potential advantages for further clinical trials.

DISCUSSION

Since, PEGylation of a protein can increase the size of the protein and decrease the clearance rate of the protein, conjugation of proteins with PEG is known to be one of very useful methods to improve the half-lives of proteins and a few PEGylated protein drugs are now approved for humans. Abuchowski *et al*. (1977) were the first researcher to prove the extension of half-life of bovine liver catalase could be accomplished with PEGylation. Also, PEGylation can raise the solubility and stability of the protein and diminish the immunogenicity of the protein.

Initially, the most common method for PEGylation of proteins adapted the modification of the N-terminus free amine modification using N-hydroxysuccinimide (NHS)-PEG. However, this way has a limitation that PEGylation occurs randomly at any residues of the free amines in the protein and causes a heterogeneous product such as multiple PEGylated byproducts. These heterogeneous PEGylated products might lose its original biological activities.

Thus, the novel PEGylation to overcome the heterogeneity and loss of biological activity of amine-PEGylated protein has been developed, which can allow protein to be modified with the selective modification of PEG at a specific site. As one of these approaches, PEGylation of a free cysteine residue in proteins has been tried in the protein formulation filed. This way has an advantage such as the homogeneous production by the specific reaction with cysteines and minimization a loss of biological activity by the pre-determined selection of the candidate sites in protein.

Recently, Kunstelj *et al*. (2013) reported a new PEGylation reagent that allows the selective modification of free cysteine at the position 18 in recombinant human G-CSF. They used (PEG) N-hydroxysuccinimide to selenocystamine reacting with thiols via thiol/diselenide reaction. Also, they mentioned that the reaction was very fast and resulting in over 95% yield. Compared to using maleimide (PEG-Mal), it was almost same in terms of the reaction rate and yield. However, *in vitro* biological activity of the

conjugate prepared with PEG-Se was higher than that of the maleimide conjugate. Thus, we might need to adopt this new PEGylating agent to our current mutant G-CSF and compare the production yield, biological activity and pharmacokinetic/dynamics later (Kunstelj *et al*., 2013).

As another example, Scaramuzza *et al*. (2012) showed the novel mono-20 kDa PEGylation of recombinant G-CSF reacted by microbial transglutaminase enzyme site-specifically at glutamine 135 residue. Using recombinant G-CSF as model system, they demonstrated the advantage of the site-specific enzymatic PEGylation by showing the simple and efficient purification step/yields and a panel of physicochemical analyses of the product. They concluded that this new site specific mono-20 kDa PEGylated G-CSF is a promising candidate drugs for preclinical and clinical tests targeting oncological patients under chemotherapy treatments. Although this novel concept of PEGylation was working for the recombinant G-CSF to show the homologous and biologically same active product, the use of the specific enzyme from microorganism might cause more attention to be paid to controlling the overall preparation process since, it is necessary to have another checking step, the removal of the used microbial enzymes in the regard of the regulatory agent for the commercialization of the PEGylated protein drugs. Thus, this study might have the academic approach than the industrial application (Scaramuzza *et al*., 2012).

The present study reports on the preparation and pharmacokinetics of PEG 20K mrHuG-CSF to provide an experimental basis before clinical trials. Introduction of a free Cys residue in rHuG-CSF and derivatization with PEG-maleimide allowed a very specific conjugation yielding homogeneous product. The Cys-substituted rHuG-CSF was produced in sufficiently large amounts, reacted site-specifically with PEG-maleimide (5 or 20 kDa) and formulated for the evaluation of their pharmacokinetic potencies in rats. The 20K PEG-mrHuG-CSF had an original biological activity that confirmed Thr134 residue is not involved in binding to its receptor. The average elimination half-life of 20K PEG-mrHuG-CSF was longer than that of rHuG-CSF with the same dose and the other main pharmacokinetic parameters of 20K PEG-mrHuG-CSF including C_{max} and T_{max}, were much greater than those of rHuG-CSF. However, we still need to evaluate the pharmacodynamic study of the 20K PEG-mrHuG-CSF and compare this with other possible modifiable derivatives for substitution of a free Cys to find the better candidates with both of the pharmacokinetic and pharmacodynamic properties in human.

ACKNOWLEDGMENTS

This study was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) Funded by the Ministry of Education, Science and Technology (NRF-2014R1A6A3A04054307).

REFERENCES

Abuchowski, A., J.R. McCoy, N.C. Palczuk, T. van Es and F.F. Davis, 1977. Effect of covalent attachment of polyethylene glycol on immunogenicity and circulating life of bovine liver catalase. J. Biol. Chem., 252: 3582-3586.

Baldwin, E., J. Xu, O. Hajiseyedjavadi, W.A. Baase and B.W. Matthews, 1996. Thermodynamic and structural compensation in size-switch core repacking variants of bacteriophage T4 lysozyme. J. Mol. Biol., 259: 542-559.

Benhar, I., Q.C. Wang, D. FitzGerald and I. Pastan, 1994. Pseudomonas exotoxin a mutants. Replacement of surface-exposed residues in domain III with cysteine residues that can be modified with polyethylene glycol in a site-specific manner. J. Biol. Chem., 269: 13398-13404.

Chaffee, S., A. Mary, E.R. Stiehm, D. Girault, A. Fischer and M.S. Hershfield, 1992. IgG antibody response to polyethylene glycol-modified adenosine deaminase in patients with adenosine deaminase deficiency. J. Clin. Invest., 89: 1643-1651.

Chaiken, I.M. and W.V. Williams, 1996. Identifying structure-function relationships in four-helix bundle cytokines: Towards de novo mimetics design. Trends Biotechnol., 14: 369-375.

Choi, S.J., D.H. Park, S.I. Chung and K.H. Jung, 2000. Plasmid stability in Long-term hG-CSF production using L-arabinose promoter system of *Escherichia coli*. J. Microbiol. Biotechnol., 10: 321-326.

Demetri, G.D. and J.D. Griffin, 1991. Granulocyte colony-stimulating factor and its receptor. Blood, 78: 2791-2808.

Fahnert, B., H. Lilie and P. Neubauer, 2004. Inclusion bodies: Formation and utilisation. Adv. Biochem. Eng. Biotechnol., 89: 93-142.

Fontana, A., B. Spolaore, A. Mero and F.M. Veronese, 2008. Site-specific modification and PEGylation of pharmaceutical proteins mediated by transglutaminase. Adv. Drug Delivery Rev., 60: 13-28.

Kuan, C.T., Q.C. Wang and I. Pastan, 1994. Pseudomonas exotoxin A mutants. Replacement of surface exposed residues in domain II with cysteine residues that can be modified with polyethylene glycol in a site-specific manner. J. Biol. Chem., 269: 7610-7616.

Kunstelj, M., K. Fidler, S. Skrajnar, M. Kenig and V. Smilovic *et al*., 2013. Cysteine-specific PEGylation of rhG-CSF via Selenylsulfide bond. Bioconjugate Chem., 24: 889-896.

Lauffenburger, D.A., E.M. Fallon and J.M. Haugh, 1998. Scratching the (cell) surface: Cytokine engineering for improved igand/receptor trafficking dynamics. Chem. Biol., 5: R257-R263.

Mott, H.R. and I.D. Campbell, 1995. Four-helix bundle growth factors and their receptors: Protein-protein interactions. Curr. Opin. Struct. Biol., 5: 114-121.

Pai, L.H., M.G. Gallo, D.J. FitzGerald and I. Pastan, 1991. Antitumor activity of a transforming growth factor α-*Pseudomonas* exotoxin fusion protein (TGF-α-PE40). Cancer Res., 51: 2808-2812.

Scaramuzza, S., G. Tonon, A. Olianas, I. Messana, R. Schrepfer, G. Orsini and P. Caliceti, 2012. A new site-specific monoPEGylated filgrastim derivative prepared by enzymatic conjugation: Production and physicochemical characterization. J. Controlled Release, 164: 355-363.

Tsutsumi, Y., M. Onda, S. Nagata, B. Lee, R.J. Kreitman and I. Pastan, 2000. Site-specific chemical modification with polyethylene glycol of recombinant immunotoxin anti-Tac (Fv)-PE38 (LMB-2) improves antitumor activity and reduces animal toxicity and immunogenicity. Proc. Nat. Acad. Sci., 97: 8548-8553.

Vemula, S., R. Thunuguntla, A. Dedaniya, S. Kokkiligadda, C. Palle and S.R. Ronda, 2015. Improved production and characterization of recombinant human granulocyte colony stimulating factor from *E. coli* under optimized downstream processes. Protein Exp. Purification, 108: 62-72.

Wells, J.A. and A.M. de Vos, 1996. Hematopoietic receptor complexes. Annu. Rev. Biochem., 65: 609-634.

Yabe, Y., M. Nishikawa, A. Tamada, Y. Takakura and M. Hashida, 1999. Targeted delivery and improved therapeutic potential of catalase by chemical modification: Combination with superoxide dismutase derivatives. J. Pharmacol. Exp. Ther., 289: 1176-1184.

Zink, T., A. Ross, K. Lueers, C. Cieslar, R. Rudolph and T.A. Holak, 1994. Structure and dynamics of the human granulocyte colony-stimulating factor determined by NMR spectroscopy. Loop mobility in a four-helix-bundle protein. Biochemistry, 33: 8453-8463.