Development of a Long- Acting Granulocyte Colony Stimulating Factor

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Declaration of Authorship

I hereby declare that I am the sole author of this thesis entitled ‘’Development of a long-acting granulocyte colony stimulating factor’’. I also declare that this thesis is the result of my own research except as cited in the references, and it has not been submitted for a degree to any other university or institution.

Signature: 

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Date: 08-08-2017

Abstract

Background: Granulocyte Colony Stimulating Factor (GCSF) is a glycoprotein with the primary function of regulating neutrophil production. GCSF is used in the treatment of chemotherapy-induced neutropaenia, and stem cell mobilisation. First generation GCSF therapies have short half-lives and the necessity for daily injections. Because of this, a number of technologies are in development to generate a long-acting GCSF. Pegylated GCSF (Pegfilgrastim) and a site-specific glycol-pegylated GCSF (Lipegfilgrastim) are the only licenced second generation GCSF's available but the long-term side effects of using PEG products remain unknown. Therefore, there is a need for new long-acting GCSF molecule. Previously it was shown that fusion of growth hormone (GH) at its *C*-terminus to growth hormone binding protein (GHBP) generated a fusion protein with delayed clearance and this thesis asked the question could the fusion of GHBP to GCSF also delay clearance.

Aim: To use protein fusion technology to generate a long-acting GCSF molecule by linking GCSF to an inactive growth hormone binding protein (GHBP) via a flexible glycine-serine linker. To prevent GH binding to GHBP in the circulation, a tryptophan-104 to alanine (W104A) change is introduced.

Hypothesis: That a GCSF fusion protein will retain biological activity and have a prolonged circulating half-life through a fusion with GHBP at its *C*-terminus.

Methods: Two fusions, one with and one without the W104A change, GCSF-W104-GHBP and GCSF-GHBP, were constructed using recombinant DNA techniques and expressed as secreted products from a Chinese hamster ovary (CHO) cell line. Protein expression was determined by ELISA and western blotting. Proteins were purified using Immobilised Metal Affinity Chromatography (IMAC). *In vitro* bioactivity was tested in an AML-193 proliferation assay and *in vivo* activity in BDF1 strain mice.

Results: Both fusions had increased molecular weight of ~50-70 kDa compared to GCSF alone of 18.8kDa, as judged by SDS-PAGE. Both molecules showed increased *in vitro* bioactivity (EC_{50} = 0.02nM) compared to native rhGCSF (EC_{50} = 0.05nM). In the *in vivo* study, GCSF-W104-GHBP displayed a reduced rate of clearance compared to Filgrastim (rhGCSF). In addition, the maximum plasma concentration (C_{max}) for Filgrastim was 16.4 nM with a time of maximum concentration (T_{max}) of approximately 2 hours. Whereas GCSF-W104-GHBP showed a delayed T_{max} of approximately 12 hours with a C_{max} of 35.4 nM. A single dose of GCSF-W104-GHBP produced a prolonged haematopoietic effect in mice with an increase in the number of blood neutrophils for up to 72 hours, and haematopoietic progenitor cells for up to 48 hours compared to 24 hours after a single dose of Filgrastim

Conclusions: A fusion protein of GCSF to inactive GHBP has enhanced biological activity *in vitro* and delayed clearance and prolonged biological activity *in vivo*. A GCSF fusion with GHBP may provide a novel long-acting therapeutic for the treatment of neutropaenia and stem cell mobilisation.

Acknowledgements

After an intensive time of three and half years, today is the day: writing this few paragraphs of thanks is the crowning achievement on my thesis. It has been a time of exceptional learning for me, in the scientific field, as well as on an individual level. I want to consider the people who have supported me lots all through this period.

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Abbreviations

acBSA:	Acetylated bovine serum albumin
AML:	Acute myeloid leukaemia
APS:	Ammonium persulphate
BHK:	Baby hamster kidney
BM:	Bone marrow
bp:	Base pair
BSA:	Bovine serum albumin
CBC:	Complete blood count
cDNA:	Complementary deoxyribonucleic acid
CHR:	Cytokine-binding homology region
CHO:	Chinese hamster ovary
CIAP:	Calf intestinal alkaline phosphatase
CMV:	Cytomegalovirus
CNS:	Central nervous system
CRH:	Cytokine receptor homology
CSF:	Colony stimulating factor
***C*-terminus:**	Carboxyl-terminus
Cys:	Cysteine
E.coli:	Escherichia coli
D:	Domain
DMSO:	Dimethyl sulfoxide
dNTP:	Deoxynucleotide triphosphates
DTT:	Dithiothreitol
EB:	Elution buffer
EDTA:	Ethylenediaminetetraacetic acid
ELISA:	Enzyme-linked immunosorbent assay
EMA:	European Medicines Agency
ERK:	Extracellular factor regulated kinases
FCS:	Foetal calf serum
FN:	Febrile neutropaenia
FNIII:	Fibronectin type III
FRT:	Flp recombinase target
F/T:	Freeze-thaw
GCSF:	Granulocyte colony stimulating factor
GCSFR:	Granulocyte colony-stimulating factor receptor
GH:	Growth Hormone

GHBP:	Growth hormone binding protein
GMCSF:	Granulocyte and macrophage colony stimulating factor
Grb2:	Growth factor receptor binding 2
HEK:	Human embryonic kidney
HF:	High Fidelity
hGCSF:	Human granulocyte colony stimulating factor
HPC:	Haematopoietic progenitor cells
HST:	Haematopoietic cell transplantation
HRP:	Horseradish peroxidase
IFN:	Interferon
Ig:	Immunoglobulin-like domain
IGF-I:	Insulin-like growth factor-I
IL:	Interleukin
IMAC:	Immobilized Metal Affinity Chromatography
IP3:	Inositol 1, 4, 5-triphosphate
JAKs:	Jak tyrosine kinases
Kb:	Kilobase pair
KC:	Keratinocyte chemoattractant
KDa:	Kilo Dalton
LB:	Luria-Bertani
LPS:	Lipopolysaccharide
LT:	leukotriene
MAP:	Mitogen-activated protein
MCSF:	Macrophage colony-stimulating factor
MDS:	Myelodysplastic anaemia
µg:	Microgram
mGCSF:	Murine granulocyte colony stimulating factor
MIP:	Macrophage inflammatory protein
µl:	Microliter
mRNA:	Messenger ribonucleic acid
MW:	Molecular weight
MWCO:	Molecular weight cut-off
ng:	Nanogram
***N*-terminus:**	Amino-terminus
P:	Proline
PBSCT:	Peripheral blood stem cell transplantation
PCR:	Polymerase chain reaction
PD:	Pharmacodynamics

PI-3K-PKB:	Phosphoinositide 3-kinase- protein kinase B
PK:	Pharmacokinetics
PES:	Polyethersulfone
PVDF:	Polyvinylidene fluoride
RB:	Roller bottle
rGCSF:	Recombinant granulocyte colony stimulating factor
r-methHuG-CSF:	Recombinant methionyl human granulocyte colony stimulating factor
RT:	Room temperature
SC:	Subcutaneous
SCF:	Stem cell factor
SCN:	Severe congenital neutropaenia
SDS-PAGE:	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEAP:	Secreted alkaline phosphatase
Ser:	Serine
SHIP:	SH2-containing inositol phosphatase
SOCS:	Suppressor of cytokine signaling
STAT:	Signal transducers and activators of transcription
TEMED:	Tetra-methyl-ethylene-diamine
Th 17:	T helper 17
Thr:	Threonine
TMB:	Tetramethylbenzidine
TNF α:	Tumour necrosis factor-alpha
Trp:	Tryptophan
TYK2:	Tyrosine kinase 2
VEGF:	Vascular endothelial growth factor
WBCs:	White blood cells
W/V:	Weight / volume
XIAP:	X-linked inhibitor of apoptosis
Y:	Tyrosine

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Chapter 1. Introduction

1.1. Discovery of colony stimulating factors

Before the 1960s, several animals' experiments had been completed to find the answer to how white blood cells (WBCs) regulate homoeostasis. However, these regulators remained unknown until 1966, when semi-solid agars were developed for growing of WBCs colonies from mouse spleen cells, and bone marrow (BM) (Ichikawa et al., 1966, Bradley and Metcalf, 1966). The colonies derived from single progenitor cells contained either granulocytes or macrophages or both. In addition, the growth of these colonies relied upon the amount of tissue extract, cells or medium 'conditioned' by other cells that were added to the cultures. Robinson et al, claimed that the stimulation of cell division in the culture system was dependent on the present of an unknown colony stimulating factor(s) (CSFs) (Robinson et al., 1967). In the 1970's, pre-clinical work succeeded in growing similar colonies from human BM cells using WBCs as a feeder layer to provide a source of CSF (Pike and Robinson, 1970, Metcalf, 2010).

In the middle of the 1980's, the efforts of many laboratories to biologically identify, and biochemically purify these CSF proteins revealed that there are four different CSF proteins with different colony-stimulating activities (Metcalf, 2010). Their names were given dependent on the most common type of colony stimulated; GMCSF stimulated both granulocyte and macrophage colony formation (Burgess et al., 1977); MCSF stimulated macrophage colony formation (Stanley and Heard, 1977); GCSF stimulated granulocyte colony formation (Nicola et al., 1983), also referred to as CSF3 (Beekman and Touw, 2010); and multi-CSF, also known as interleukin 3 (IL3) stimulated a wide range of haematopoietic cell colonies (Ihle et al., 1982).

1.2. Purification and cloning of GCSF

Murine GCSF (mGCSF) and human GCSF (hGCSF) were first purified in 1983 and 1984, respectively (Nicola et al., 1983, Welte et al., 1985). Purification of the

mGCSF was from medium conditioned by mouse lung cells, while purification of the hGCSF was from medium conditioned by the human bladder carcinoma cell line 5637 (Welte et al., 1985, Metcalf, 2008, Welte, 2012). In 1986, molecular cloning of the complementary deoxyribonucleic acid (cDNA) for GCSF led to the first expression of this protein from Escherichia coli (E*. coli*) (Nagata et al., 1986). These achievements enabled the production of recombinant GCSF (rGCSF), which facilitated the study of its biologic characteristics (Zsebo et al., 1986, Panopoulos and Watowich, 2008).

1.3. Structure of GCSF

There are two splice variants of hGCSF which are encoded by two different messenger ribonucleic acids (mRNA) from the same gene (i.e. CSF3 gene): hGCSFa is 177 amino acids (19.6 kDa), and hGCSFb which has lost the N-terminal 3 amino acids and is 174 amino acids (18.8 kDa). hGCSFb is more active (~20 times) than hGCSFa, although they have similar biological activities(Nagata, 1989, Naghoosi et al., 2008, Molineux, 2011, Welte, 2012).

The central structural feature of hGCSF is similar to other helical cytokine family members. As shown below (Figure 1), hGCSF contains four antiparallels, left-handed α-helical bundles, in an 'up-up-down-down' structure (i.e., both helices A and B extend up, while both helices C and D extend down). The helix A contains 29 amino acids, the helix B contains 21 amino acids, the helix C contains 24 amino acids, and the helix D contains 30 amino acids (Arvedson and Giffin, 2012). The molecule consists of five cysteine (Cys) residues. Four of them form two disulphide bonds, which are located between Cys 36-42 and Cys 64-74, while the fifth cysteine residue at 17th position is free (Hill et al., 1993, Werner et al., 1994). However, Cys 17 is only partially available to solvent; therefore, it is less likely to react with other proteins (Arakawa et al., 1993). hGCSF does not contain *N*-linked glycosylation sites but has a single *O*-linked glycosylation site on Threonine (Thr)-

133. However, glycosylation is not necessary for biological activity, as the form expressed in E*. coli,* which lacks this structure, is fully active (Dale et al., 1993) Arvedson & Giffin, 2012).

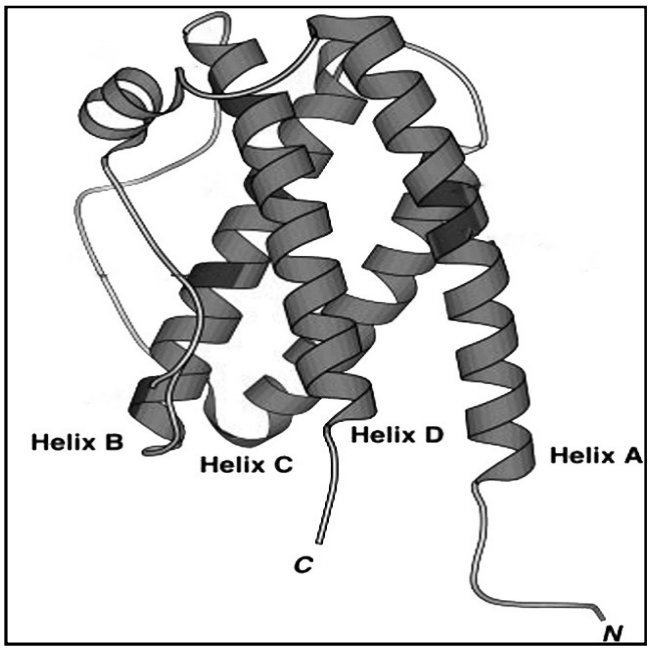


Figure 1. The structure of human GCSF

The molecular structure of hGCSF consists of four antiparallel, lift-handed α-helical bundles. Both helices A and B extend up, while both helices C and D extend down (Modified from Naghoosi et al., 2008, pp. 230).
N= amino-terminus
C= carboxyl-terminus

1.4. Biology of GCSF

GCSF is expressed by a variety of cells, including; BM stromal cells, endothelial cells, fibroblasts, monocytes, macrophages, astrocytes, and a number of immune cells (Demetri and Griffin, 1991, Knudsen et al., 2011, Adusumilli et al., 2012). Furthermore, it is expressed by myeloblastic leukaemia cells, and carcinoma cells (Adusumilli et al., 2012). The physiological roles of GCSF have been determined

by gene deletion studies in mice. These studies demonstrated that GCSF is a key regulator of neutrophil production and activity, under both normal physiological and 'emergency' conditions, such as infections and BM aplasia. Both GCSF and GCSF receptor (GCSFR) deficient mice are characterised by severe neutropaenia, and a dramatically decreased ability of neutrophils to control fungal and bacterial infections (Lieschke et al., 1994).

In keeping with the mouse model data, the circulating GCSF levels in healthy humans are very low (<30-163 pg/ml) and commonly undetectable. These low levels are important to maintain the daily production rate of neutrophils. However, in conditions such as neutropenia, infection, and aplastic anaemia, the serum GCSF levels are markedly elevated (>100,000 pg/ml) and subsequently decline with recovery (Barth et al., 2002, Molineux, 2011).

GCSF regulates neutrophil production and enhances neutrophil function by a number of mechanisms (Roberts, 2009). It induces proliferation in all granulocytic lineage cells (i.e., from haemopoietic stem cells to myelocyte). However, to maintain stem cell proliferation, co-stimulation of other factors is required such as stem cell factor (SCF), thrombopoietin, fibroblast growth factors, and insulin-like growth factor 2 (Zhang and Lodish, 2008). In addition, GCSF alone can stimulate common myeloid progenitor cells to produce huge numbers of progeny. Collaterally, it stimulates neutrophil differentiation and accelerates metamyelocytes maturation (Figure 2). Moreover, GCSF prolongs the survival-promoting activity of neutrophils and their precursors.

Many tissues when stimulated by inflammatory mediators such as tumour necrosis factor (TNF)-a, vascular endothelial growth factor (VEGF), interferon (IFN)-b, lipopolysaccharide (LPS), IL-17 and IL-1, can produce and release GCSF into the bloodstream resulting in neutrophil production within, and mobilisation from, the

BM (Bendall and Bradstock, 2014). Additionally, the locally produced GCSF within tissues enhances the neutrophils function at the infection site by inhibiting neutrophil apoptosis (Colotta et al., 1992, Bendall and Bradstock, 2014), and increasing survival within infected tissues (Gregory et al., 2007, Bendall and Bradstock, 2014). Furthermore, GCSF has chemokinetic activity on neutrophils although it does not directly induce the chemotaxis of these cells. It promotes non-directional motility, which increases neutrophils response to chemotactic factors, such as the bacterial agent N-Formyl-Methionine-Leucine-Phenylalanine. (Smith et al., 1994, Bendall and Bradstock, 2014). Under both normal physiological and 'emergency' conditions, GCSF is a prototypical neutrophil-mobilising cytokine (Dale, 2012, Bendall and Bradstock, 2014, Bajrami et al., 2016). The number of peripheral blood neutrophils increases significantly after a single injection of GCSF, peaking at 6 hours, and returning to near normal levels at 24 hours (Kim et al., 2006, De La Luz Sierra et al., 2007, Bajrami et al., 2016). Many other neutrophil-mobilising agents contribute to stress-induced neutrophil mobilisation, such as leukotriene B4 (LTB4), and CXCR2 ligands (Martin et al., 2003, Eash et al., 2010, Bajrami et al., 2016). CXCR2 ligands (e.g., macrophage inflammatory protein 2 [MIP-2] and keratinocyte chemoattractant [KC] in mice, and IL-8 in humans) induce neutrophil mobilisation more rapidly than GCSF, with a 10-fold increase in neutrophilia occurring 30 minutes after injection (Fibbe et al., 1999, Bajrami et al., 2016). After an initial acute fast phase of inflammation, neutrophil mobilisation slows despite increases in serum CXCR2 ligand concentrations. In other words, after the acute phase of inflammation, the neutrophil response to the CXCR2 ligand is suppressed. This is due to expression of GCSF later in the acute inflammatory response which leads to inhibition of CXCR2-mediated rapid neutrophil mobilisation, initiating more controlled regulation, and slow neutrophil mobilisation from the BM.

Shortly after GCSF administration, transient neutropaenia often occurs even though GCSF is routinely used to treat neutropaenia. Recent studies indicate that the main cause of this transient neutropaenia might be due to either neutrophil accumulation in the pulmonary vasculature (DeJesus et al., 2011), or to leukocyte integrin activation after GCSF administration (Donahue et al., 2011), or to GCSF induced inhibition of CXCR2 signalling (Bajrami et al., 2016).

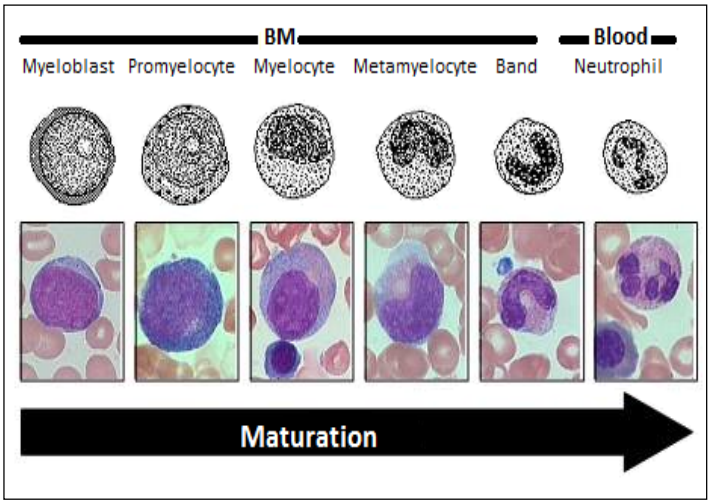


Figure 2. Maturation stages of neutrophils and their precursors

Myeloblast is an immature cell, which is found in the BM and not in peripheral blood. It has a large round nucleus with fine diffuse immature chromatin, and a prominent nucleolus. Promyelocyte is slightly larger than myloblast. It has a round or oval nucleus with slightly condensed chromatin, and less prominent nucleolus. Myelocyte is smaller than promyelocytes. It has eccentric round-oval nuclei with coarse chromatin, and small or even invisible nucleoli. Metamyelocyte is smaller than myelocytes. It has many secondary granules. Band is smaller than metamyelocytes. It characterised by a U-shaped or deeply indented nucleus, with heavily clumped chromatin and secondary granules. Neutrophils is characterised by definite lobation joined by thin filaments of coarsely clumped chromatin (Modified from Greer et al., 2013).

1.5. Regulation of GCSF expression

As described in the previous section, many inflammatory factors in the extracellular microenvironment, such as LPS, TNFα, and IL1β are elevated during infection, which in turn stimulate the production of GCSF (Figure 3). The circulatory levels of GCSF stimulate the production of neutrophils in the BM and mobilise them to the peripheral circulation (Demetri and Griffin, 1991, Panopoulos and Watowich, 2008).

The expression of GCSF is regulated by transcriptional and post-transcriptional mechanisms (Ernst et al., 1989, Falkenburg et al., 1991, Panopoulos and Watowich, 2008). NF-IL6 and NF-κBp65 are transcription factors which participate in several inflammatory pathways. Some of the binding sites for these factors are present in the promoter region of the GCSF gene and regulate its expression and its constitutive production in carcinoma cells (Nishizawa et al., 1990, Panopoulos and Watowich, 2008, Shannon et al., 2009).

In addition to promoter elements, IL-17 is also considered an essential regulator of the production of GCSF and neutrophils *in vivo* (Langrish et al., 2005, Stark et al., 2005). It is synthesised by T helper 17 (Th17) cells, which are located in the intestinal *lamina propia*, and they can be produced during infection by specific bacteria such as Staphylococcus & Candida albicans (Ivanov et al., 2006, LeibundGut-Landmann et al., 2007, Denning et al., 2007). Production of IL-17 activates IL-17 receptor signal transduction, which enhances GCSF production (Figure 3). The key stimulator of IL-17 production is IL-23 which is produced by tissue macrophages and dendritic cells (Harrington et al., 2005, Roberts, 2005). Therefore, phagocytosis of circulating neutrophils by tissue cells leads to suppression of the production of IL-23, resulting in a decreased production of IL-17 and GCSF (Stark et al., 2005).

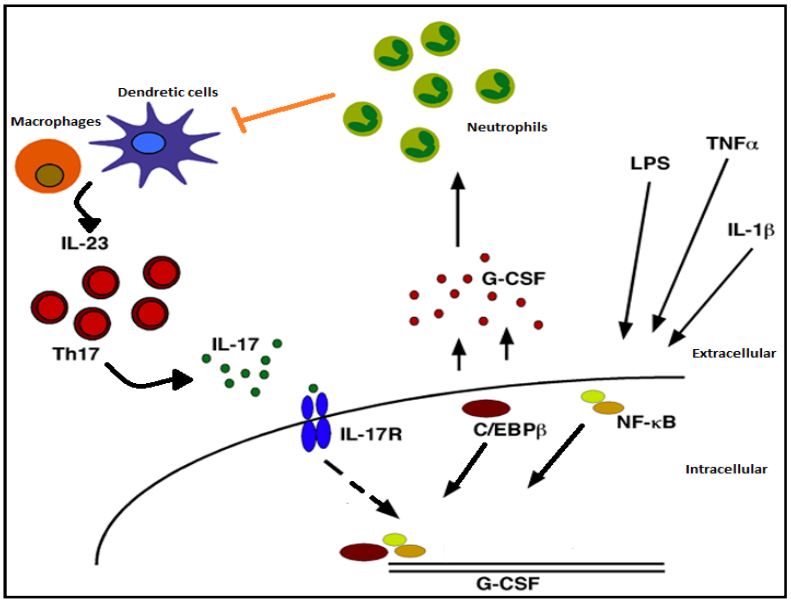


Figure 3. Regulation pathways of GCSF and neutrophil production.

(Right) Many inflammatory stimuli are elevated during infection, such as LPS, TNFα and IL-1β. They act on target cells to induce expression of GCSF by intracellular signalling molecules such as NF-κB and C/EBPβ. The promoter region of GCSF gene contains binding sites for these molecules, which in turn mediates inducible expression of GCSF. (Left) IL-23, which is produced by tissue macrophages and dendritic cells, stimulates Th17 cells to synthesis IL-17. IL-17 induces GCSF expression through the IL-17 receptor. (Centre) Neutrophil production is stimulated by GCSF synthesis. Phagocytosis of circulating neutrophils by macrophages and dendritic cells inhibits their IL-23, thus decreasing synthesis of IL-17, leading to decrease GCSF synthesis (Modified from Panopoulos & Watowich 2008).

1.6. GCSF clearance mechanisms

There are many, parallel elimination pathways to clear proteins including; receptor-mediated endocytosis, reticuloendothelial system, renal clearance, and non-specific proteolysis and degradation. Clearance of proteins by the kidneys is the main elimination pathway for those which are small enough to pass through glomerular filtration, but their ability to be filtered also depends on many physical factors such as molecular weight (MW), charge, structure, and hydrodynamic size of the molecule. Large proteins (i.e. > 70 kDa) are not able to be filtered, but

smaller proteins can have negligible renal clearance depending on their biophysical characteristics (Molineux, 2011).

Clearance by the receptor-mediated endocytosis pathway, which is a saturable clearance pathway, is a crucial homeostatic mechanism regulating granulopoiesis for GCSF. During neutropaenia, decreased GCSFR expression results in increasing concentrations of GCSF, which stimulate neutrophil production and mobilisation. During neutrophilia, the clearance of GCSF is increased due to the increased numbers of GCSFR-bearing cells. The role of GCSFR clearance has been studied in GCSFR knock-out mice ($GCSFR^{-/-}$). These studies demonstrated a markedly reduced clearance of Filgrastim (short acting GCSF, MW: 18.8 kDa) and Pegfilgrastim (long-acting GCSF, MW: 38.8 kDa) compared to wild type control (Molineux, 2011).

In addition to neutrophil-mediated clearance, two other clearance pathways for GCSF are present. One of them is renal clearance which is the main characteristic that differentiates Filgrastim from Pegfilgrastim. Filgrastim is readily excreted by the kidneys (>75%) as its clearance is lowered in nephrectomised rats than in sham-operated rats (Yang et al., 2004, Yang, 2006), whilst the higher hydrodynamic radius of Pegfilgrastim prevents glomerular filtration. Extracellular proteolytic degradation by neutrophil elastase is potentially another clearance pathway of GCSF compounds (El Ouriaghli et al., 2003, Hunter et al., 2003, Piper et al., 2010, Abdolzade-Bavil et al., 2016). The effect of neutrophil elastase on Lipegfilgrastim (long-acting GCSF, MW ~ 38-39 kDa) and Pegfilgrastim was investigated by incubating the drugs with human neutrophil elastase followed by SDS-PAGE. The results showed that Lipegfilgrastim was more resistant to degradation with human neutrophil elastase than Pegfilgrastim, which explain the longer *in vivo* half-life of (i.e. 5-10 hours longer) compared to Pegfilgrastim (Median half-life ~42 hours) (Abdolzade-Bavil et al., 2016).

The liver role in GCSF clearance seems to be insignificant, as the pharmacokinetics of Filgrastim was similar between healthy individuals and hepatic patients, after subcutaneous administration (Johnston et al., 2000, Molineux, 2011).

1.7. GCSF receptor

When GCSF is produced, it binds to the extracellular domain GCSFR, this, in turn, stimulates continuous intracellular signalling (Avalos, 1996). In the following paragraphs, details about GCSFR and its signalling pathway will be discussed.

1.7.1. Expression of GCSFRs

The expression of GCSFRs has been detected in haematopoietic cells, including all neutrophils and their precursors (i.e., myeloid stem cells, myeloblasts, promyelocytes, myelocytes and metamyelocytes), monocytes, normal B & T lymphocytes, and myeloid & lymphoid leukaemia (Boneberg et al., 2000, Manz et al., 2002, Morikawa et al., 2002, van de Geijn et al., 2003, Touw, 2007, Touw and Bontenbal, 2007, Panopoulos and Watowich, 2008). Also, GCSFRs have been found on non-haematopoietic tissues, including vascular endothelial cells (Bussolino et al., 1989), cardiomyocytes (Harada et al., 2005), adult neural stem cells, neurons of the central nervous system (CNS) (Schneider et al., 2005), many of non-haematopoietic tumors cell lines (Roberts, 2005), the placenta (McCracken, 1999), and many of fetal organ tissues (Calhoun et al., 1999).

1.7.2. Cloning and structure of GCSFR

The first cloning of GCSFR was done by Fukunaga and his colleges in 1990. GCSFR is a transmembrane protein encoded by a single gene on human chromosome 17q21 (Fukunaga et al., 1990, Boulay et al., 2003). It consists of 836 amino acids, arranged as the follows; 604 amino acids in the extracellular region,

23 and 183 amino acids for transmembrane and intracellular regions, respectively (Arvedson and Giffin, 2012).

The extracellular region contains 6 domains, which are commonly called D1-D6, with approximately 100 amino acid residues in each domain. D1 is an immunoglobulin-like (Ig) domain, while D2-D6 are fibronectin type III (FNIII) domains (Figure 4).

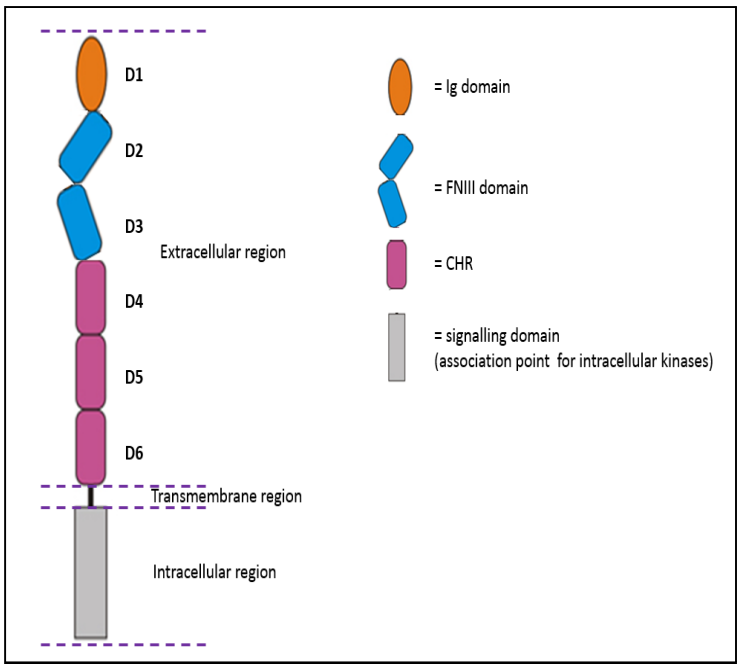


Figure 4. The domains of GCSFR

GCSFR consists of extracellular, transmembrane and intracellular regions. The extracellular region contains 6 domains (D1-D6). D1 is an immunoglobulin domain, while D2-D6 are fibronectin III domains. D2 & D3 form the cytokine-binding homology region. The intracellular region is signalling domain, which can associate with intracellular kinase, and initiate a signalling response
D= domain
Ig= immunoglobulin
FNIII= fibronectin type III
CHR= cytokine-binding homology region

Both domains (i.e., Ig & FNIII), contain two β-sheets with 7 β-strands (A-G), forming a β sandwich. For the Ig domain, the first sheet contains A, B, and D strands, while the second sheet contains C, F and G strands. The FNIII domains are similar, but D strand is switched from the first sheet to the second sheet (Figure 5).

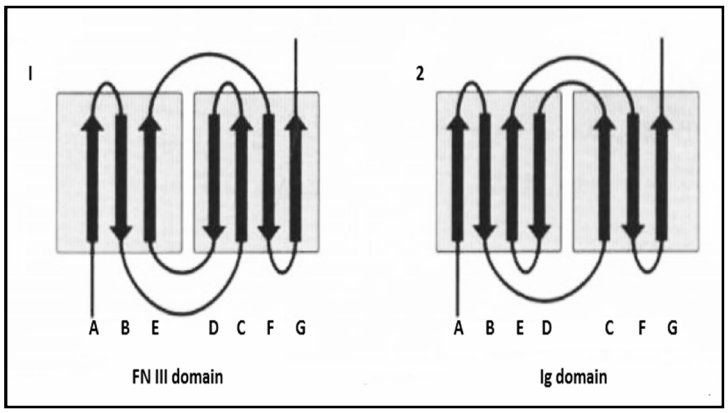


Figure 5. Topology map of β-strands for fibronectin type III and immunoglobulin domains.

(1)FNIII & (2) Ig domains consist of seven β-strand which form a β-sandwich. The *blue-grey* shading of β-sandwich faces indicates strands that cluster together on each face, and each *arrow* depicts an individual strand (Modified from Arvedson & Giffin, 2012, pp.68).
FNIII= fibronectin type III
Ig= immunoglobulin

D2 and D3 form the cytokine-binding homology region (CHR). This region contains two FNIII domains, six conserved cysteine residues, and a conserved tryptophan (Trp)-serine (Ser) (WSXWS) sequence motif (Arvedson and Giffin, 2012). The FNIII domains are connected together by a proline-rich linker, which is relatively rigid, holding them at a fixed angle (~70° to 110°). This angle, which is commonly called the ‘‘elbow’’ region, is the site of ligand binding. In terms of the conserved cysteines, there are four conserved cysteine residues located within the CHR *N*-terminal domain. They form two disulphide bonds between the faces of the β-sandwich to stabilise its structure. The other two conserved cysteine residues are located within the CHR *C*-terminal domain and they form one disulphide bond

(Figure 6). The conserved WSXWS motif still has an unclear function; however, it is thought to participate in receptor folding (Hilton et al., 1996, Arvedson and Giffin, 2012). Regarding the three membranes-proximal domains (D4-D6), there is very little information about their function, but it seems that they are not involved in ligand binding (Layton and Hall, 2006).

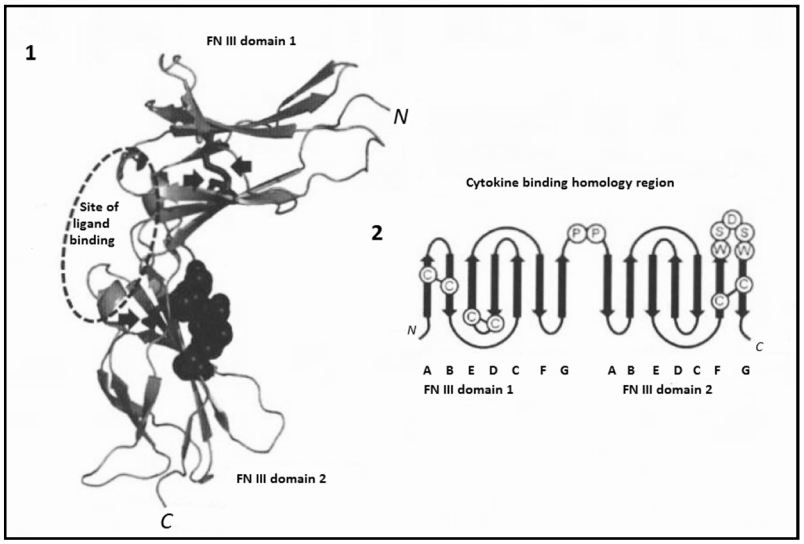


Figure 6. The cytokine-binding homology region's features.

The cytokine-binding homology region contains many conserved elements, including two fibronectin type III domains connected by a proline-rich linker, six conserved cysteines forming disulphide bonds, and a WSXSW (Trp-Ser-X-Trp-Ser) motif. (1) The structure of the cytokine-binding homology region of GCSFR. The *black arrows* depict the disulphide bonds formed by the conserved cysteines, the *space-filling spheres* indicate the WSXSW motif, and the *dotted circled area* indicates the ligand binding site in the elbow region. (2) A β-strands topology map of GCSF cytokine-binding homology region illustrates the conserved features (Modified from Arvedson & Giffin, 2012, pp.69).
FNIII= fibronectin type III
C = cysteine
P= proline
W= tryptophan
S= serine
N= amino-terminus
C= carboxyl-terminus

The intracellular region of the GCSFR contains two motifs, called box 1 and box 2, and they are located in the membrane-proximal region. Box 1 is highly conserved proline motifs (P-X-P), while box 2 is less conserved proline motifs and contains acidic residues (Touw and van de Geijn, 2007). These regions (i.e. box 1 & box2) are important for the binding of Jak tyrosine kinase (JAKs) to the receptor region, and then transduction of the proliferation signals. The carboxy-terminal (*C*-terminal) region of GCSFR contains a third conserved motif (box 3) and is involved in the induction of myeloid progenitor cell line proliferation and in the transduction of phagocytic signals (Santini et al., 2003, Touw and van de Geijn, 2007). As will be described in this review, the cytoplasmic region of GCSFR also contains four tyrosines which are important in both positive and negative signalling pathways.

1.7.3. Structural interaction of GCSF and its receptor

Although two crystal structures of GCSF and portions of GCSFR with 2:2 stoichiometry have been determined, only one of them, which was determined by Tamada et.al, has biological relevance and a structure consistent with other cytokine/receptor-structures (Tamada et al., 2006, Arvedson and Giffin, 2012). This structure contains hGCSF and the Ig domain (D1) and CHR (D2 & D3) from hGCSFR (Li and Nicholas, 2002). As seen below in Figure 7, and Figure 8, each GCSFR binds two different hGCSF ligands and each ligand binds to two different receptors.

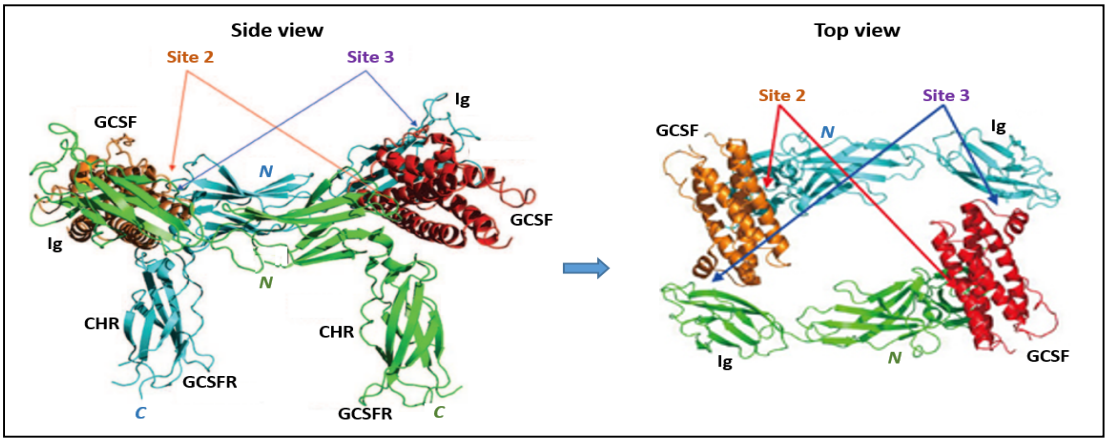


Figure 7. Crystallographic structure between human GCSF and portions of GCSFR.

The structure of GCSF/GCSFR complex contains human GCSF, and the Ig domain & CHR from human GCSFR. Each GCSF ligand binds to two different GCSFR molecules, and each receptor binds to two different ligands. The GCSF molecules are coloured in red and orange, and GCSFR molecules are coloured in green and cyan. In general, there are three binding sites of interaction between the cytokine and its receptor. Sites 1 & 2 occur between the cytokine and the receptor CHR, while site 3 occurs between the cytokine and the receptor Ig domain. However, on the GCSF molecule, only sites 2&3 are used. Site 2 binds the GCSF ligand to the CHR of one GCSFR, and site 3 binds the same ligand to the Ig domain of another GCSFR (Modified from Tamada et. al, 2006, pp.3136).
GCSF= granulocyte colony stimulating factor
GCSFR= granulocyte colony stimulating factor receptor
Ig= immunoglobulin
CHR= cytokine-binding homology region
N= amino-terminus
C= carboxyl-terminus

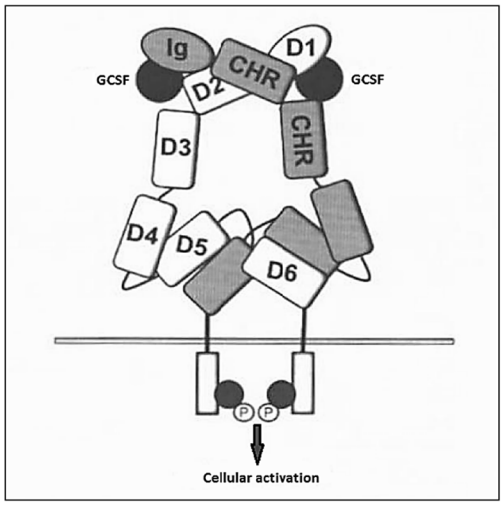


Figure 8. The model of GCSF/GCSFR signalling complex.

A crossover between domain 5 and domain 6 results a conformation where the extracellular domains end would be positioned. Dimerisation of two GCSFRs is required to initiate a signalling response (Modified from Arvedson and Giffin, 2012, pp.79).
GCSF= granulocyte colony stimulating factor
D= domain
Ig= immunoglobulin
CHR= cytokine-binding homology region
P= phosphorylation

1.8. GCSF signal transduction pathways

The binding of GCSF to its receptor causes activation of multiple intracellular signalling cascades. The known pathways activated via GCSFR are Jak/STAT, p21Ras/MAP kinase and PI-3K/PKB (Figure 9). In addition, the GCSFR complex is internalised to the endosomal compartments and either degraded or recycled. It is believed that only a minority of GCSFR needs to be occupied to induce a maximal biological response (Wiczling et al., 2009).

1.8.1. Jak/Stat pathway

After receptor ligation, Jak 1, Jak 2 and tyrosine kinase 2 (TYK 2) are activated, which in turn leads to phosphorylation of the signal transducer and activator of transcription 1, 3 and 5 (STAT1, STAT3, and STAT5) proteins. Subsequently, STATs form dimers and translocate to the nucleus, where they activate target gene transcription. GCSF weakly and transiently activates STAT 1 but strongly and robustly activates STAT3 (Tian et al., 1994, Nguyen-Jackson et al., 2010).

As mentioned previously, the cytoplasmic tyrosine (Y) residues of GCSFR, which are phosphorylated by Jak kinase, play a crucial role in the intracellular signalling. Both Y704 and Y744 are the main docking sites for STAT3. However, for the maximal phosphorylation of STAT3, the availability of Y704 is required (Tian et al., 1994, Roberts, 2005). STAT3 is required for cell cycle exit, differentiation, and proliferation of myeloid progenitor (Nguyen-Jackson et al., 2010). Similar to STAT1, STAT5 is also weakly and transiently activated by GCSF, however, its activity does not depend on tyrosine phosphorylation (Dong et al., 1998, Touw and van de Geijn, 2007). It has a maximum level of activity around 10 to 30 minutes only, after activation of GCSFR (McCracken et al., 1996, Ward et al., 1999b). In the granulocytic lineage cells, STAT5 induces anti-apoptotic mechanisms in circulating neutrophils and their progenitors to maintain homeostatic regulation of

peripheral blood neutrophil numbers, especially during the inflammatory response (Fievez et al., 2007).

1.8.2. Ras/MAP Kinase & PI-3K/PKB Pathways

Another tyrosine residue of GCSFR, which is important for a maximal proliferative signal transmission, is Y764 (Akbarzadeh, 2002, Hermans et al., 2003, Roberts, 2005). Phospho-Y764 binds to both SH2 domains of Shc and growth factor receptor-bound protein 2 (Grb_2), which are signalling intermediate molecules of the p21 Ras pathway (van de Geijn et al., 2003). The latter (i.e. Grb_2), can also be recruited by binding to Shc. In *vitro*, a significant decrease of p21 Ras activation, neutrophil differentiation, and proliferation of myeloid progenitors is observed when Y764 is absent and vice versa (Hermans et al., 2003, Touw and van de Geijn, 2007).

Mitogen-activated protein (MAP) kinase pathways are activated by MAP kinase, resulting in cell proliferation, and differentiation. Extracellular signal-regulated kinases 1 & 2 (Erk 1/2) are considered as the main downstream effectors from p21 Ras, which are involved in the proliferation of myeloid progenitor cells (Koay et al., 2002, Kendrick et al., 2004). In neuronal cells, Erk's (also known as BMK1) are also strongly activated by GCSF. Finally, GCSF also activates the phosphoinositide-3-kinase-protein kinase B (PI-3K-PKB) pathway which stimulates cell survival by inhibiting the apoptotic cascades (Dong and Larner, 2000, Hunter and Avalos, 2000, Touw and van de Geijn, 2007).

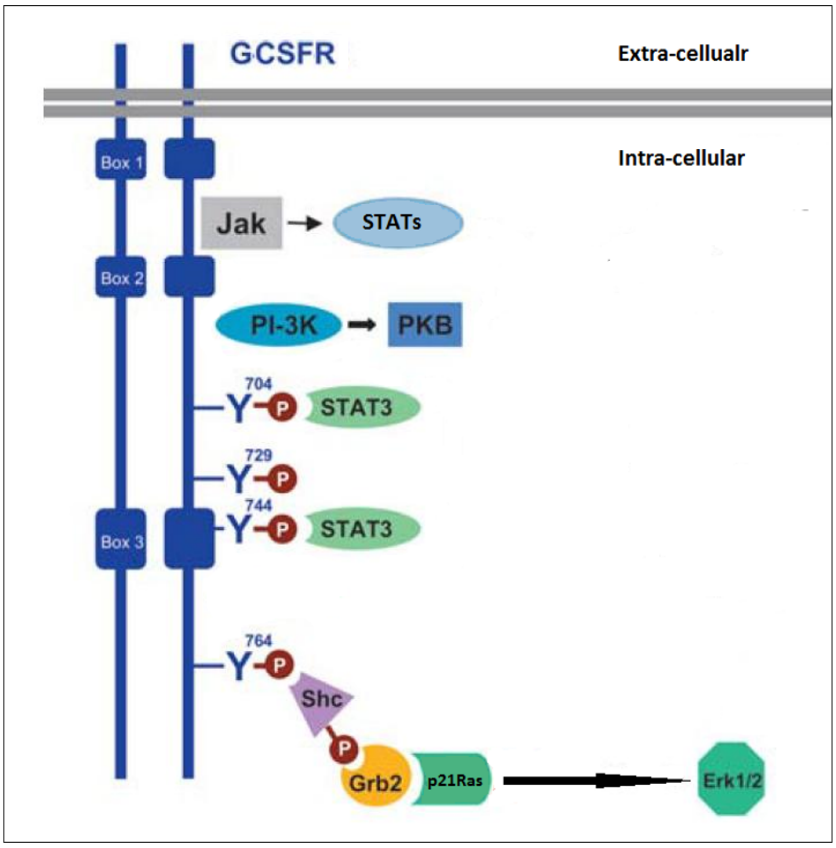


Figure 9. Intracellular signalling pathways of GCSFR.

GCSFR acts directly via three signalling pathways, which are Jak/STAT, the p21Ras/MAP kinase (Erk 1/2), and the PI-3K/PKB, resulting in activation of nuclear transcription factors and regulation gene transcription. This in turn promotes growth, differentiation, proliferation, and survival of cells. GCSFR tyrosines are phosphorylated by Jak kinase, and then function as docking sites for many signalling molecules. However, many other signalling routes are activated independent of GCSFR tyrosines. Please, see the text for further discussion (Modified from Touw and van de Geijn, 2007, pp.802).
GCSFR= granulocyte colony stimulating factor receptor
Y= tyrosine
Jak= Janus kinase
STAT= signal transducers and activator of transcription
Grb2=growth factor receptor-bound protein 2
PI-3K= phosphoinositide 3-kinase
PKB= protein kinase B
ERK= extracellular signal-regulated kinase
P= phosphorylation

1.9. Negative regulation of GCSFR signalling

Activation of GCSFR signalling is transient as there are many inhibitory mechanisms that have been implicated in downregulation of the signalling. The SH2-contaning inositol phosphatase (SHIP) protein is essential for regulating haematopoietic signalling. It binds to Y764 of the GCSFR via Shc, and dephosphorylates PI-3K/PKB molecules, resulting in down-regulation of myeloid cells proliferation (Touw and van de Geijn, 2007).

The SH2- containing protein tyrosine phosphatase SHP-1 is also considered as a negative regulator of haematopoietic signalling, in both myeloid and lymphoid lineages. However, none of the tyrosine residues of GCSFR serves as a docking site for this regulator (Ward et al., 2000, Touw and van de Geijn, 2007). It has been shown that SHP-1 binds to Jak2 independent of its SH2 and dephosphorylate it *in vitro* (Jiao et al., 1996).

The members of suppressor of cytokine signalling (SOCS) protein family (i.e. SOCS 1-7 and CIS) down-regulate the cytokine signalling by different mechanisms, for example; CIS competes with signalling substrates for tyrosine docking of GCSFR, SOCS1 and SOCS3 inhibit kinase activity. SOCS1 binds directly to Jak kinases with high affinity, while SOCS3 needs to bind selectively to the phosphorylated Y729 of the GCSFR for efficient signal inhibition (Hortner et al., 2002, Hermans et al., 2003, van de Geijn et al., 2004, Zhuang et al., 2005).

1.10. GCSFR mutations in myeloid disorders

A considerable number of mutations in the GCSFR gene (i.e. CSF3R) have been identified in myeloid disorders. They can be classified into intracellular truncations, transmembrane, extracellular and other mutilations. Some details about these mutations and their related disorders are described in the following paragraphs and illustrated in Figure 10.

1.10.1. Intracellular receptor truncations

These truncations occur between 82 and 98 amino acids from the *C*-terminus of GCSFR. Although the truncated receptor binds normally to GCSF, its strong growth signal fails to induce maturation when the receptor is expressed in myeloid cell lines. Some patients with severe congenital neutropaenia (SCN), which is characterised by a severe decrease in neutrophil count (< 0.5 x 10^9/L) with maturation arrest of progenitor cells at the promyelocyte/myelocyte stage, have this mutation (Freedman and Alter, 2002, Ward, 2007).

1.10.2. Transmembrane mutations

In *de novo* acute myeloid leukaemia (AML), activating Thr-617>Asn mutations in the transmembrane region of GCSFR have been reported (Forbes et al., 2002). These mutations cause constitutive phosphorylation of the receptors and the signalling substrates (JAK2, STAT3, and ERK), which eventually leads to the development of AML.

1.10.3. Extracellular receptor mutation

This mutation changes a conserved proline residue between the *N*-&*C*- terminal barrels of the CRH domain of the extracellular domain of GCSFR, leading to prevention of the 2:2 ligand /receptor complex formation. It was found in 10% of SCN patients who failed to respond to GCSF therapy (Druhan et al., 2005).

1.10.4. Other mutations

There are a few other mutations of GCSFR that have been found in AML, and myelodysplastic syndrome (MDS). For example, in MDS, deletion of 3 nucleotides leads to prolong signaling activation (Awaya et al., 2002, Wölfler et al., 2005), and also deletion of single-nucleotide polymorphism predisposes patients to high-risk MDS, however; the exact mechanism of action stills unknown (Wölfler et al., 2005).

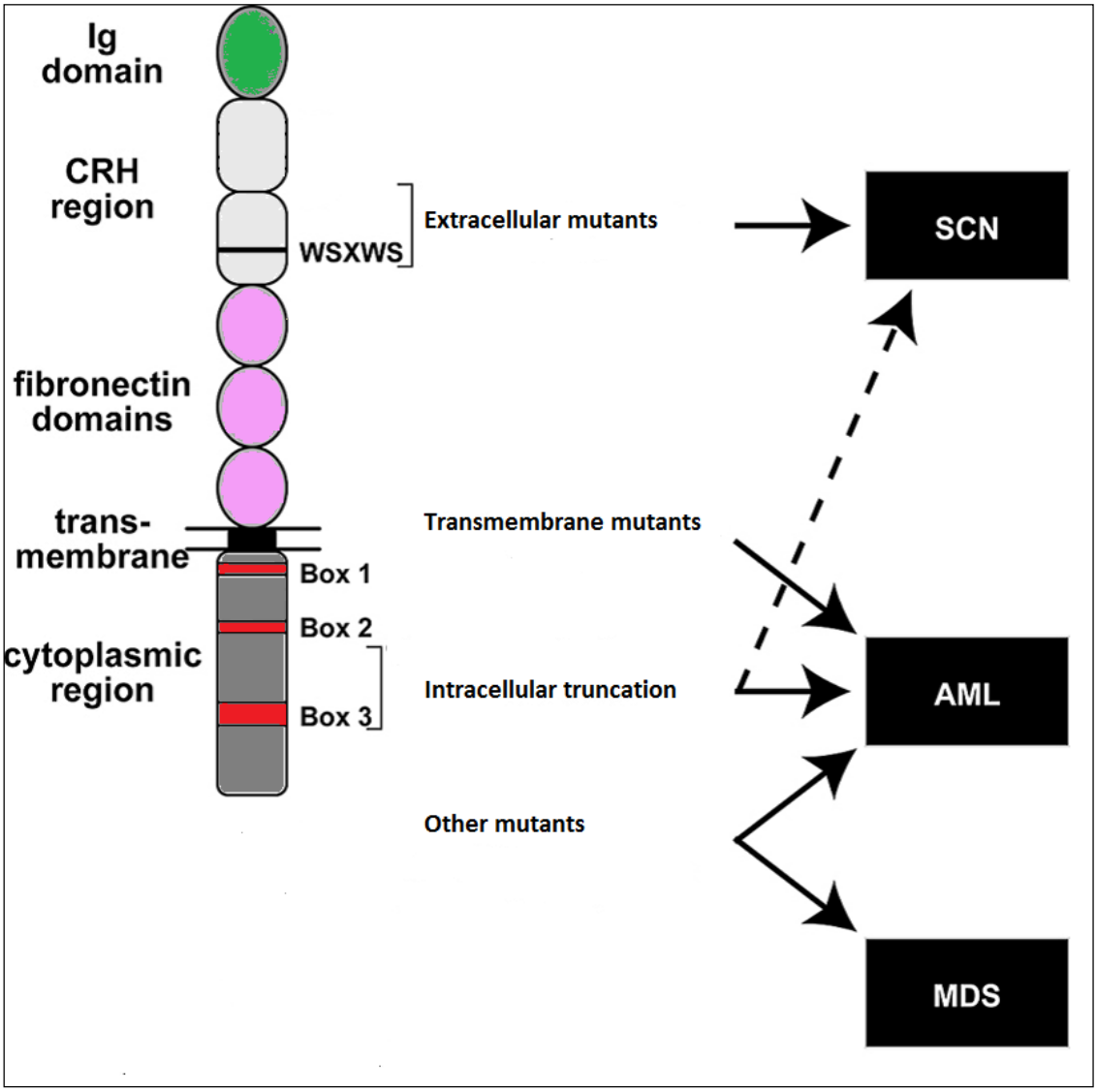


Figure 10. Mutations of GCSF receptor myeloid disorders.

Schematic representation of the GCSFR, showing the relative positions of mutations, and their clinical manifestations (Modified from Ward, 2007, pp.610).
Ig = immunoglobulin
CHR = cytokine-binding homology region
AML = acute myeloid leukemia
SCN = severe congenital neutropaenia
WSXWS = conserved tryptophan -serine sequence motif

1.11. The main clinical uses of GCSF

1.11.1. Prophylaxis of febrile neutropaenia

Febrile neutropaenia (FN) is a major adverse effect of myelosuppressive chemotherapy in cancer patients (Kelly and Wheatley, 2009). It is defined as an axillary temperature > 38.0˚C for more than one hour, or > 38.5˚C in a single occasion, with an absolute neutrophil count < $1.0x10^6$/L (Shah and Welsh, 2014). Prophylactic administration of GCSF prevents or reduces the incidence of FN, and accelerates the recovery of neutrophil numbers. In addition, it reduces the incidence of inflammation, the use of antibiotics, and the length of hospitalisation (Clark et al., 2005, Cooper et al., 2011, Shah and Welsh, 2014).

1.11.2. AML and SCN management

Pre-treatment of AML patients with GCSF therapy prior to chemotherapy has been shown to improve the patient condition by increasing the susceptibility of myeloid leukaemia blast cells to chemotherapy, and also by rapidly increasing the neutrophil counts (within 4 to 24 hours) (Löwenberg et al., 2003, Roberts, 2005). Furthermore, GCSF could have beneficial direct effects on some types of leukaemia, for example; a study was done by Nimubona et al. showed that the patients with hypoplastic AML, who were treated with GCSF alone, achieved complete remission (Nimubona et al., 2002).

Similar to FN, the use of GCSF in SCN increases the neutrophil numbers and prevents or decreases the recurrent infections and the frequency of fever (Dale et al., 2003). However, as discussed previously, some of the SCN patients (around 20%) have mutations of the GCSFR (i.e. intracellular receptor truncations), and these patients have a high risk of developing AML/MDS during GCSF therapy (Ancliff et al., 2003). Furthermore, once SCN patients develop AML, the GCSFR mutation clones become overt in more than 80 % of these patients. Therefore, these truncations are considered a crucial step in the expansion of the

preleukaemic clones (Ward et al., 1999a, Touw and van de Geijn, 2007). Hence, the questions about the possibility of GCSF administration expanding these GCSFR mutant clones and therefore trigger the development of AML have been raised. However, preliminary data are complicated and the conclusions are still debatable (Touw and Bontenbal, 2007, Ward, 2007). For example, Freedman and Alter claimed that there were no significant relationships between the dose or duration of GCSF therapy (Freedman and Alter, 2002), and the onset of AML and MDS, while Donadieu and his colleges showed that the long duration of GCSF therapy was associated with high risk of leukaemia development (Donadieu et al., 2005). Resolving these conflicting conclusions will require further large retrospective, and formal comprehensive studies (Ward, 2007). Other questions about whether GCSFR mutations are useful predictors for leukaemic progression of SCN or not has been considered, as most of SCN patients who develop AML/MDS have a poor therapy outcome. Regular monitoring of GCSFR mutations has been considered to be helpful to detect malignant transformation signs at the earliest possible stage (Zeidler et al., 2009, Beekman and Touw, 2010). This can create the opportunity to timely consider alternative treatments, such as allogeneic stem cell transplantation. However, GCSFR mutations can easily be missed in direct sequencing protocols when they present in minor clones. Hopefully, this problem will be resolved by next-generation sequencing technologies which allow mutation detection in smaller subsets of cells (Beekman and Touw, 2010). The time intervals between the first detection of GCSFR mutations and the onset of leukaemic transformation is still a major dilemma which makes the decision to perform transplantation on patients with SCN who respond favourably to GCSF treatment without other additional evidence of leukaemic progression, difficult. The most acceptable option is “watchful” waiting, with taking into account that all patients with GCSFR mutations will progress to AML eventually (Freedman and Alter, 2002, Beekman and Touw, 2010).

1.11.3. Haematopoietic stem and progenitor cell mobilisation

One of the oldest forms of anti-cancer immunotherapy is haematopoietic cell transplantation (HST) (Bendall and Bradstock, 2014), which is previously called as peripheral blood stem cell transplantation (PBSCT). It includes haematopoietic stem and progenitor cells transplantation from self (autologous) or histocompatible allogeneic healthy donors. In autologous HST, cryopreserved haematopoietic cells are used to restore BM function, commonly for patients with multiple myeloma (Hari and McCarthy, 2013) or lymphomas (Gascoyne et al., 2013) after the delivery of high dose chemotherapy (Bendall and Bradstock, 2014). In allogeneic HST, patients receive stem cells from healthy donors. It is also used to restore immune function and haematopoiesis in patients with immunodeficiency syndromes and BM failure. Recently, it was used widely to treat patients with haematological malignancies as cellular immunotherapy (Cavazzana-Calvo et al., 2013, Parmar and Ritchie, 2014).

Conventionally, autologous and allogeneic HST were carried out by aspirating large volumes of BM from the pelvic crests under general anaesthesia. However, many clinical data demonstrated that GCSF can mobilise large numbers of haematopoietic cells from the BM into the circulation. Moreover, sufficient numbers of these cells could be collected from normal donors or cancer patients to allow successful allogeneic and autologous HST, respectively (DeLuca et al., 1992, Sheridan et al., 1992, Grigg et al., 1995, Bendall and Bradstock, 2014). These data has led to extensive use of GCSF to mobilise hematopoietic cells in the majority of allogeneic and autologous transplants (Bendall and Bradstock, 2014).

1.12. General adverse effects of GCSF administration

Besides AML and MDS, there are other adverse events reported in patients who receive GCSF. These events are summarised in Table 1.

Table 1. Side effects related to the administration of GCSFs.			
System	**Adverse events**	**Possible mechanism/s**	**References**
Musculoskeletal & autoimmune	Osteopaenia & osteoporosis (common)	GCSF administration leads to increase bone resorption by increasing the activity of osteoclasts, resulting in a significant bone loss.	(D'Souza et al., 2008)
	Joint pain & generalised weakness (common).	1)Bone marrow expansion ,2) Stimulation of GCSF-receptors on primary afferent nerve fibres, 3) Stimulation of inflammatory cells, 4) Activation of osteoclasts and osteoblasts (Figure 11)	(Lambertini et al., 2014)
	Reactive rheumatoid arthritis (uncommon).	Increase the number & activity of circulating neutrophils.	(Hayat et al., 1995, Vidarsson et al., 1995)
	Pseudogout exacerbation (uncommon)	Increase the cytokines production in joints by the presence of a large number of neutrophils.	(Sandor et al., 1996)
Renal	Reversible renal impairments (uncommon)	GCSF induced leukocytosis in the kidneys.	(Hirokawa et al., 1996)
Cutaneous	Erythematous rash & urticaria (common), Sweet syndrome, erythema nodosum, pyoderma gangrenous, exacerbation of psoriasis, & neutrophilic dermatosis (uncommon).	Neutrophilic infiltration into epidermis and dermis	(Nomiyama et al., 1994, Dereure et al., 2000, Prendiville et al., 2001, George et al., 2005, White et al., 2006, Llamas-Velasco et al., 2013)
	Maculopapular rash & granulomatous dermatitis (uncommon)	Macrophages accumulation in skin.	(Glass et al., 1996, Alvarez-Ruiz et al., 2004)
Respiratory	Acute respiratory distress syndrome (uncommon)	Accumulation of activated neutrophils in the lung due to chemoattractant molecules released during infection. Then these cells release a variety of injurious substances such as platelet –activating factors, proteases, oxidants, and leukotrienes, which lead to damage the alveolar epithelium and endothelium.	(van Woensel et al., 1994, Niitsu et al., 1997, Hierholzer et al., 1998, Inano et al., 1998, Yokose et al., 1998, Couderc et al., 1999, Gertz et al., 2000, Azoulay, 2001, Yamaguchi et al., 2012, Inokuchi et al., 2014)
Cardiovascular	Acute arterial thrombosis (uncommon)	GCSF induces platelet activation & aggregation.	(Kawachi et al., 1996, Hüttmann et al., 2006)
	Myocardial infection (uncommon)	GCSF induces inflammatory cells aggregation and angiogenesis within the atherosclerotic plaque.	(Darie et al., 2004, Kang et al., 2004, Hill et al., 2005, Wang et al., 2005, Haghighat et al., 2007)
Haematological	Sickle cell crisis in haemoglobinopathies patients (uncommon)	An increase in circulating activated neutrophils with enhanced endothelial attachment.	(Abboud et al., 1998, Wei, 2001)
	Splenomegaly & extramedullary haematopoiesis (common)	Stimulation of myelopoiesis.	(Litam, 1993, O'Malley et al., 2003)
GCSF = Granulocyte colony stimulating factor			

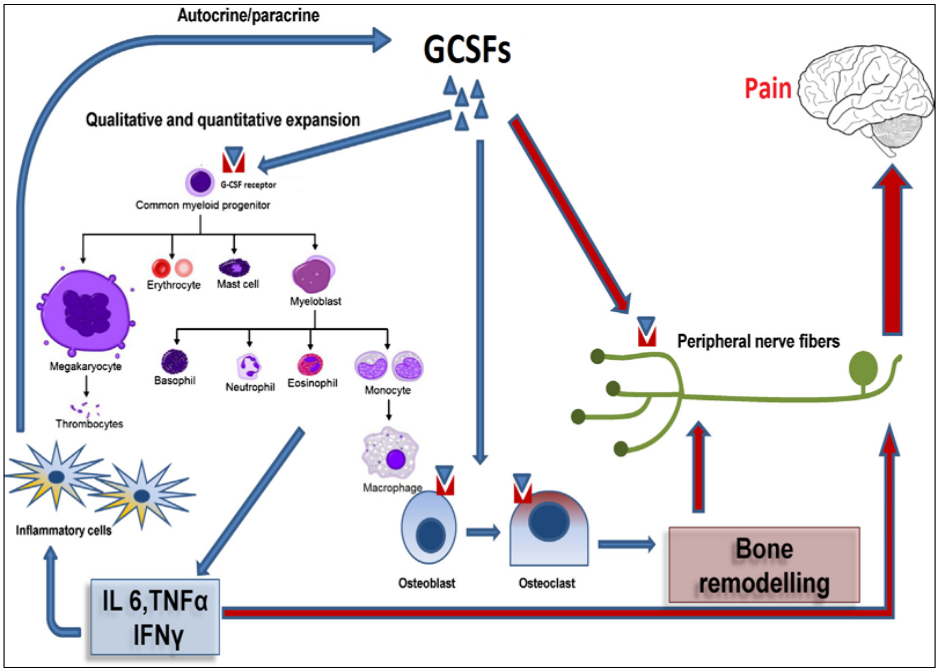


Figure 11. Causes of bone pain related to GCSF administration.

There are four possible causes for bone pain due to administration of GCSF. The first and important one is the quantitative and qualitative expansion of the BM. Mature cells stimulation followed by cytokines secretion (such as IL6, &TNFα) and inflammatory mediators production (such as arachidonic acid & leukotrienes) could also contribute to pain. The second one is stimulation of GCSFR on afferent nerves leads to produce peripheral nociceptor sensitization. The third one is stimulation of inflammatory cells (such as monocytes & macrophages) contributes to nerve remodelling by releasing of inflammatory cytokines. The fourth one is activation of osteoclasts and osteoblasts by GCSF induces bone resorption (Modified from Lambertini et al., 2014, pp.119).
GCSFs= colony stimulating factors
IL-6= interleukin 6
TNFα= tumour necrosis factor-alpha
IFN=interferon

1.13. Available forms of rhGCSF

Many different preparations of rhGCSF are available from different manufacturers. The most common forms are, Filgrastim (Neupogen®, Amgen, Inc.), Lenograstim (Granocyte®, Chugai Pharmaceutical Co.), Pegfilgrastim (Neulasta®, Amgen, Inc.), and more recently Lipegfilgrastim (Lonquex®, TEVA Ltd). More details about each one of them are described below.

1.13.1. Short-acting GCSF

1.13.1.1. Filgrastim (Neupogen®)

Filgrastim is a recombinant methionyl hGCSF (r-metHuGCSF) produced in E. *coli*. It consists of 175 amino acids with a molecular weight (MW) around 18.8 kDa. Its amino acid sequence is similar to the native GCSF produced in human cells, except for the addition of an *N*-terminal methionine, which is important for providing stability in the bacterial expression system (Roskos, 2012). Filgrastim is rapidly eliminated from the circulation (half–life ~ 3.5 hours), by renal clearance, and GCSFR-mediated- endocytosis (Kuwabara et al., 1996). Because of the short half-life, it is given as daily subcutaneous injections to maintain an effective concentration, which is associated with poor patient compliance (Roskos, 2012, Hoggatt and Pelus, 2014).

1.13.1.2. Lenograstim (Granocyte®)

Lenograstim is an *O*-linked, glycosylated form of rhGCSF, which is expressed in Chinese hamster ovary (CHO) cells. It consists of the same 174 amino acids as the natural hGCSF sequence. *In vitro*, the carbohydrate residues of the glycosylated GCSF (i.e. Lenograstim) play an important role to protect the protein from proteolytic degradation, resulting in an increase in stability (Dunn and Goa, 2000). This was explained by the greater pH resistance of the glycosylated molecule (Bonig et al., 2001). However, *in vivo* comparative studies, comparing Filgrastim to Lenograstim demonstrated that the activity of Lenograstim was similar to Filgrastim with respect to maximum WBC counts and time to neutrophil recovery after myelosuppressive chemotherapy (Nohynek et al., 1996, Tanaka et al., 1997, Hüttmann et al., 2005). Moreover, it needs frequent daily injections to maintain its activity (half-life ~3-4 hours), which are similar to Filgrastim (Pedrazzoli et al., 1996). In addition, a study showed that there was no clinical superiority of Lenograstim compared to Filgrastim in any of the approved indications, including treatment of neutropaenia (Ataergin et al., 2008), in haematopoietic peripheral

stem cells mobilisation (Baumann et al., 1993, Welte et al., 1996, Hüttmann et al., 2005). However, previous studies have shown that a lower dose of Lenograstim is as effective as the standard dose of Filgrastim for haematopoietic peripheral stem cells mobilisation in patients undergoing autologous HST. Additionally, a more rapid mobilisation has been observed in patients receiving Lenograstim compared to those receiving Filgrastim (Median time to the collection: 12 days, 13 days; respectively) (Ataergin et al., 2008). A more recent study investigated the differences in haematopoietic peripheral stem cell mobilisation in response to Filgrastim, Lenograstim, and Pegfilgrastim. The results showed that high-dose chemotherapy plus Lenograstim resulted in more rapid, adequate mobilisation with fewer leukapheresis compared to Filgrastim and Pegfilgrastim. In the setting of WBC and platelet recovery, no differences were observed between the three regimens (Ria et al., 2015).

1.13.2. Long-acting GCSF

The key problems with the first generation GCSF therapies (Filgrastim & Lenograstim) are rapid elimination from the circulation, short half-lives, and daily injections. During neutropenia, GCSFR-mediated- endocytosis is significantly reduced, and renal clearance becomes dominant. Thus, if the renal clearance could be reduced or eliminated while the GCSFR-mediated- endocytosis was retained, the drug would remain in circulation during neutropenia and be cleared only when the neutrophils start to recover (Arvedson et al., 2015). This results in a self-regulating therapeutic. A variety of technologies has been either developed or are in development (Table 2) that are designed to improve the current limitations of GCSF therapy. Some of these technologies are designed to make the protein larger, more elongated, or even more negatively charged, which results in reduced or eliminated renal clearance (Ohlson et al., 2001, Arvedson et al., 2015).

1.13.2.1. Pegfilgrastim (Neulasta®)

PEGylated Filgrastim (Called; Pegfilgrastim), was approved globally in 2002. It was the only second generation GCSF available on the market until 2013 when the European Medicines Agency (EMA) approved Lipegfilgrastim. Pegfilgrastim is produced by covalently conjugating a 20-kD polyethene glycol (PEG) molecule to the *N*- terminal methionine of Filgrastim (i.e., only one PEG molecule attached per GCSF) (Roskos, 2012). In general, the biological effects of Pegfilgrastim are similar to the parent molecule; however, Pegfilgrastim has greater pharmacokinetic properties than Filgrastim because the PEG molecule associates with two or three water molecules which create a hydrophilic shield. This shield protects the protein from proteolytic digestion and immunologic recognition (Milla et al., 2012). Furthermore, the PEG molecule of Pegfilgrastim results in an increase in the molecule size (~38.8 kD), resulting in prevention of the renal clearance of the drug, and reduced neutrophil receptor-mediated clearance (Galluppi, 2001, Yang, 2013). Serum levels of Pegfilgrastim are sustained with a median half-life of 42 hours and could be dosed either by body weight or as a fixed dose, subcutaneously, once or twice weekly to treat severe neutropenia or once per chemotherapy cycle (Molineux, 2004, Knudsen et al., 2011). As a result of less frequent and fixed dosing, Pegfilgrastim simplifies the management of neutropenia, minimises dosing errors, and improves patient quality of life (Molineux, 2004). However, the high cost of the PEGylation process is considered as one of its drawbacks, because Pegfilgrastim needs post-expression chemical modification and many steps of purification (Pisal et al., 2010).

1.13.2.2. Lipegfilgrastim (Longuex®)

Lipegfilgrastim is site-specific glycol-pegylated recombinant methionyl GCSF (MW~ 38-39 kDa), which is PEGylated by conjugation of a single 20-kDa PEG to the natural *O*-glycosylation site (i.e. threonine^{134}) using a novel glycoPEGylation technology (Abdolzade-Bavil et al., 2013). Because the glycosylation site is empty

as the recombinant GCSF is produced in E. *coli*, the addition of the *O*-glycan was achieved enzymatically using two recombinant glycosyltransferase enzymes and activated sugar nucleotide donor substrates. Finally, the PEG molecule is covalently attached to the *O*-glycan (Hoggatt and Pelus, 2014). Binding of Pegfilgrastim and Lipegfilgrastim to GCSFR was evaluated in preclinical studies using an NFS-60 cell-based [^{125}I]-GCSF competitive GCSFR binding assay. In this assay, binding of GCSFR to Pegfilgrastim and Lipegfilgrastim was similar (Abdolzade-Bavil et al., 2013, Hoggatt and Pelus, 2014). Furthermore, Lipegfilgrastim had a 30% greater neutrophil response and a 60% higher bioavailability compared to Pegfilgrastim in healthy volunteers. Additionally, Lipegfilgrastim exhibited better time-dependent resistance to neutrophil elastase degradation and greater retention of functional activity than Pegfilgrastim, which might explain the longer *in vivo* half-life of Lipegfilgrastim (i.e. 5-10 hours longer) versus Pegfilgrastim (Abdolzade-Bavil et al., 2013, Buchner et al., 2014). Similar to Pegfilgrastim, Lipegfilgrastim is cleared mainly by neutrophil-mediated clearance involving internalisation via GCSFRs and degradation within the neutrophil.

Table 2. Long-acting GCSF formulations in development.

Drug	Description	Stage of development	references
Balugrastim	A recombinant fusion protein of human serum albumin and GCSF produced in yeast	Phase III study ongoing	(Gladkov et al., 2016)
Empegfilgrastim (BCD-017)	PEGylated GCSF with 30-kDa PEG residue	Phase III study ongoing	(Hoggatt and Pelus, 2014)
Maxy-G34	PEGylated GCSF with 3 units of 5-kDa PEG residue attached to 3 amino acids	Completed phase IIa study	(Jevsevar et al., 2010)
Benefilgrastim (F-627)	A recombinant fusion protein of human Fc and GCSF produced in mammalian cells	Phase II study ongoing	(Glaspy et al., 2014)
Pegnartograstim (Ro 25-8315)	Mutant GCSF with replacement of 5 amino acids at the *N-terminal*. PEG added to *N-terminus* and lysine residues: 1–3 units of 20 kDa PEG per GCSF	Completed phase I study	(Bowen et al., 1999, van der Auwera et al., 2001)
BK0026	Single 20 kDa PEG conjugated to glutamine 135	Phase I study ongoing	(Scaramuzza et al., 2012)
StimuXen (Lipoxen)	Polysialylation (attach poly Sialic acid to *N-terminal* of GCSF)	Preclinical	(Zhang et al., 2014)
3DHSA-G-CSF	Fusion of the domain III of human serum Albumin to GCSF.	Preclinical	(Zhao et al., 2013)
GCSF/IgG-Fc & GCSF/IgG-C_H	Fusion of GCSF to IgG1& IgG4 (Fc & C_H domains), respectively.	Preclinical	(Cox et al., 2004)

GCSFs= granulocyte colony stimulating factors
PEG = polyethylene glycol
Ig = immunoglobulin

1.14. General potential drawbacks of PEGylation

Despite PEGylation having many positive impacts on biological therapies such as increasing drug stability, reduced renal excretion, and reduced dosing frequency, it has some drawbacks. Besides the high cost of manufacture, the chemical alteration of the protein surface and amino acid charge in proteins by this process can lead to impaired protein activity (Fishburn, 2008, Veronese and Mero, 2008,

Jevsevar et al., 2010, Carter, 2011). In addition, the PEG polymer is polydispersed and manifests as different molecular weights which potentially could make the formulation of the PEG-conjugated proteins more susceptible to aggregation (Pisal et al., 2010). One major drawback of any drug treatment is the formation antibodies to the drug product. The presence of anti-PEG antibodies have been reported in some studies, suggesting that PEG is both antigenic and immunogenic (Armstrong, 2009, Garay et al., 2012), although generally PEG is considered to be non-immunogenic by itself, which may be due to rapid renal clearance of the unconjugated polymer. Some PEGylated proteins, especially uricase (Jun-ichi et al., 1985, Garay et al., 2012) and ovalbumin (Richter AW, 1984, Garay et al., 2012) have been associated with a strong anti-PEG immune response. Many studies reported that PEGylated liposomes induce a strong immune response which results in the rapid blood clearance of subsequent PEG-liposome doses (known as accelerated blood clearance) in monkeys, rabbits, rats, and mice. This phenomenon is due to an anti-PEG immune response (Semple et al., 2005, Judge et al., 2006, Wang et al., 2007), which was sufficient to induce significant morbidity (Semple et al., 2005). In 44% of patients with hepatitis C, PEG antibodies were detected but this did not affect the response to PEG-IFN in patients (Tillmann et al., 2010), and hyposensitisation therapy of allergic patients with PEG-modified allergens (Richter AW, 1984). However, rapid clearance of PEG-asparaginase was shown to directly correlate with the presence of anti-PEG antibodies in acute lymphoblastic leukaemia patients and may decrease therapeutic efficacy (Armstrong et al., 2007). Furthermore, a phase III clinical trial in patients with severe chronic gout showed that the use of Pegloticase resulted in the formation of PEG antibodies, leading to loss of efficacy and increased risk of subsequent infusion reactions (Sundy et al., 2011).

The Prevalence of PEG antibodies in up to 25% of healthy blood donors with no known previous exposure to PEG has been reported (Armstrong, 2009) which is

higher than reported in 1984 with an estimated occurrence of 0.2% (Richter AW, 1984). This increase in the prevalence of PEG antibodies may be due to an improvement in the limit of detection of antibodies and to greater exposure to PEG in processed foods, pharmaceuticals and cosmetics (Garay and Labaune, 2011).

1.15. Asterion's ProFuse ™ technology

Asterion uses protein fusion technology (ProFuse ™) to generate long-acting biopharmaceuticals using one-step manufacturing and completely native components. This technology retains and prolongs the potent bioactivity of proteins. Therefore, Asterion's 3rd generation proteins reduce the risk of immunogenicity, delay clearance, require less frequent injection, limit side-effects, improve patient's compliance, and cost-of-good advantages over 1st and 2nd generation proteins.

Wilkinson and colleges succeeded in generating a long-acting growth hormone (GH) by fusing the human GH to its extracellular receptor (i.e.GH binding protein: GHBP). They observed that the clearance of this ligand-receptor GH fusion molecule was ~300 times less than the native GH in rats (Wilkinson et al., 2007, Cawley et al., 2013).

Furthermore, a number of long-acting GCSF molecules have been designed, cloned, and expressed by linking GCSF to its extracellular receptor via flexible linkers. The main generated prototypes were 4A1 and 4D1 (Appendix A & B, respectively), with the lead molecule, 4A1 (Figure 12), being used in the majority of the preclinical work. It showed excellent delayed clearance properties and extremely long half-life (~60 hours) in Sprague-Dawley rats. One of the features of this molecule is the potential ability of GCSF to have both intra and intermolecular interactions with the associated receptor in the fusion which may

provide a mechanism of protection against proteolysis and prolong the action of GCSF.

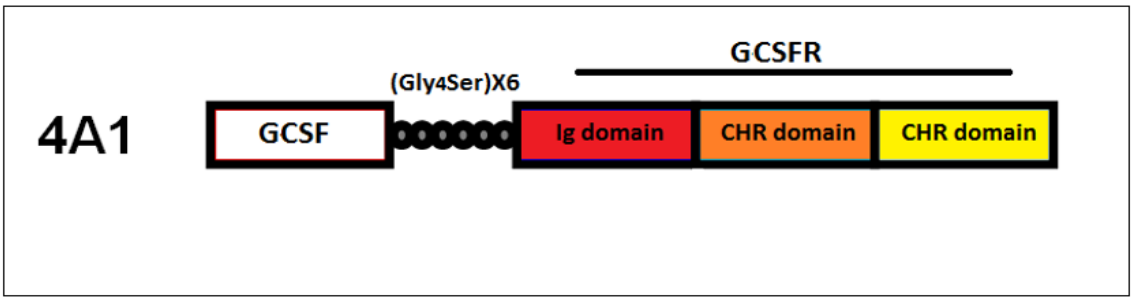


Figure 12: A schematic diagram of 4A1 (GCSF-L-GCSFR) construct.

GCSF molecule binds to the Ig domain CRH domains of one receptor chain and to the CRH domains of the second receptor chain by infusion fixable Gly_4Ser X6 linker.
GCSF= granulocyte colony stimulating factor
GCSFR= granulocyte colony-stimulating factor receptor
Ig = immunoglobulin
CHR = cytokine-binding homology region

Using the concept of this fusion technology, we propose to generate a long-acting GCSF molecule by linking GCSF to an inactive GH binding protein (GHBP). The fusion of GCSF to GHBP increases the molecular weight from 18.8kDa for GCSF alone to 60kDa for the fusion. GHBP contains tryptophan-104 to alanine change that prevents binding to GH in the circulation. The inactive GHBP would be expected to prolong the circulating half-life of GCSF through increased protein size, which will help to reduce *in vivo* clearance, whilst GCSF will be free to bind and activate its receptor. Furthermore, this molecule may be hypothesised to have increased biological activity due to less intra and intermolecular interactions compared to previously tested GCSF fusions (i.e. 4A1).

1.16. Rationale for fusing GCSF to GHBP

- GCSF is a 4 alpha helical cytokine similar to GH.
- The GH receptor is very similar structurally to the GCSF receptor containing two cytokine binding homology domains.
- Linking proteins such as albumin, antibody F_C fragment and the carboxy terminal peptide to the *C*-terminus of cytokines helps to delay clearance. GHBP, when fused to GH, delays the clearance of the molecule a may have a similar action when linked to GCSF.

1.17. Main aims

The aims of the current project are listed below:

- Construct and express the fusion protein in a mammalian system.
- Compare the biological activity of the fusion protein to commercial GCSF using an AML *in vitro* proliferation assay.
- Produce proof of concept data by studying the pharmacokinetic and pharmacodynamic properties of the fusion molecule in a mouse model.

Chapter 2. Materials and Methods

2.1. Materials

2.1.1. Suppliers

Abcam	Cambridge, UK
Amgen	Thousand Oaks, USA
ATCC	Middlesex, UK
BD Biosciences	San Jose, USA
BDH Laboratories	Poole, UK
Beckman Coulter	Fullerton, USA
Beckton Dickinson	Oxford, UK
Biolegend	London, UK
Bio-Rad Laboratories	Hemel Hempstead, UK
BioTek	Bedfordshire, UK
Charles River	Freiburg, Germany
Costar	Cambridge, UK
ECACC	Porton Down, UK
ENM	Enfield, UK
Eppendorf UK Ltd.	Cambridge, UK
Fisher-Scientific	Loughborough, UK
Fujifilm	Tokyo, Japan
GE Healthcare	Little Chalfont, UK
Geneflow Ltd.	Staffordshire, UK
Gibco (Invitrogen Corp.)	Paisley, UK
Greiner Bio-one	Stonehouse, UK
Ilford	Mobberley, Cheshire, UK
Invitrogen	Paisley, UK
Iwaki SLS	East Riding of Yorkshire, UK
Labtech International Ltd.	East Sussex, UK
Marvel	Dublin, Ireland
Melford laboratories	Ipswich, UK

Merck BDH	Lutterworth, UK
Mirus Bio LLC	Wisconsin, USA
Nalgen Nunc International	Roskilde, Denmark
New England Biolabs	Massachusetts, USA
Nuve	Istanbul, Turkey
Olympus Optical Co. Ltd.	Tokyo, Japan
Oxoid Ltd.	Basingstoke, UK
Pharmacia & Upjohn	Milton Keynes, UK
Promega	Southampton, UK
ProSci	Poway, USA
Qiagen	West Sussex, UK
R&D systems	Minneapolis, USA
Roche Diagnostics	Mannheim, Germany
Sanyo	Moriguchi, Japan
Sarstedt	Numbrecht, Germany
Sartorius	Göttingen, Germany
Sigma-Aldrich	Poole, UK
Source BioScience	Nottingham, UK
Stuart	Staffordshire, UK
VWR	Lutterworth, UK

2.1.2. Plasmid vector

pSecTag	Invitrogen

2.1.3. Oligonucleotide primers

Name	Sequence	Target DNA sequence
GCSF_Nhe1F	5' AAGCTGGCTAGCCACCATGGC 3'	GCSF signal sequence
GCSF_Not1	5'AATTAATAGCGGCCGCCGGGCTGGGCAAG 3'	
GCSF-F	5'TGCCCCAGCCAGGCCCTGCA3'	GCSF
GHBP -R	5' TCCAGGGTGCTCTGCTAAGG 3'	GHBP
BGH-R	5' TAGAAGGCACAGTCGAGG 3'	GHBP
CMV	5' TATTACCATGGTGATGCGGTTTTGG 3'	GCSF

2.1.4. Bacterial cells strains and mammalian cell lines

Cells	Uses	Characterizations	Supplier
XL1-Blue *E.coli* strain	Transformation of plasmid DNA and for plasmid preparation	Genotype; recA1, endA1, gyrA96, thi-1, hsdR17, supE44, relA1, lac, [F', proAB, $lacI^qZM15$, Tn10 (tet^r)].	Stratagene
CHO-Flp In	Expression of both pSecTag GCSF-GHBP-W104-Histag, & pSecTag GCSF-GHBP- Histag	Express the lacZ-Zeocin™ fusion gene & contains a single integrated Flp Recombination Target (FRT) site.	Invitrogen

2.1.5. DNA cloning

100bp ladder	New England Biolabs
1kb ladder	New England Biolabs
Acetylated bovine serum albumin (acBSA)	Promega
Agar	Sigma-Aldrich
Agarose	Sigma-Aldrich
Ampicillin	Sigma-Aldrich
Beckman Avanti J-20I centrifuge	Beckman Coulter
CIAP Buffer	Promega
$CaC1_2$	Sigma-Aldrich
Carbenicilin	Melford laboratories
Digestion buffers	Promega
DNA Loading Dye (6X)	Promega

dNTPs	Pharmacia & Upjohn
EDTA	Beckton Dickinson
Ethidium bromide	Sigma-Aldrich
Expand high fidelity PCR system	Roche
GenElute, gel extraction kit	Sigma-Aldrich
Glycerol	Beckton Dickinson
Ligase buffer	Promega
$MgCl_2$ (hydrous or anhydrous)	Sigma-Aldrich
Minispin centrifuge	Eppendorf UK Ltd.
NaCl	Beckton Dickinson
Nanodrop spectrophotometer (ND-1000)	Labtech International Ltd.
QIAprep spin mini-prep kit	Qiagen
Restriction enzymes	Promega & New England Biolabs
Taq polymerase	Promega
T4 ligase	Promega
Tris-Base	Sigma-Aldrich
Tryptone	Melford Laboratories
Yeast extract	Melford Laboratories

2.1.6. Bacterial Cell Culture

2.1.6.1. Media

Media	Content
Luria-Bertani (LB) medium*	1% Tryptone 0.5% Yeast extract 85.6 mM NaCl
SOC medium	Pre-made: 2% Tryptone 0.5% Yeast extract 20 mM glucose 10 mM NaCl 2.5 mM KCl 10 mM $MgCl_2$ 10 mM $MgSO_4$

*In order to make LB agar plates for selection of transformants, 5% (w/v) of agar was added to LB media with a selective antibiotic.

2.1.6.2. Antibiotics

Carbenicillin	Sigma-Aldrich
Hygromycin B	Gibco (Invitrogen Corp)
Zeocin	Invitrogen

2.1.7. Mammalian cell culture

6-well plates	Costar
12-well plates	Costar
96-well Cell Culture Plate	Costar
T25 & T75cms filtered flasks	Nalgen Nunc intl
AML-193 cell line	(ATCC;Lot#:347526; Cat.#: CRL-9589)
CellTitre 96 AQueous Proliferation Assay Reagent	Promega
Cryogenic vials	Nalgen Nunc intl
Foetal Calf serum (FCS)	Labtech
Fugene-6 transfection reagent	Roche Diagnostics
GMCSF (5ng/ml)	Source BioScience
Ham F12 mix	Gibco (Invitrogen Corp)
Hyclone	Fisher-Scientific
Insulin	Sigma-Aldrich
Iscove's Modified Dubellco Media (IMDM)	Gibco (Invitrogen Corp)
Labtech 2 wells chamber slides	Nalgen Nunc intl
Labtech chambers coverglass	Nalgen Nunc intl
L-Glutamine	Gibco (Invitrogen Corp)
Mirus Trans	Mirus Bio LLC
Olympus CK2 microscope	Olympus Optical Co. Ltd.

Penicillin-Streptomycin	Gibco (Invitrogen Corp)
Phosphate Buffer Saline 10X	Gibco (Invitrogen Corp)
Sanyo CO_2 Incubator	Sanyo North America Corp
Tally counter	ENM
Thermostated water bath (NB9)	Nuve
Transferrin	Sigma-Aldrich
Trypan blue	Sigma-Aldrich
Trypsin-EDTA	Gibco (Invitrogen Corp)

2.1.8. Protein detection

Acetic acid	Fisher-Scientific
Acrylamide (30%)	Geneflow Ltd.
Ammonium persulphate (APS)	Merck BDH
Avidin- Horseradish Peroxidase (HRP)	Biolegend
Bovine gamma-globulin	Sigma-Aldrich
Bovine serum albumin (BSA)	Sigma-Aldrich
Bradford reagent	Bio-Rad Laboratories
Bromophenol Blue	Sigma-Aldrich
Clarity™ western ECL substrate	Bio-Rad Laboratories
Coomassie Blue reagent	Sigma-Aldrich
Cuvettes	Sarstedt
Dithiothreitol (DTT)	Sigma-Aldrich
Dried skimmed milk	Marvel
Ethanol	Fisher-Scientific
Glycine	Fisher-Scientific
H_2SO_4	Merck BDH
Iso-propanol	Fisher-Scientific
2X Laemmli sample buffer	Bio-Rad Laboratories

Methanol	VWR
Microplate reader	Labtech
Microwave	Sanyo
Mini orbital shaker	Stuart
Mini Protean II	Bio-Rad Laboratories
NaCl	Melford Laboratories
$NaHCO_3$	Sigma-Aldrich
NaN_3	Merck BDH
Phosphate buffered saline (PBS) tablets	Oxoid Ltd.
pH Meter	Geneflow Ltd.
Polyvinylidene fluoride (PVDF) membrane	Bio-Rad Laboratories
Precision plus protein standards	Bio-Rad Laboratories
Recombinant human GCSF	Biolegend
Sodium dodecyl sulfate (SDS)	Sigma-Aldrich
3, 3', 5, 5'-tetramethylbenzidine (TMB)	Sigma-Aldrich
Tetra-methyl-ethylene-diamine (TEMED)	Merck BDH
TRIS Base	Sigma-Aldrich
Tris-HCl	Melford Laboratories
Tween20	Sigma-Aldrich
Vortex mixer	Stuart
X-Ray developer & fixer solutions	Ilford
X-ray films	Fujifilm
96-well microtiter plate	Costar

2.1.9. Antibodies

Proteins detection method	1ry antibody	2ry antibody	Supplier
Western blot & Coomassie stain	Rabbit polyclonal to GCSF	Goat anti-rabbit IgG, pAb	Abcam
ELISA	Purified anti-human GCSF [BVD13-3A5]	Biotin anti-human GCSF [BVD11-37G10]	Biolegend
***In vivo* study**	**Antibody**	**Isotype**	**Supplier**
	FITC Rat Anti-Mouse Ly-6G & Ly-6C (0.5 mg/ml)	FITC Rat IgG2b, Isotype Control (0.5 mg/ml)	BD Biosciences
	PE Rat Anti mouse CD117 (0.2 mg/ml)	PE Rat IgG2b, Isotype Control (0.2 mg/ml)	BD Biosciences
	PE-Cy5 Rat Anti mouse CD11b (0.2 mg/ml)	PE-Cy5 Rat IgG2b, Isotype Control (0.2 mg/ml)	ProSci

2.1.10. Protein purification and concentration

5ml IMAC HiTrap column	GE Healthcare
AKTA prime rig	GE Healthcare
Avanti centrifuge J-26XP	Beckman Coulter
Imidazole	Sigma-Aldrich
Millipore filter unit (0.22µm)	Fisher-Scientific
NaOH	BDH Laboratories
Nickel Chloride	Sigma-Aldrich
Roller bottle (2L)	Greiner Bio-one
Sodium acetate (anhydrous)	Fisher -Scientific
Sodium phosphate dibasic	BDH Laboratories

Sodium phosphate monobasic	BDH Laboratories
Viva flow 200 concentrator	Sartorius

2.1.11. *In vivo* study

1ml syringes and 25-G, 16mm needles	Beckton Dickinson
BDF1 mice	Charles River
Cell strainer (70µm)	Fisher –Scientific
Children EDTA tubes	Beckton Dickinson
Dissection kit	
Erythrocytes lysing kit	R&D systems
Flow cytometry machine (LSRII)	Beckton Dickinson
Isoflurane	GE Healthcare
Neupogen (Filgrastim) injection 30 MU (0.6 mg/ml)	Amgen

2.2. General methods

2.2.1. Growth and storage of cells

2.2.1.1. Growth and storage of bacterial cells

For growing Escherichia coli (*E.coli*) strains, frozen glycerol stocks of these strains (either transformed with the plasmid of interest or non-transformed), were initially streaked onto LB agar plates containing selective antibiotic(s) when required. The plates were then incubated overnight at 37°C. The next day, one well-isolated colony was inoculated into LB medium containing an appropriate antibiotic, and grown during the day at 37°C for 6-8 hours. This was then seeded into fresh LB medium (1/1000 dilution) and grown overnight at 37°C in an orbital shaker. For long-term storage, glycerol stocks were made by mixing 800µl of an overnight grown bacterial culture with 200 µl of 50% sterile glycerol in a 1.5 ml Eppendorf tube. The cells were then frozen at -80°C.

2.2.1.2. Preparation of chemically competent cells

XL1-Blue cells were used for plasmid DNA transformation. The following protocol was followed to prepare these cells. On a fresh LB/ Tetracycline agar plate, 10µl of an XL1-Blue glycerol stock was plated and incubated at 37°C overnight. The next day, one well-isolated colony was picked and used to inoculate 2 ml LB media and allowed to grow for at least 6 hours at 37°C in an orbital shaker at 37°C. Then, 50 µl of this bacterial culture was seeded into 50 ml LB media, and grown overnight at 37°C with shaking shaker. The next day, approximately 20-25 ml of the overnight culture was used to inoculate 250 ml of a pre-warmed LB media and grown at 37°C with shaking until an OD600 of approximately 0.9 was reached. After that, the culture was placed on ice and chilled for 10 minutes before centrifugation at 2400 x g for 30 minutes at 4°C to pellet the cells. The media was discarded, and the cell pellet resuspended in 100 ml of a sterile, ice-cold 0.1M $MgCl_2$ solution and centrifuged as described previously. The supernatant was discarded, and the pellet resuspended in 100 ml of a sterile, ice-cold 0.1M $CaCl_2$ and kept on ice for 60 minutes before centrifuging again. The supernatant was once again removed and the pellet finally resuspended in 12.5 ml of 85mM $CaCl_2$ plus 15% glycerol. The cell suspension was aliquoted (~450 µl) into pre-chilled, sterile 1.5 ml Eppendorf tubes and immediately placed in liquid nitrogen. The cells were finally stored at -80°C.

2.2.1.3. Growth and maintenance of adherent mammalian cells

Non-transfected Chinese hamster ovary cells (CHO) were grown in Ham's/F12 medium supplemented with 10% FCS, 100µg/ml Streptomycin / Penicillin, and 2mM L-glutamine. The same media was used to grow the CHO-Flp-In cell line supplemented with 100 µg/ml Zeocin. Cells were grown in a 5% CO_2 incubator at 37°C in a 95% humidified atmosphere. For passaging adherent cells, the medium was removed from the flask when the cells were 80-90% confluent. After that, the cells were washed once with 10 ml PBS to remove excess medium and serum

before incubation with 5 ml Trypsin-EDTA for 2 minutes at 37°C. The cells were then dislodged by gently tapping, and checked under a microscope to confirm detachment. Thereafter, 10ml of the complete medium were added to stop trypsinization (as prolonged cell incubation with trypsin results in damaged cell membranes and cell death) and cells were centrifuged for 5 minutes at 150 x g. The resulting pellet was resuspended in the appropriate medium and counted using a haemocytometer before plating out at the required cell density. Usually a dilution of 1:5 to 1:10 was used for routine growth.

Stable cell lines containing GCSF-GHBP and GCSF-W104-GHBP, and carrying the hygromycin B-resistant gene were grown in the same medium as described above supplemented with 250 µg/ml hygromycin B.

2.2.1.4. Adaptation of adherent cells to serum-free suspension culture

Stable CHO Flp-In cells lines were routinely adapted to serum-free suspension culture for use in protein expression. The adaptation process ensures that high cell densities can be achieved (thus reducing volume) thus reducing the need for large numbers of adherent cell culture flasks. Furthermore, serum free media reduces contamination derived from media components.

When the confluency of adherent cells in T75 flasks reached ~ 70 to 90%, the complete growth medium (i.e. Ham's/F12 medium supplemented with 10% FCS, 100µg/ml Streptomycin / Penicillin, and 2mM L-glutamine) was removed, and replaced with, fresh, serum-free medium (Hyclone SFM4CHO Utility). The viability of any detached cells was checked every 2 days using trypan blue exclusion (Section 2.2.2). After detachment of adherent cells, (~1 week), they were collected by centrifugation (5 minutes at 150 x g), counted and re-seeded into fresh Hyclone SFM4CHO Utility media at 0.25 - 0.5 × 10^6 cells/ml. Cells were considered adapted when cell doubling was approximately every 2 days with >90% cell viability. Stocks

were immediately frozen upon adaption (Section 2.2.1.4). For routine growth, cultures were maintained at densities between 0.25 to 2 x 10^6 cells/ml in Hyclone SFM4CHO Utility media supplemented with 250µg/ml hygromycin B, with passaging every 2-3 days back to ~0.25 x 10^6 cells/ml.

2.2.1.5. Storage & resuscitation of frozen mammalian cell stocks

Genetic changes could affect the continuously growing mammalian cell lines with high passage numbers. Therefore, the cells with low passage number were stored at -196°C in the presence of dimethyl sulfoxide (DMSO) which acts as a cryoprotective agent. The freezing medium was prepared as follows and kept on ice:

Cells type	Freezing medium		
	FCS	Serum-free medium (Hyclone)	DMSO
Adherent cells	90%	-	10%
Adapted suspension cells	-	90%	10%

After trypsinization, adherent cells and adapted suspension cells were transferred to sterile centrifuge tubes and centrifuged for 5 minutes at 150 x g. The resulting pellets were resuspended in the chilled freezing medium at a density of at least 3 ×10^6 viable cells/ml. 1 mL of the cell suspension was added to appropriately labelled and chilled cryogenic vials. These vials were then wrapped in cotton wool and stored at -80°C in polystyrene storage boxes. After 72 hours, vials were transferred to liquid nitrogen (-196°C) for long-term storage. For thawing: one vial of cells was removed from liquid nitrogen, and thawed quickly in a 37°C water bath (care was taken not to submerge the top). When only a few ice crystals remained the vial was wiped clean with 10% Trigene solution and cells diluted with an appropriate volume of pre-warmed culture medium (usually 10-15 ml) and plated out in T75 flasks. Cells were expanded appropriately for use. Usually, a minimum of 2 passages had to be undertaken with cell viability >90%, before cells were used in further experiments.

2.2.2. Cell counting and viability

Counting cells is important to set up new cultures with known cell numbers as well as to establish, and monitor growth rates. A haemocytometer was used to estimate cell number, and viability using trypan blue. Briefly, the cell suspension was diluted 1:1 (vol: vol) with Trypan Blue (a dilution factor of 2) loaded into the chamber edge of a clean, dry haemocytometer. Thereafter, the haemocytometer was placed under an inverted microscope and viewed at 100x magnification. Dead cells took up and retained the trypan blue stain whereas the stain was actively excluded by viable cells. The number of cells was counted in two of the outer four "large" squares as shown below (Figure 13). These counts were added together and divided by 2 to get an average number of cells per square.

The cell concentration was calculated as follows:

Cell concentration (in cells/ml) = average viable cell count per square x Dilution Factor (2) x 10^4

The cell viability was calculated as follows:

Cell Viability (%) = [Total Viable cells / Total cells (Viable +Dead)] X 100

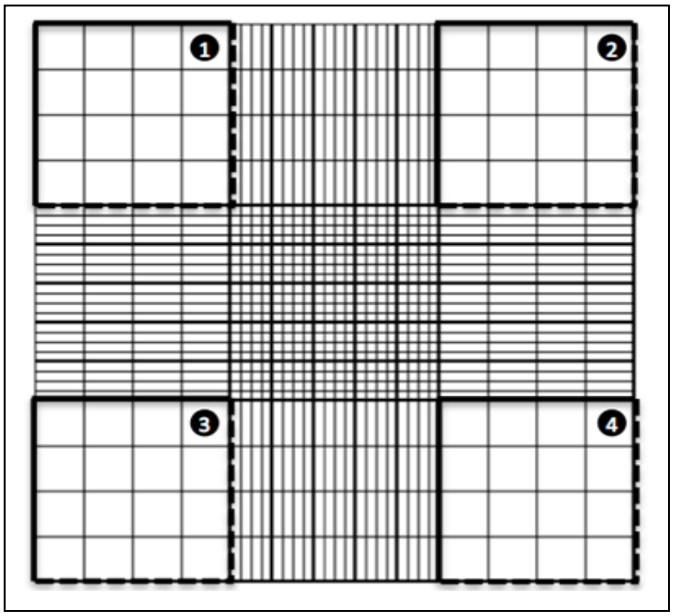


Figure 13. Grid layout of Haemocytometer.

After placing the haemocytometer under the microscope with a typical magnification of 100 x, the cells were counted in two of the corner four "large" squares (1mm x 1mm each one). The cells on the bottom and right lines (-----) were not included in the count (Adapted from Hemocytometer, 2013).

2.2.3. DNA manipulation and analysis

2.2.3.1. Polymerase chain reaction (PCR)

PCR was used in this work to amplify DNA fragments and introduce restriction enzymes sites into expression constructs. A GCSF fusion protein construct (Named: pGCSFSecTag-4A1) containing full-length GCSF and its signal sequence, which was available in our laboratory, was used to PCR the GCSF molecule.

The design of forward and reverse oligonucleotide primers to amplify the required regions of a DNA fragment was done preceding the PCR reaction. Synthesised primers were stored at -20°C at a concentration of 100pmol/µl (100µM). Reactions were carried out using Expand High Fidelity PCR system kit (Roche), and set up according to the following protocol:

Master Mix I (per reaction):

Constituent	Reaction	Control (no template)
Forward primer: GCSF)_Nhel (10pmol/µl)	1 µl	1 µl
Reverse primer: GCSF_Not1 (10pmol/µl)	1 µl	1 µl
dNTPs (10mM)	1.25 µl	1.25 µl
Sterile water	20.75 µl	21.75 µl
DNA template: 4A1 (100ng/ µl)	1 µl	-
Total Volume	25 µl	25 µl

Master Mix II (per reaction):

Constituent	Volume (x1 reaction)
10X polymerase buffer with $MgCl_2$ (1.5mM final concentration)	5 µl
Expand polymerase (10 IU/µl)	0.85 µl
Sterile water	19.15 µl
Total Volume	25 µl

Prior to loading the samples into the PCR machine, 25µl of the master mix II was added to 25µl master mix I. In addition, a separate PCR reaction without DNA template was run as a negative control.

The cycling conditions for PCR were as follows:

Cycles	Event	Temperature	Time
1 cycle	Initial Denaturation	94°C	2 minutes
25 cycles	Denaturation	94°C	30 seconds
	Primer annealing	54°C	30 seconds
	DNA extension	72°C	30 seconds
1 cycle	Final extension	72°C	10 minutes

2.2.3.2. Agarose gel electrophoresis

Agarose gel electrophoresis is the most popular method of separating and analysing a mixed population of DNA. It was used to estimate DNA concentration, to check the size of digested DNA fragments, and to allow for separation and purification of both PCR products and digested DNA prior to ligation. On each occasion, the percent of agarose used was determined by the DNA size to be analysed. Different agarose percentages that are suitable for separation of DNA of varying sizes are highlighted below:

Agarose % (w/v)	Range of DNA separation (bp)
0.6	1.000 – 30.000
0.7	800 –12.000
1	500 –10.000
1.2	400 –7.000
1.5	200 –3.000
2	50 –2.000

All agarose gels were prepared by boiling the appropriate amount of agarose in 1X TAE electrophoresis buffer (0.04M Tris-acetate, 1mM EDTA; pH 8.3), in a microwave oven for 1 minute. After that, 2.5 µl of ethidium bromide (Stock = 10 mg/ml) was added to each 50 ml of the cooled gel solution, to give a final concentration of 0.5 µg/ml. Ethidium bromide intercalates between nitrogenous

bases of DNA, therefore when it is exposed to ultraviolet light it fluoresces with an orange colour, which is intensified almost 20-fold after binding to DNA. The gel solutions were poured into casting trays along with well combs which were removed after the gels had set. Thereafter, DNA samples (including DNA markers) were diluted appropriately with 6X DNA loading dye before loading to the gels. For loading, 10% of the samples were loaded in an inner well for visualisation with ultraviolet (UV) light, while the remaining samples (90%) were loaded in a peripheral lane which would be cut away before exposure to UV. This was important to avoid potential damage to the remaining DNA samples, in the peripheral lane, by UV light during visualisation. DNA markers (100 bp or 1kb ladders) were used routinely to estimate DNA product size after migration. All gels were run at 100 volts.

2.2.3.3. DNA clean up and purification

GenElute gel extraction kit was used to clean up and purify DNA from 50 bp to 10 kb after PCR or enzymatic digestion. The kit was used as per manufacturers' instructions. Briefly, DNA was separated first by agarose gel electrophoresis. Then, a lane containing only a fifth of the total digested DNA was visualised using a UV transilluminator and the appropriate band cut out. This was then used to align with a lane containing the remaining digested DNA. The relevant band of DNA was excised from the agarose gel with a clean, sharp razor blade. The gel slice was then weighted in a 1.5 ml Eppendorf tube. After that, 300 µl of gel solubilisation solution was added for every 100 mg of agarose gel. In other words, 3 gel volumes of the gel solubilisation solution were added to the gel slice. The gel mixture was incubated at 60 °C until the gel slice was completely dissolved (approximately 10 minutes). Preparation of the binding column was completed while the agarose was being solubilised. Briefly, the binding column was placed into one of the provided 2 ml collection tubes. Then, 500 µl of column preparation solution was added to the binding column. The column was centrifuged at 13,000

rpm for 1 minute, and flow through liquid discarded. The column preparation solution increases the DNA binding affinity to the silica membrane resulting in more consistent yields. Before loading the solubilised gel mixture onto the binding column, 1 gel volume of 100 % isopropanol was added to the gel mixture to facilitate improved DNA binding to the column. The column was then centrifuged at 13,000 rpm for 1 minute, and flow through liquid discarded. Thereafter, 700 µl of wash solution was added to the binding column, centrifuged at 13,000 rpm for 1 minute, and flow through liquid was discarded. Before transferring the binding column to a fresh collection tube, it was centrifuged for an extra 1 minute without any additional wash solution to remove excess ethanol. Bound DNA was eluted by the addition 10 mM Tris-HCl buffer, pH 8.5 (preheated to 65 °C). Plasmid DNA recoveries can be improved by up to 2-3 fold when eluting at this temperature.

2.2.3.4. Restriction enzyme digestion

Restriction enzymes are enzymes that recognise specific, short DNA sequences, and they cleave double-stranded DNA at known specific sites within or adjacent to their recognition sequences. These enzymes generate either "sticky end" or "blunt end" DNA fragments. The sticky end fragments were produced when the enzymes cut asymmetrically within the recognition site, while the blunt end fragments were produced when the enzymes cut at precisely opposite sites in the two strands of DNA. All restriction enzymes and buffers which were used in this study are described below:

Restriction enzyme	Restriction enzyme buffer (10x concentration)	Restriction enzyme recognition site	End type
AvrII	**CutSmart™ buffer:** 50mM Potassium acetate; 20mM Tris-acetate; 10mM Magnesium acetate; 100µg/ml BSA	5'-C^{v}CTAGG-3' 3'-GGATC$_{\wedge}$C-5'	Sticky
EcoRV_HF	**CutSmart™ buffer:** 50mM Potassium acetate; 20mM Tris-acetate; 10mM Magnesium acetate; 100µg/ml BSA	5'-GAT^{v}ATC-3' 3'-CTA$_{\wedge}$TAG-5'	Blunt
Nhe1	**NEBuffer 2:** 50mM NaCl; 10mM Tris-HCl; 10mM MgCl2; 1mM DTT	5'-G^{v}CTAGC-3' 3'-CGATC$_{\wedge}$G-5'	Sticky
Not1	**NEBuffer 2:** 50mM NaCl; 10mM Tris-HCl; 10mM MgCl2; 1mM DTT	5'-GC^{v}GGCCGC-3' 3'-CGCCGG$_{\wedge}$CG-5'	Sticky

A typical double digestion reaction was set up as follows:

Constituent	Single digest with 1st restriction enzyme	Single digest with 2nd restriction enzyme	Control reaction (buffer only)
acBSA (1mg/ml)	1µl	1µl	1µl
DNA	500ng-1µg	500ng-1µg	500ng-1µg
Restriction enzyme buffer (10x)	1 µl	1 µl	1 µl
1st restriction enzyme (10U/µl)	1 µl	-	-
2nd restriction enzyme (10U/µl)	-	1 µl	-
Nuclease-free water to a final volume of	10 µl	10 µl	10 µl

The restriction enzyme was always added last after mixing, and its volume was less than or equal to 10% of the total reaction volume to avoid having >5% glycerol content in the reaction to avoid potential star activity. The reaction was incubated for 2 hours at 37 °C. A control reaction without restriction enzymes was also set up.

After incubation, a fifth (2.5 µL) of each single reaction was pipetted into two sterile Eppendorf tubes (these are the single digest controls). The remaining single

digests were pooled together (~15 µL) and appropriate volumes of acBSA (2 µL), restriction enzyme buffer (2 µL), restriction enzymes (1 µL of each), and nuclease-free water (made up to 35 µL) were added prior to incubation for 1 hour at 37°C. After digestion, the samples were mixed with the appropriate volumes of 6X DNA loading buffer, and analysed by agarose gel electrophoresis, to ensure correct digestion of DNA and to isolate appropriately digested DNA fragments. The relevant band of DNA was cleaned up using the GenElute kit and quantified using a nanodrop spectrophotometer at 260nm.

2.2.3.5. Calf intestinal alkaline phosphatase treatment

In DNA subcloning, calf intestinal alkaline phosphatase (CIAP) was used to catalyse the removal of 5´-phosphate groups from DNA strands, when the vector was cut with two enzymes that had different end types, i.e. sticky and blunt ends. Therefore, the linear DNA fragments which lacked the 5' phosphate groups could not recirculate and ligate. Particularly in this work, CIAP was used to remove the 5´-phosphate group from a pSecTag GCSF-GHBP-Hist plasmid, which was digested with AvrII/EcoRV restriction enzymes. Because the AvrII digest produced a 5’ recessed sticky end, while EcoRV-HF produced a blunt end, these ends potentially could re-ligate together and produce false positives or hinder the ligation process.

According to the manufacturer's instructions (Promega), to dephosphorylate of 5´ recessed or blunt end DNA fragments, CIAP was diluted first in CIAP 1X reaction buffer to a final concentration of 0.01U/µl. Then the reaction was set up as follows:

Constituent	Volume
DNA (up to 10 pmol of 5´-ends)	40 µl
CIAP 10X reaction buffer	5 µl
Diluted CIAP (0.01U/µl)	5 µl
Total	50 µl

The reaction was first incubated for 15 minutes at 37°C, and then at 56°C for 15 minutes. After that, the second aliquot of CIAP was added prior to repeating the incubations at both temperatures. The DNA was then isolated by agarose gel electrophoresis (Section 2.2.3.3) and cleaned up using the GenElute kit.

2.2.3.6. DNA Ligation

T4 DNA ligase was used to ligate the double digested vector and insert DNA fragments, with complementary “blunt “or” sticky " ends. This ligase catalysis the phosphodiester covalent bond formation between the 3' hydroxyl and 5' phosphate ends of the DNA in an ATP-dependent reaction. The molar ratio of vector to insert in the ligation reactions was 1:3 in a total volume of 10 µl. As a negative control in all ligation experiments, separate reactions were set-up that included all ingredients except the insert and/or T4 DNA ligase. The ligation reactions were incubated at 37°C for 3 hours before transformation into chemically competent bacterial cells.

A typical DNA Ligation reaction is described below:

Constituent	Ligation	Control 1	Control 2
Vector DNA (20-100ng)	X µl	X µl	X µl
Insert DNA	Y µl	-	-
10X Ligase Buffer	1 µl	1 µl	1 µl
T4 DNA ligase (10U/µl)	1 µl	1 µl	-
Nuclease-free water to a final volume of	10 µl	10 µl	10 µl

The amount of fragment to use per ligation was calculated as follows to give a 3:1 ratio (insert: vector):

ng of insert = (ng vector x kb size of insert)/ Kb size of vector X molar ratio of (insert/ vector).

2.2.3.7. Bacterial transformation

The heat-shock method was used to transform *E. coli* chemically competent cells with plasmid preparations or ligation reactions. Firstly, a 200 µl aliquot of these cells was thawed on ice. The ligation mixture (~10 µl) was added to the cells and left on ice for 20 minutes (As a positive control in each transformation reaction, 50 ng of a defined plasmid was also transformed). Cells were then heated shocked at 42°C for 1 minute and returned immediately to the ice for a further 10 minutes. Thereafter, 800 µl of the SOC medium was added to the cells, and incubated for 45 minutes in an orbital shaker at 37°C, in order to allow expression of the antibiotic resistance genes. The samples were centrifuged at 4000 rpm for 10 minutes, and the formed pellet re-suspended in 100µl of SOC medium and further dilutions carried out at 1:10 and 1:100 using SOC media. Dilutions were spread on LB agar plates containing carbenicillin (100 µg/ml) and left overnight at 37°C. The next day, three isolated colonies were selected from the agar plates, and grown up during the day at 37°C in 1ml LB media. The colonies were then seeded at 1/1000 into 6ml LB media containing carbenicillin, and grown overnight. The next day, cultures were used to produced plasmid mini-preparations and/or glycerol stock.

2.2.3.8. Preparation of plasmid DNA (DNA mini-prep)

The QIAprep spin miniprep kit protocol was followed to purify small amounts (up to 20µg) of a plasmid DNA from 1-6 ml overnight bacterial cell cultures. Firstly, one well-isolated colony from a freshly streaked LB/ carbenicillin agar plate was inoculated with 1ml of LB medium containing carbenicillin, and grown during the day. The culture was seeded then into 5ml of LB medium containing carbenicillin, and grown overnight in an orbital shaker at 37°C. Next day, the culture was centrifuged at 13,000 rpm for 10 minutes at 4°C. After that, the supernatant was discarded, and the pellet was resuspended in 250µl of resuspension buffer. After resuspension of the pellet, 250 µl of lysis buffer was added, and the sample gently mixed by inverting the tube 4-6 times until the solution became viscous and clear.

Then, 350 µL of neutralisation buffer was added, and the sample immediately mixed again by inverting the tube until the solution became cloudy. The sample was centrifuged at 13,000 rpm for 10 minutes in a table-top microcentrifuge to remove bacterial debris and the supernatant loaded on a QIAprep spin column containing a silica gel membrane to bind the DNA. The column was centrifuged at 13,000 rpm for 60 seconds, and the flow-through discarded. The column was washed with 750 µl of wash buffer and centrifuged again at 13,000 rpm for 60 seconds. After discarding the flow-through, the column was centrifuged for an additional 1 minute at 13,000 rpm to remove residual wash buffer. Finally, to elute the DNA, the column was placed in a clean 1.5ml Eppendorf tube and 50 µl of the elution buffer was added to the centre of the column and kept for 1 minute before centrifuging for 1 minute at 13,000 rpm. DNA was quantitated using a Nanodrop spectrophotometer, and used in other manipulations such as stable and transient transfection of CHO Flp-In cells.

2.2.3.9. Quantification of DNA using a Nano-drop spectrophotometer

The Nanodrop spectrophotometer (ND-1000) was used to check the DNA concentration. Simply, 1µl of DNA was pipetted on the lower optical surface of the spectrophotometer, and the absorbance was read at 260nm. The absorbance ratio at 260 and 280 nm was used to assess the sample purity. In general, a ratio of ~1.8 usually indicates pure DNA sample, while less than 1.8 indicates contamination of the sample with protein. The concentration of double-stranded (ds) DNA was calculated as follows:

ds DNA (ng/ ul) = OD260 x dilution factor x 50ng/ µl

As 1 OD for ds DNA at 260 nm = 50 ng/µl

2.2.3.10. DNA sequencing

DNA sequencing was carried out at Sheffield University by the Genetic Core Facility of the medical school using an ABI automated system. Oligonucleotide

sequencing primers and DNA templates (PCR products or plasmids) were provided to the service at 10µl/reaction of 1pmol/µl and 10µl/reaction of 100ng/µl, respectively. SeqMan software (Lasergene version 8; DNAStar, Madison, WI) was used to align the sequencing data against the correct sequence template.

2.2.4. Mammalian cell transfection

2.2.4.1. Invitrogen Flp-In system

A modified Invitrogen vector pSecTag-V5/FRT-Hist was used in a mammalian expression system. This vector is used in Invitrogen's Flp-In system to direct integration of the target gene into the host cell line (in our case Flp-In CHO), allowing rapid generation of stable clones into specific sites within the host genome for high expression. Flp-In CHO cell lines have a single Flp recombinase target (FRT) site located at a transcriptionally active genomic locus. Stable Flp-In CHO cell lines were generated by co-transfection of the vector (Containing FRT target site), and pOG44 (transiently expresses flp recombinase) into a Flp-In cell line.

2.2.4.2. Transient transfection

Transient transfection is a quick method for testing protein expression without using antibiotics to select stable cells. Therefore, this type of transfection allowed for the introduction of multiple plasmid copies into the transfected cells leading to high levels of expressed protein within a relatively short period of time.

Briefly, CHO cells were seeded at a cell density of 0.25 $x10^6$ cells into wells of a 24 well-plate, using 1ml per well. They were incubated overnight at 37°C with 5% CO_2. The next day, in four, sterile 1.5ml Eppendorf tubes each containing 100 µl of serum free media (without antibiotics), 6 µl of Mirus reagent (ratio of 3:2 to DNA) was added by slowly dropping onto the surface of media in each tube, and gently mixed by flicking. In two tubes, designated as transient transfection tubes, 4 µg of the pSecTag GCSF-GHBP- His were added into the first tube, and 4 µg of the

pSecTag GCSF-GHBP-W104-His were added into the second tube. As a positive control, the third tube contained 4 µg of 4A1 plasmid. The fourth tube was left without plasmid addition as a negative control. All tubes were gently mixed by flicking or rolling and left for 15 minutes at room temperature. The transfection mixture was then pipetted dropwise into individual wells of a 24 well plate in duplicate. The cells were incubated at 37°C, 5% CO_2 for 24 hours before changing the media to serum free media. Media was harvested after 3 days and tested for protein expression using Western blotting and/or ELISA.

2.2.4.3. Stable transfection

This method was used to generate stable clones of both pSecTag GCSF-GHBP-His, and pSecTagGCSF-GHBP-W104A-His in CHO Flp-In cells. Briefly, CHO Flp-In cells were cultured in Ham's/F12 medium containing 10% FCS, 100µg/ml Streptomycin, 100U/ml Penicillin, 2mM L-glutamine, and 100µg/ml Zeocin, and passaged twice before continuing with experiment. After washing and trypsinization, the cells were transferred to sterile centrifuge tubes and spun down for 5 minutes at 1000 rpm. Mirus was used as the transfection reagent at a ratio of 3:2 Mirus (µl) to DNA (µg) as per manufacturer's instructions, and was warmed to room temperature, and vortexed before use. The cells were plated at $0.25x10^6$ cells per well of a 6 well plate in a total volume of 2ml media and incubated overnight at 37°C, at which point they were ~ 60- 70% confluent. On the next day, the media was replaced on the cells with fresh media containing no antibiotics. For the transfection, into four sterile, pre-labelled 1.5ml Eppendorf tubes each containing 92.5 µl of serum free media (DMEM/F12 without antibiotics), 7.5 µl of Mirus reagent was added dropwise onto the surface of the media to each tube followed by gently mixing by flicking. Then, 0.25µg of each target plasmid or a positive control plasmid (4A1 plasmid, which had been used previously to prepare a stable cell line), were pre-mixed with 5µg of the pOG44 plasmid and added to the appropriate tube. One tube was left without plasmid addition as a negative

control. All tubes were gently mixed by flicking or rolling and left for 15 minutes at room temperature. Then, the DNA/Mirus mixture and the Mirus alone (negative control) samples were carefully pipetted dropwise into individual wells of a 6 well plate and gently mixed by swirling. The cells were left for 24 hours at 37°C with 5% CO_2 prior to changing the media to a selective media containing 600µg/ml hygromycin B with media changed every 2 days. The majority of cells were observed to be dead after 4 days. Cells were continually grown in 6 well plates until a significantly clustered cell growth was observed. At this point, cells were transferred to T25 flasks to break up cell clusters and allowed to grow until confluency before transferring to T75 flasks. Cells were deemed to be stable when all control cells were dead, ~ 2 weeks from transfection. Stable cells were immediately frozen as described (Section 2.2.1.4) or grown on for testing protein expression by Western blot and ELISA.

2.2.5. Methods of proteins detection

2.2.5.1. Sodium Dodecyl Sulphate (SDS)-Polyacrylamide gel Electrophoresis (PAGE)

SDS-PAGE was used to separate proteins according to their molecular weight using Mini Protean II (Bio-Rad), which was set up according to manufacturer's instructions. The following tables describe the preparation of a 10% acrylamide resolving gel, 4% stacking gel, and 1X Tris-glycine-SDS running buffer:

10 ml of 10% resolving gel:

Constituent	Volume
Acrylamide 30% (w/v)	3.33 ml
1.5M Tris-HCL (pH 8.8) containing 10 % SDS	2.5 ml
Deionized H_2O	4.06 ml
APS (10%,w/v)	100 µl
TEMED	10 µl

10 ml of 4% stacking gel:

Constituent	Volume
Acrylamide 30% (w/v)	1.3 ml
0.5M Tris-HCL (pH 6.8) containing 10 % SDS	2.5 ml
Deionized H_2O	6.1 ml
APS (10%,w/v)	50 µl
TEMED	10 µl

1L of 1X Tris-glycine-SDS running buffer:

Constituent	Volume
10 x Tris-glycine SDS	100 ml
Deionized H_2O	900 ml

Samples to be analysed were mixed with equal volumes of 2X Laemmli sample buffer, and heated for 15 minutes at 65°C, before loading on the gel. After setting up the gel cassette in the electrophoresis tank, the running buffer was added. The voltage was held constant throughout a separation (i.e.100 volt per gel) until the dye-front reached the gel base. The samples were run alongside the pre-stained marker (precision plus protein standard) for protein size determination.

2.2.5.2. Western blotting

After separation of samples by SDS-PAGE, the gel was trans-blotted onto PVDF membrane using a Bio-Rad gel transfer kit and carried out according to the manufacturer's instructions. Importantly, the PVDF membrane was firstly pre-wetted with methanol for 30 seconds due to its hydrophobicity and then equilibrated for 15 minutes in a transfer buffer (5.8g of Tris-Base, 2g of Glycine and 1L of deionized H_2O). Electrophoresis carried out for 45 minutes at 100 volts, and the membrane was then blocked in PBS-Tween (PBST)-5% milk solution overnight at 4°C. The next day, the membrane was washed briefly in an excess volume of PBST and incubated at room temperature for 1 hour with primary antibody, polyclonal rabbit anti-human GCSF (Biolegend, catalogue no. 910801) , at a dilution of 0.75 µl per 10ml of 1% milk protein/PBST. Thereafter, the membrane was washed again in an excess volume of PBST and incubated at room

temperature for half an hour with secondary antibody, Goat anti-rabbit IgG-HRP (Abcam, ab6721) at a dilution of 0.5 µl per 10ml in 10ml of 1% milk protein/PBST. The membrane was washed 3 times for 15 minutes per wash in an excess volume of PBST to reduce background contamination. In order to visualise the protein bands, the membrane was incubated for 2 minutes with freshly mixed ECL (Bio-Rad) detection reagents (ECL reagents contain luminol and hydrogen peroxide solution). HRP reacts with hydrogen peroxide to produce reactive oxygen, this, in turn, reacts with luminal to produce light. The membrane was placed in an X-ray cassette and exposed to sensitive X-ray film to detect any expressed proteins. The exposure time was varied between 2 to 5 minutes, and relied on the amount of antigen on the membrane, the concentration of the antibodies used, and varied between experiments. Finally, the films were developed and fixed in the X-ray developer & fixer solutions, respectively.

2.2.5.3. Coomassie staining

Coomassie Brilliant Blue is the most popular anionic protein dye, which stains almost all proteins with good quantitative linearity. In the staining reaction, ionic interactions between sulfonic acid groups of the dye and positive protein amine groups lead to protein-dye binding. In this project, Coomassie staining was used to detect purified GCSF proteins.

Briefly, after running the samples on SDS-PAGE gel, the gel was incubated in Coomassie blue staining solution (0.25% Coomassie Brilliant Blue dye, 10 % acetic acid, 50% methanol, and 40% distilled water) for 30 minutes at room temperature with gentle shaking on an orbital shaker. The gel was destained in destain solution (10% methanol, 5% acetic acid, and 85% distilled water) with gentle shaking on an orbital shaker at room temperature. The destain solution was replenished when required, and heated in a microwave oven at full power for 1

minute, to speed up the destaining process, until the desired background on the gel was achieved.

2.2.5.4. Enzyme-linked immunosorbent assay (ELISA)

A sandwich ELISA was performed to detect the presence of proteins in media, plasma samples and to quantify purified protein. The sandwich ELISA measures the antigen amounts between capture and detection antibodies; therefore, it has a high specificity. The preparation of buffers used in this assay are outlined below:

Buffers	Preparation
Coating buffer	0.1M $NaHCO_3$ (4.2g), pH to 9.6 with NaOH Make up to 500ml with deionized H_2O
Wash buffer (PBST)	PBS (5 tablets) 0.05% (v/v) Tween 20 (250 µl) Make up to 500ml with deionized H_2O store at 4°C
LKC Assay buffer	5 M NaCl (15 ml) 0.5 M Tris (50 ml), pH to 7.75 with HCl 0.05% (w/v) NaN_3 (0.25 g) 0.01% (v/v) Tween 20 (50 µl) 0.5% (w/v) BSA (2.5 g) 0.05% (w/v) Bovine gamma-globulin (0.25 g) pH 7.75 Make up to 500ml with deionized H_2O store at 4°C
Blocking buffer	20 ml of PBST 3% (w/v) BSA (0.6 g)
Stop Solution	5% (v/v) Sulphuric acid (25 ml) Make up to 500ml with deionized H_2O
Streptavidin-HRP buffer	1µl Streptavidin-HRP in 10 ml PBST/ 0.5% BSA

Briefly, the wells of a 96- well plate were coated with 100 µl of capture antibody (Purified anti-human GCSF, Biolegend, and BVD13-3A5) at a concentration of 1 µg/ml in coating buffer, covered and incubated at 4°C overnight. Next day, the coating solution was removed, and the plate washed 3 times with 200 µl of PBST and patted dry on a paper towel to remove excess wash solution. The remaining protein-binding sites were blocked with 200 µl of blocking buffer per well, and the covered plate incubated at room temperature for 1 hour before washing 3 times with 200 µl of PBST as previously described. GCSF standard concentrations were

prepared as described in Table 3 and 100µl added in duplicate to selected wells together with appropriately diluted protein samples (Table 4) and left for 2 hours at room temperature with mixing. The plate was then washed 3 times with 200 µl of PBST before the addition to each well of 100 µl of detection antibody (Biotin anti-human GCSF, Biolegend, BVD11-37G10), at a concentration of 2µg/ml in the LKC buffer. The plate was left for 2 hours at room temperature with mixing prior to washing 3 times with 200 µl of PBST. Thereafter, 100 µl of streptavidin-conjugated HRP diluted in PBST (1:1000) and BSA (1:10000) was added to each well. The plate was incubated for 30 minutes at room temperature with mixing prior to washing 6 times with 200 µl of PBST. Then, 100 µl of the detection substrate (TMB solution) was added to each well until sufficient colour development was observed when the reaction was stopped by the addition of 100 µl of stop solution. The absorbance of each well was read at 450 nm with background correction at 630 nm using a Biotech LT4500 plate reader and Gen5 software. The background absorbance was subtracted from all data points prior to plotting the standard concentrations (x-axis) against absorbance (y-axis). The concentrations of the unknown protein samples were interpolated from the standard curve using GraphPad Prism.

Table 3. Preparation of GCSF standard curve.

Sample (µl)	**LKC assay buffer (µl)**	**[GCSF] ng/ml**	**Dilution**
Stock at 10µg/ml	-	10000	-
10 of 10000 ng/ml	990	100	100x
500 of 100 ng/ml	500	50	5.35x
500 of 50 ng/ml	500	25	2x
500 of 25 ng/ml	500	12.5	2x
500 of 12.5 ng/ml	500	6.25	2x
500 of 6.25 ng/ml	500	3.125	2x
500 of 3.125 ng/ml	500	1.56	2x
500 of 1.56 ng/ml	500	0.8	2x
500 of 0. 8 ng/ml	500	0.4	2x
500 of 0.4 ng/ml	500	0.2	2x
500 of 0.2 ng/ml	500	0.1	2x
500 of 0.1 ng/ml	500	0.05	2x
0	500	0	0

Table 4. Preparation of protein samples.		
Sample (µl)	**LKC assay buffer (µl)**	**Dilution**
50 of neat	450	1:10
50 of 1:10	450	1:100
50 of 1:100	450	1:1000
50 of 1:1000	450	1:10000
50 of 1:10000	450	1:100000

2.2.5.5. Bradford Protein Assay

The Bradford assay is a colorimetric protein determination method. It depends on the protein binding to Coomassie Brilliant Blue G-250 dye (Bradford, 1976). When the dye binds to protein under acidic conditions, it is converted from the protonated red cationic form (A_{max} = 470 nm) to a stable unprotonated blue form (A_{max} = 595 nm) (Fazekas de St Groth et al., 1963, Reisner et al., 1975, Sedmak and Grossberg, 1977). The dye binds more readily to the cationic residues such as arginine and lysine, thus, the response of the assay would depend on the amino acid composition of the protein (Noble and Bailey, 2009). This assay was used in this project to measure the concentration of the purified proteins and rhGCSF in solution using BSA as a protein standard. 10mg/ml solution of BSA in distilled water was prepared and diluted firstly to 1mg/ml, and then to 100µg/ml by 10-fold dilutions. From this 100µg/ml stock, the following standards were prepared:

Standard BSA (µg/ml)	**Final concentration in assay (µg/ml)**	**Dilution**	
25	20	x4	2ml 100µg/ml + 6ml ddH_2O
12.5	10	x2	2.5ml 25µg/ml + 2.5ml ddH_2O
6.25	5	x2	2.5ml 12.5µg/ml + 2.5ml ddH_2O
2.5	2	x2.5	2ml 6.25µg/ml + 3ml ddH_2O
1.25	1	x2	2ml 2.5µg/ml + 2ml ddH_2O

Standards were prepared as follows: 0.2ml dye reagent was pipetted into separate1.5ml Eppendorf tubes followed by 0.8ml of each standard. The solutions were mixed by gentle inversion and incubated at room temperature for 5 minutes before reading at 595 nm. A separate blank by substituting sample for distilled water was prepared at the same time. For analysis of unknowns, appropriately

diluted samples were diluted into a final volume of 0.8 ml distilled water followed by 0.2 ml dye reagent, then mixed gently and incubated for 5 minutes. All standard and unknown samples were carried out in duplicate and transferred into plastic disposable cuvettes before reading at 595nm. For analysis of the results, OD595nm readings of standards were plotted against concentration and the regression line equation was obtained. This equation was used to calculate the concentration of samples.

2.2.5.6. Native-PAGE

Native-PAGE is run in the absence of SDS and samples are not heated. Therefore, proteins mobility is dependent on their charge, hydrodynamic size, and shape instead of just their molecular mass. Native-PAGE was used in this work as part of the stability study to detect any alterations in a protein conformation or formation of oligomers, dimers, and aggregates (See section 2.2.9). It was set up using a Mini-Protean II apparatus (Bio-Rad). The following tables describe the preparation of an 8% acrylamide separating gel, running buffer, and 5X native loading buffer.

Separating gel:

Constituent	Volume
H_20	6.9ml
30% Acrylamide(Protogel)	4.0ml
1.5M Tris, pH 8.8	3.8ml
APS (10%)	150µl
TEMED	9µl

1L of running buffer:

Constituent	Weight/L
H_20	1 litre
Tris base	3g
Glycine	14.4g
pH	8.3

10 ml of 5X native loading buffer:

Constituent	Volume
H_20	2.9ml
1M Tris, pH 8.8	3.1ml
Glycerol	4ml
Bromophenol Blue	0.25% (w/v)

The required concentrations of samples to be analysed were adjusted by diluting with PBS followed by mixing with an equal volume of 1X native loading buffer. After setting up the gel cassette in the electrophoresis tank, the running buffer was added. The voltage was held constant throughout the separation at 100 volts as described previously (Section 2.2.5.1). The samples were run alongside precision plus protein standard. The standard was not used for molecular weight determination only but also as an aid to gauge the running of the gel and as a control for the transfer of protein to PVDF membranes for western blotting. After separation gels were either stained using coomassie blue (Section 2.2.5.3) or transferred to PVDF membrane for western blotting (Section 2.2.5.2).

2.2.6. Concentration of media using a Vivaflow 200

Before purification of media samples, a Vivaflow 200 concentrator was used to concentrate samples to save time and make the purification process easier. The Vivaflow 200 is a tangential flow filtration device which can be used to concentrate media samples. It comprises of plexiglass, a polyethersulfone (PES) membrane filter with 10kDa molecular weight cut-off (MWCO), pumping tubing, and Masterflex variable speed peristaltic pump. To start the concentration process, all tubing was cleaned first with 0.5M NaOH followed by 500 ml of distilled water.

Media was circulated until the volume was concentrated down by ~10-fold. Importantly, the media sample was kept on ice during this process to avoid the degradation of the protein. To clean the apparatus post use, the concentrator was

flushed with distilled water followed by recirculating 250ml of 0.5M NaOH for 40 minutes at 100ml/min. The device was then drained and washed through with 500-1000ml deionized water followed by filling with 20% ethanol and refrigerated at 4°C. Concentrated samples were stored at -40°C until ready for purification.

2.2.7. Purification of Histidine-tagged proteins using IMAC

Immobilized Metal Affinity Chromatography (IMAC) is a powerful method for purifying poly-histidine tagged recombinant proteins. This method works by using the natural tendency of histidine to form a complex with divalent metals such as nickel, cobalt, copper, around neutral pH. Separation of the histidine-tagged proteins from most untagged proteins is achieved by immobilising the metal ion on a chromatographic resin. The binding interaction between the resin and tagged protein is pH dependent and bound protein can be eluted by reducing the pH or by the addition imidazole (analogue of Histidine) or by the addition of the metal chelator EDTA (Bornhorst and Falke, 2000).

Initially, all tubing was cleaned with 0.2M NaOH (20 ml at 1ml/min) followed by distilled water (50 ml at 1-2ml/min) prior to running column. A 5ml HiTrap IMAC column (GE Healthcare) was set-up in the cold room attached to a P1 peristaltic pump. The column was charged with 4mg/ml Nickel Chloride and equilibrated with 50 ml of equilibration buffer (20 mM Sodium phosphate, pH 7.4, 0.5M NaCl, and 10% glycerol). Media samples were defrosted quickly and cleared by centrifugation at ~20k x g using a JA 25-50 for 20 minutes at 4°C. The samples were diluted 1:1 with 40 mM of Sodium phosphate pH 7.4, 1M NaCl, 20 % glycerol, and 20 mM imidazole, and loaded onto the column at 2ml/min. The flow through (unbound fraction) was collected and the column washed to remove contaminants with 50 ml of equilibration buffer with the addition of 10mM imidazole followed by 50 ml of 20mM Na acetate, 0.5M NaCl, pH 6.0, and 10% glycerol.

At this stage, the column was attached to an AKTA prime rig for gradient elution at 4°C. Briefly, around 30 ml of 500 mM Imidazole, 20mM Na acetate, 0.5M NaCl, pH 6.0, and 10% glycerol was prepared. The bound protein was eluted using a gradient elution from 0- 500 mM imidazole in pH 6.0 buffer over 50 ml at a flow rate of 0.5ml/min collecting 2ml fractions. The eluted fractions were analysed by SDS-PAGE and Bradford protein assayed. Relevant fractions were pooled together and dialysed against 1L PBS buffer at 4°C. The dialysis was performed for 1hour, 2hours and overnight using 1L fresh PBS each time to ensure adequate buffer exchange and removal of all salts and imidazole. To clean and store the column after each use, the column was washed with 50 ml water followed by 50 ml of 0.2M NaOH and 50 ml of water, and finally 50 ml of 20% ethanol.

2.2.8. AML-193 cell-based proliferation assay

AML-193 is established from a childhood monocytic leukaemia. It is a GMCSF-dependent cell line. *In vitro*, other cytokines including interleukin-3, and GCSF can sustain the growth of these cells without inducing maturation (Valtieri et al., 1991). AML-193 cell-based proliferation assay was used to assess the *in vitro* bioactivity of GCSF, and to determine the ability of our protein constructs to stimulate proliferation of AML-193 cells in a manner comparable to available certified reference GCSF.

2.2.8.1. Preparation of the cells

AML-193 cells were removed from liquid nitrogen storage and placed into a 37°C water bath for two minutes. The contents of the vial were then transferred to a 15 ml tube containing 9 ml of culture medium (5% FBS, 100 mg/ml streptomycin, 100 U/ml penicillin, 4mM L-glutamine, 5 ng/ml GMCSF, 5 µg/ml transferrin, 5 µg/ml insulin in Iscove's modified Dulbecco's medium). Cells were centrifuged at 150 x g for 5 minutes and the resulting cell pellet was resuspended in culture medium at a density of 0.23 x 10^6 cells /ml.

2.2.8.2. Cell culture

Cells were cultured in 5% CO_2 incubator at 37°C, in culture medium at a density of 3 x 10^5 – 2 x 10^6 cells/ml. Passages were performed twice a week ensuring cell density did not exceed 2.5 x 10^6 cells/ml. Cell viability was assessed by trypan blue exclusion. Prior to the assay cells were washed 3 times with PBS by spinning for 5 minutes at 150 x g. The pellet was resuspended in assay medium (5% FBS, 100 mg/ml streptomycin, 100 U/ml penicillin, 4mM L-glutamine, 5 µg/ml transferrin, 5 µg/ml insulin in Iscove's modified Dulbecco's medium), at a density of 0.5 x 10^6 cells /ml.

2.2.8.3. Standard/sample preparation

A commercial GCSF (0.2mg/ml, Biolegend) was reconstituted in a 50 % solution of phosphate buffered saline and water (both sterile) to a concentration of 10 µg /ml (10,000 ng/ml), divided into 10 µl aliquots and stored at -80°C. On each day of assay, 1 vial was removed from the freezer and working concentrations were prepared, ranging from 0 ng/ml to 10,000 ng/ml, as shown in Table 5. Protein samples were prepared as described in Table 6.

Table 5. Preparation of GCSF standards.

Sample (µl)	Assay media (µl)	[GCSF] nM (pM)	ng/ml	Dilution
Stock at 10µg/ml	-	10.68 (1068)	10000	-
5 of 10000 ng/ml	495	5.34 (5340)	100	100x
250 of 100 ng/ml	250	2.67(2670)	50	2x
250 of 50 ng/ml	375	1.068 (1068)	20	2.5x
250 of 20 ng/ml	250	0.534 (534)	10	2x
250 of 10 ng/ml	250	0.267 (267)	5	2x
250 of 5 ng/ml	250	0.133 (133)	2.5	2x
250 of 2.5 ng/ml	250	0.06675 (66.75)	1.25	2x
250 of 1.25 ng/ml	250	0.0333 (33.37)	0.625	2x
250 of 0.625 ng/ml	250	0.01668 (16.68)	0.3125	2x
250 of 0.3125 ng/ml	250	0.00834 (8.34)	0.1562	2x
250 of 0.1562 ng/ml	500	0.00278 (2.78)	0.052	3x
250 of 0.052 ng/ml	500	0.00926 (0.926)	0.0173	3x
250 of 0.0173 ng/ml	500	0.000308 (0.308)	0.00578	3x

Table 6. Preparation of protein samples.		
Sample (µl)	**Assay media (µl)**	**Dilution**
50 of neat	450	1:10
50 of 1:10	450	1:100
50 of 1:100	450	1:1000
50 of 1:1000	450	1:10000
50 of 1:10000	450	1:100000

2.2.8.4. AML-193 bioassay

First, 50 µl of each protein under test or GCSF standards were added into appropriate wells of a 96-well microplate in triplicate followed by 50 µl of cell suspension. The plate was shaken softly to allow cells and standard/test samples to mix. Control wells contained 50 µl assay media and 50 µl cell suspension whilst blank wells contained 100 µl assay media only. Cells were exposed to different concentrations of the test sample or GCSF standard for 72 hours at 37ºC, 5% CO_2. After incubation, 20 µl of cell titre reagent was added to each well and the plate placed back at 37ºC/5% CO_2. Plate readings were taken after 40, 80, and 120 minutes at 490nm. Results from control wells containing cells only were subtracted from other samples. Based on the respective dose-response curves (logarithmic, 4-parametric) the EC_{50} values (the concentration which causes 50% of maximal response) for each protein were calculated.

2.2.9. Protein stability studies

2.2.9.1. Short-term stability

Over an 8 day period, stability studies were obtained on protein constructs to look for any degradation or formation of higher order structures. Samples of each construct were kept at different temperatures (i.e. room temperature, 4˚C and -80˚C). The analysis was done using SDS-PAGE and/or Native-PAGE followed by coomassie staining and/or western blotting. To prevent contamination, all sample

manipulations were carried out in a vertical laminar flow hood. In addition, all samples were filter sterilised before use.

i) Preparation of samples for analysis

Before setting up the experiment, a concentrator was filled up with 2ml of 1X PBS & spin at 2500 rpm for 10 minutes. The flow through was then discarded. Protein samples were first concentrated, and filtered (0.22µM) inside the vertical laminar flow hood. The protein concentration was then determined by Bradford assay (Section 2.2.5.5). Final concentrations of 0.45 mg/ml, 0.916 mg/ml for GCSF-W104-GHBP & GCSF-GHBP, respectively were obtained. The concentration of all proteins was adjusted to 0.45 mg/ml using sterile PBS. Samples were then pipetted into labelled, sterile Eppendorfs on day 0 as shown in Table 7.

Table 7. Samples set-up on Day 0.

Sample label	**Volume**	**Next step**
2 x Control (T0) samples	10µL	10µL Laemmli buffer were added, heated for 15 minutes at 65°C, and stored in -20°C
Room temperature (RT)	120µL	Stored on lab bench (~22-25°C)
4°C	120µL	Stored in fridge (4°C)
-80°C Freeze/Thaw (F/T)	120µL	Stored in -80°C
Untreated day 8 (UD8)	10µL	Stored in -80°C
Untreated 3 months (U3M)	10µL	Stored in -80°C

As described in Table 7, T0 samples were made up with 10µL Laemmli buffer, heated for 15 minutes on a heating block at 65°C, and then stored at -20°C. These are the untreated control samples for analysis. Remaining samples were stored at 4ºC in the fridge, room temperature on the bench, and for the F/T, UD8 and U3M samples, stored at -80°C. At each subsequent time point of 1, 4 and 8 days, 10 µL of sample was removed and treated the same as the T0 sample.

ii) Day 1, 4 and 8 samples analysis

For SDS-PAGE analysis on days 1(1D), 4 (4D) and 8 (8D), 10µL (~5µg) of each sample stored at RT, 4˚C and F/T were taken, and pipetted into labelled Eppendorf tubes containing 10µL of Laemmli buffer before heating for 15 minutes at 65˚C. These were called samples 'A'. These samples were stored at -20˚C until analysed.

The stored "A" samples (~0.225 µg/µL) were defrosted and centrifuged for 10 seconds. For western blot analysis, 1.5µL (~225ng) of each sample "A" was pipetted into new Eppendorfs containing 18.5µL of (1x) Laemmli buffer, creating samples "B" (~17ng/µL). The remaining "A" samples (~4µg) were used for coomassie staining analysis.

Analysis by SDS-PAGE was set up using four gels, two for coomassie staining samples "A", and two for western blotting samples "B" (Table 8, 9, 10 & 11). For the western blotting gel, 6µL (~100ng) of samples "B" were loaded per lane, while all of sample "A" (~4µg) were loaded for the coomassie staining gel.

Table 8. 1st gel layout (RT, SDS-PAGE of coomassie stain samples).

Lane	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Treatment			**RT**							
	Standard	0	1D	4D	8D					

Table 9. 2nd gel layout (4˚C and , F/T, SDS-PAGE of coomassie stain samples).

Lane	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Treatment			**4˚C**			**F/T**				
	Standard	0	1D	4D	8D	1D	4D	8D		

Table 10. 3rd gel layout (RT, SDS-PAGE of western blot samples).

Lane	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Treatment			**RT**							
	Standard	0	1D	4D	8D					

Table 11. 4th gel layout (4°C and , F/T, SDS-PAGE of western blot samples).										
Lane	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Treatment			**4°C**			**F/T**				
	Standard	0	1D	4D	8D	1D	4D	8D		

The native-page analysis was carried out only on day 8 samples. 10µL (~5µg) of each sample stored at RT, 4°C and -80°C were taken along with 10µL of the UD8 sample which acted as an untreated control and pipetted into labelled Eppendorfs containing 10µL of (5x) Native-PAGE loading buffer creating samples "a" (~0.225µg/µl). Importantly, theses samples were analysed on the same day as storage conditions might modify the protein.

For western blotting analysis, 1µL of each sample "a" (~337ng) was pipetted into a new Eppendorf containing 18.5µL of (1x) Native-PAGE loading buffer, creating samples "b" (~17ng/µL). The remaining samples from "a" were used for analysis by coomassie staining (~4µg).

Native-PAGE was set up with two gels at 8% acrylamide, one for coomassie staining and one for the western blotting (Table 12 & 13, respectively). For western blotting, 6µL (~100ng) were loaded per lane of sample "b", while all of sample "a" (~4µg) were loaded for coomassie staining. Methods for western blotting, coomassie staining, and Native-PAGE, have been described previously (Sections 2.2.5.2, 2.2.5.3, & 2.2.5.6, respectively).

Table 12. 1st gel layout (Day 8, Native-PAGE: Coomassie staining).										
Lane	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Treatment			**RT**	**4°C**	**F/T**					
	Standard	UD8	8D	8D	8D					

Table 13. 2nd gel layout (Day 8, Native-PAGE: Western blotting).										
Lane	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Treatment			**RT**	**4°C**	**F/T**					
	Standard	UD8	8D	8D	8D					

2.2.9.2. Long-term stability

The stability of protein samples was also assessed over a longer period of time (3 months incubation at RT, 4˚C, and -80˚C). However, in this experiment, the samples were only analysed by SDS-PAGE followed by coomassie staining (Table 14). 10 µl of each sample was pipetted into Eppendorfs containing 10 µl of Laemmli buffer and heated for 15 min at 65˚C. A total of 17 µl of each sample were analysed (~4µg of each protein), in addition, to the control sample (U3M), which had been stored at -80˚C for 3 months.

Table 14. Gel layout (3 months, SDS-PAGE of coomassie staining samples).

Lane	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Treatment		**GCSF-W104-GHBP**	**RT**	**4°C**	**F/T**		**GCSF-GHBP**	**RT**	**4°C**	**F/T**
	Standard	U3M	3M	3M	3M	Standard	U3M	3M	3M	3M

2.2.10. *In vivo* determination of both pharmacokinetic and pharmacodynamic properties

To evaluate the pharmacokinetics (PK) and pharmacodynamics (PD) effects of GCSF-W104-GHBP in mice compared to Filgrastim (rGCSF), the following methods were used.

2.2.10.1. Animals

A total of 34 Specific pathogen-free (BDF1) male mice, 7-9 weeks of age, were used for PD and PK studies. In these studies, BDF1 mice were chosen specifically because the PK of GCSF is known to depend on the strain of mice used (de Haan et al., 2000, Halpern et al., 2002), and BDF1 mice have been shown to have a strong response to GCSF (Molineux et al., 1999, Lord et al., 2001). Mice were acclimatised for one week prior to the start of the experiment. All procedures involving mice were conducted at the University of Sheffield, UK and were approved by the Home Office (PPL 70/8799) and the University of Sheffield's Animal Ethics Committee in accordance with the Animal [Scientific Procedures] Act 1986.

2.2.10.2. Protein preparations and administrations

The working concentrations of Filgrastim (rGCSF), and GCSF-W104-GHBP were obtained by diluting as necessary in 5% dextrose. Control mice (n=10) received a

single injection of 5% dextrose, and experimental mice (n=8 for Filgrastim, n=16 for GCSF-W104-GHBP) received a single injection of test proteins at a dose level of 13 nmol/kg. All injections were given subcutaneously (SC) at the mid-scapular region at 0 hours. It was important to weight each mouse at the same day of experience, and given the correct volume of test proteins, depending on its weight, in a total volume of 100 µl.

Molar equivalence of the test proteins is based upon an average molecular weight of 46.9 kD for GCSF-W104-GHBP and reported MW of 19 kD for Filgrastim. Table 15 summarises the dose administration used in these studies.

Table 15. Molar equivalence.

Protein	Dose administrated (mg/kg)	Molar Equivalent (nmol/kg)
Filgrastim	0.25	13
GCSF-W104-GHBP	0.61	13

2.2.10.3. General sampling procedures

A total of 4 mice (1 from control group, 1 from Filgrastim group, and 2 from GCSF-W104-GHBP group) were sacrificed per time points (2, 6, 12, 24, 36, 48, 72, and 96 hours), in addition, 2 mice from control group were sacrificed immediately after receiving the injection at 0 hour. Generally, the mice were euthanised by Isoflurane inhalation, and blood was withdrawn slowly by the cardiac puncture from the heart left ventricle when the animal under deep anaesthesia. The cardiac puncture was chosen as a terminal bleeding method in this experiment because it yields large amounts of aseptic blood (~500 µl-1ml). It was performed using 25-G, 16-mm needles with a 1-ml syringe, and inserted ~10 mm deep. When a sufficient amount of blood was collected, or the blood flow had stopped, the needle was removed from the syringe (as pressing the blood through the needle can cause cell lysis), and the blood was expelled into EDTA tube. The animal then was culled by neck dislocation and placed in a fridge. If bone marrow (BM) samples were required, after sacrificing the animal, it was placed on its back on a dissecting board. The front legs were fixed with needles and cut with scissors along the tibia and femur of one leg. The muscles were then open until the bone was visible. After cleaning the bones of muscle tissue, the femur was removed by cutting with the scissor at the hip and knee joint. The femur was then

cleaned with scissors, and laboratory tissues to remove all flesh from the bone. After cleaning the femur, it was placed in ice-cold PBS. The same bone removal/cleaning procedure was repeated on the other leg.

2.2.10.4. Analysis of blood and BM samples for PD studies

The number of neutrophils and haematopoietic progenitor cells in mice was evaluated daily for 5 consecutive days.

A) Blood counting

Approximately 180-200µl of blood, which was collected into EDTA tubes, were sent to the haematological lab for complete blood count (CBC) at 2, 6, 24, 36, 48, 72, and 96 hours after injections.

B) Flow cytometry

The number of neutrophils and haematopoietic progenitor cells was assessed in blood, and BM samples by flow cytometry using FITC- conjugated Gr-1 (Ly-6G/Ly-6C)[Clone: RB6-8C5], PeCy5- conjugated MAC-1 (CD11b) [Clone: M1/70], and PE-conjugated CD117 (c-kit) [Clone: 2B8] antibody markers. The blood and BM samples were collected at 24, 48, 72, and 96 hours after injections.

The RB6-8C5 antibody binds to a common epitope on mouse Ly-6G and Ly-6C. The level of antigen expression is directly correlated with neutrophils differentiation and maturation in the BM. It is also expressed transiently in monocytes during their differentiation in the BM. In the periphery, it recognises neutrophils, and monocytes (BD Pharmingen). The M1/70 monoclonal antibody reacts with mouse CD11b. It is expressed by neutrophils, macrophages, myeloid-derived dendritic cells, activated lymphocytes, and mouse B-1 cells (ProSci). The 2B8 reacts with mouse CD117 which is expressed on haematopoietic progenitor stem cells, mast cells; AML cells (BD Pharmingen).

Rat IgG2b isotype controls conjugated with the same fluorochrome (FITC-, PeCy5-, and PE-) as the test antibodies were used to measure the level of non-specific background signal which might cause by primary antibodies. All the isotype controls

were used at the same concentrations as the test antibodies (i.e. 1:500 for FITC- Gr-1, PeCy5- MAC-1, and their isotypes, while 1:250 for PE- CD117 and its isotypes).

i) blood flow cytometry

For staining, 30µl of whole blood, which was collected into EDTA tubes, was added to FACS pre-labelled tubes (unstained, Gr-1 antibody, MAC-1 antibody, antibody cocktail containing Gr-1 + MAC-1, isotype cocktail containing Gr-1 +MAC-1, CD117 antibody, and CD117 isotype). Then, 1µl of each antibody and isotype was added to its corresponding tube and incubated at room temperature for 30 minutes. At the end of the incubation, the tubes were vortexed vigorously for 10 seconds, and 2 ml of lysing buffer (1X) was then added to the cells before vortex again for 10 seconds. The cells were incubated at room temperature until red cell lysis was completed (~10-15 minutes). Completion of red cell lysis was easily observed by a darkening in colour of the fluid, and clearing of turbidity. The leukocytes were then pelleted by centrifugation for 5 minutes at 1000 rpm. The supernatant was then aspirated, and the cells were washed by adding 2 ml of washing buffer (1X). After that, the cells were vortexed for 5 minutes and centrifuged for 5 minutes at 1000 rpm. After centrifugation, the supernatant was aspirated, and the cells were resuspended in 300µl of FACS buffer (PBS with 5% BSA). Finally, flow cytometric analysis of the cells was accomplished with an LSRII.

ii) BM flow cytometry

After removing, and cleaning the femurs, and placed in ice-cold PBS, the BM cells were obtained by cutting ends with scalpels, and then sucking and flushing the cells out using a needle filled with 1ml cold PBS. The cells were then filtered by cell strainers (pore size 70µm), to remove bone fragments and cell clumps. The cells were pelleted by centrifugation at 1500 rpm for 3 minutes. After centrifugation, the supernatant was removed and 1 ml of lysing buffer (1X) was added and vortexed vigorously for 10 seconds. The cells were incubated at room temperature until red cell lysis was completed (~10-15 minutes). At the end of the incubation, the cells were pelleted by centrifugation for 3 minutes at 1000 rpm, and then the supernatant was removed. The cells were resuspended in 700µl of FACS buffer. For staining, 30µl of cells suspension was added to FACS pre-labelled tubes as described in the previous section. The cells were stained for 30 minutes by using the same method as blood

and then washed twice with 1 ml of washing buffer (1X). After removing the supernatant, the cells were resuspended in 300µl of FACS buffer, and flow cytometric analysis was accomplished with an LSRII.

2.2.10.5. PK studies

The remaining volume of the blood in EDTA tubes, which was collected at 0, 2, 6, 12, 24, 36, 48, 72, and 96 hours after injection, was centrifuged using a refrigerator centrifuge at 2000 rpm for 15 minutes at 4˚C. Following centrifugation, the plasma was transferred into clean Eppendorf tubes using a Pasteur pipette. The plasma was apportioned into 100-200 µl aliquots, stored at -80˚C until analysis. The plasma concentrations of Filgrastim and GCSF-W104-GHBP were determined by human GCSF ELISA kit in the presence of 2% mouse plasma.

Table 16. The plan of *in vivo* study

Time points (hrs)	Number of animals per time points			Type of study
	Control group	Filgrastim group	GCSF-W104-GHBP group	
0	2	0	0	PK & PD
2	1	1	2	PK
6	1	1	2	PK
12	1	1	2	PK
24	1	1	2	PK & PD
36	1	1	2	PK
48	1	1	2	PK & PD
72	1	1	2	PK & PD
96	1	1	2	PK & PD
Total number	9	7	14	34

Chapter 3. Cloning of GCSF-GHBP & GCSF-W104-GHBP

3.1. Summary

In this chapter, ProFuse™ technology was used to link full-length GCSF to inactive GHBP via a flexible glycine-serine linker. The inactive GHBP includes an amino acid change at tryptophan-104 (A tryptophan to alanine substitution). This change prevents GH binding to GHBP in the circulation. The results showed that fusion proteins were successfully constructed and expressed in mammalian cell lines as judged by ELISA and western blot analysis.

3.2. Introduction

Many strategies to extend the half-life of therapeutic molecules have been used. One of the most common methods is protein fusion technology. In the preclinical development programme, Asterion has already designed, cloned, and expressed a GCSF protein fusion construct by linking full-length GCSF to its extracellular receptor domain via a flexible glycine-serine linker (code name: 4A1) using ProFuse™ technology. The pharmacokinetic performance of 4A1 showed longevity of action, and excellent delayed clearance properties over commercially available GCSF products in a mouse model (unpublished data). Furthermore, growth hormone binding protein (GHBP) has also been used in growth hormone (GH)-GHBP fusions to great effect by increasing the longevity of action of GH whilst retaining biological activity and increasing molecular weight as mentioned earlier in this work (Section 1.15). Because of these promising results, ProFuse™ technology was used in this project to link full-length GCSF to inactive GHBP via a flexible glycine-serine linker. The inactive GHBP includes an amino acid change at tryptophan-104 (A tryptophan to alanine substitution). This change prevents GH binding to GHBP in the circulation. We hypothesise that this novel construct will have increased molecular weight, and a delayed *in vivo* clearance time. Furthermore, it may also show increased biological activity over 4A1, as described above, due to less intra- and inter- interactions.

3.3. Aims

The aims of this chapter are:

1. Construction of pSecTag GCSF-GHBP-Hist (Called: GCSF-GHBP)
2. Construction of pSecTag GCSF-W104-GHBP-Hist (Called: GCSF-W104-GHBP)
3. Mammalian expression of the GCSF constructs

4. Detection of protein expression

3.4. Construction of GCSF-GHBP

To construct GCSF-GHBP, it was necessary to initially PCR up full-length GCSF. Then the GCSF molecule was ligated into an already existing plasmid that contained the GHBP molecule with a Hist tag. The whole process and results are described in the following paragraphs.

3.4.1. PCR product generation

Using the primers GCSF-Nhe1 (forward primer) and GCSF-Not1 (reverse primer), the full-length GCSF molecule including its natural signal sequence was PCRed from the 4A1 template (the novel construct of GCSF linked to the extracellular domain of the GCSF receptor via a flexible $(Gly_4Ser)_6$ linker. The full nucleotide and protein sequence of the pSecTag GCSF-4A1 molecule is highlighted in Appendix A. The PCR was set up according to the protocol which is described in section 2.2.3.1.

3.4.2. Analysis, cleanup, & quantification of PCR product

The PCR product was separated on a 1% agarose/TAE gel (Section 2.2.3.2) alongside a 1kb ladder as standard. As shown in Figure 14 below, full-length GCSF containing the signal sequence was produced (~0.6 kb). The PCR fragment was excised from the gel and cleaned up using a GenElute kit (Section 2.2.3.2). The amount of DNA was quantified using the nanodrop at A260nm. Eluting bound DNA in 50µl and 25 µl gave a concentration of 15.4 ng/ µl and 8.2 ng/ µl respectively.

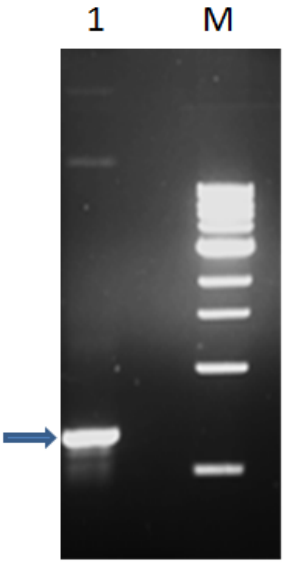


Figure 14. PCR product.

Lane 1: PCR fragment of GCSF (~0.6 kb)
Lane M: 1kb DNA Ladder: Molecular weight marker: Bands starting at the bottom 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, 5.0, 6.0, 8.0, 10.0 Kb.

In summary, the PCR was successful showing the presence of a fragment at ~0.6kb, which is the expected size.

3.4.3. Digestion and clean-up of PCR product

The PCR product was digested with Nhe1 and Not1 restriction enzymes to generate a 5' end and a 3' end prime restriction sites respectively. The digestion reaction was carried out as follows overnight at 37°C.

Constituent	Volume
acBSA (1mg/ml)	5 µl
NEBuffer 2 (10x)	5 µl
PCR product (~0.5 microg)	35 µl
Nuclease-free water	2 µl
Not1(10U/ µl)	2 µl
Nhe1 (10U/ µl)	1 µl
Total volume	50 µl

As can be observed in the setting up of the digestion reaction, double the amount of units of Not1 restriction enzyme were used in order to increase activity in NEBuffer 2 which was 50% efficient compared to Nhe1 in the same buffer which was 100%. After digestion, the PCR product was separated on a 1% agarose/TAE gel alongside a 1kb ladder as standard. The digested PCR product (~0.6 kb), shown in Figure 15, was excised from the gel and cleaned up using a GenElute kit, and quantified using the nanodrop at A260nm. Eluting bound DNA in 50µl and 25µl gave a concentration of 12.9 ng/ µl and 5.8 ng/ µl respectively.

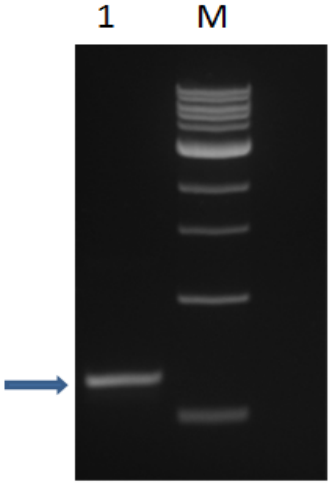


Figure 15. Digested PCR product.

Lane 1: Digested PCR fragment of GCSF (~0.6 kb)
Lane M: 1kb DNA Ladder: Molecular weight marker: Bands starting at the bottom 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, 5.0, 6.0, 8.0, 10.0 Kb.

As shown in Figure 15, the digested PCR product is the correct size (~0.6kb). However, it is quite difficult to see a difference in size between digested and non-

digested PCR product on the gel as the base difference is only ~10 bp in a 0.6kb fragment. The PCR product was taken forward for ligation.

3.4.4. Digestion and clean-up of pSecTag leptin-GHBP-Hist

Within the laboratory, we already had a plasmid containing leptin linked to GHBP (See appendix C for nucleotide and protein sequence). Therefore to construct GCSF-GHBP, it was a simple task to remove the leptin molecule by digestion with Nhe1 and Not1 restriction enzymes and replace with GCSF. The digestion reaction was set up as follows:

Constituent	Single digest with Not1	Single digest with Nhe1	Control reaction (buffer only)
acBSA (1mg/ml)	2 µl	1 µl	1 µl
NEBuffer 2 (10x)	2 µl	1 µl	1 µl
Plasmid (~0.5 mg) (0.702 ng/ µl)	1 µl	1 µl	1 µl
Nuclease-free water	14 µl	6 µl	7 µl
Not1(10U/ µl)	2 µl	-	-
Nhe1(10U/ µl)	-	1 µl	-
Total volume	20 µl	10 µl	10 µl

The reactions were left at 37°C for 2 hours. After that, a fifth of each single digest was pipetted into two sterile Eppendorf tubes; the remaining single digests were pooled together to give 18 µl in total. Then, 3 µl of acBSA (1mg/ml), 2 µl of Buffer 2, 21 µl of Nuclease-free water, 2 µl of Not1, and 1 µl of Nhe1 were added to give 30 µl in total. The reactions were left at 37°C for 1 hour. All reaction mixtures (i.e. single digests, double digest, and control reaction) were run on 1% agarose gel alongside 1kb ladder as standard (Figure 16). Gel-isolated digested plasmid (pSecTag-GHBP-Hist) was cleaned up using the GenElute kit and quantified using the nanodrop at A260nm. 50, and 25 µl of the elution solution produced 9.4, and 6.7 ng/ µl, respectively. The plasmid was taken forward for ligation.

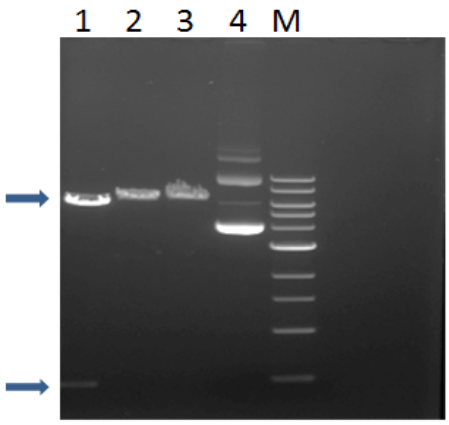


Figure 16. Double digest of pSecTag leptin-GHBP-Hist.

Lane 1: The lower smaller fragment represents the leptin molecule as a result of a successful double digest by both restriction enzymes. The upper band represents the remaining digested plasmid without the leptin insert.
Lane2: The band represents Leptin-GHBP-Hist as results of single digest using Not1.
Lane3: The band represents Leptin-GHBP-Hist as a result of single digest using Nhe1.
Lane 4: Undigested pSecTag leptin-GHBP-Hist
Lane M: 1kb DNA Ladder: Molecular weight marker: Bands starting at the bottom: 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, 5.0, 6.0, 8.0, 10.0 Kb.

As shown in Figure 16, double digestion of pSecTag leptin-GHBP-Hist using Nhe1 and Not1 restriction enzymes produced 2 bands in lane 1. The upper band is the digested plasmid at ~7.0 kb (pSecTag-GHBP-Hist), while the lower band is the leptin insert at ~0.5 kb which indicates that the double digest was successful. The single digests have worked and both enzymes have efficiently linearized the plasmid. They are of higher molecular weight compared to the double digested plasmid as they have retained the leptin insert.

3.4.5. Ligation of full-length GCSF to pSecTag-GHBP-Hist

The ratio of plasmid to insert was set up at 1:3 (molar) in a total volume of 10 µl. The amount of the PCR fragment of GCSF to use was calculated as described in section 2.2.3.6.

The ligation reaction was set up as follows:

Constituent	Ligation	Control 1	Control 2
20 ng pSecTag link, vector at 9.4ng/ µl	2 µl	2 µl	2 µl
Digested PCR fragment (containing GCSF) at 12.9ng/ µl	4 µl	-	-
10X Ligase Buffer	1 µl	1 µl	1 µl
T4 DNA ligase (10U/ µl)	1 µl	1 µl	-
Nuclease-free water to a final volume of	10 µl	10 µl	10 µl

The reactions were left at 37°C for 3 hours prior to transforming into E*. coli* chemically competent cells (Section 2.2.3.7). The ligation plates contained ~308 colonies, while the control plates contained no colonies. Plasmid preparations were made from 3 isolated colonies which were selected from the ligation plate and grown overnight at

37°C in LB media containing carbenicillin (100 µg/ml). The plasmid was purified using a Qiagen mini plasmid kit, and DNA eluted using 50 µl and 25 µl of the elution buffer (EB) for each colony (see below).

	50 µl of EB	**25 µl of EB**
1st colony	518.9 ng/ µl	261.5 ng/ µl
2nd colony	517 ng/ µl	283.8 ng/ µl
3rd colony	550 ng/ µl	229 ng/ µl

Nucleotide sequences of all three colonies were confirmed as positive using primers GCSF-F, GCSF-R, CMV-F, and GHBP-R. Lasergene software was used to align sequences. In summary, construction of GCSF-GHBP was successfully achieved and confirmed by sequencing. Full nucleic acid and amino acid sequences of GCSF-GHBP are shown in Appendix D.

3.5. Construction of GCSF-W104-GHBP

The second aim of this chapter was to construct GCSF-W104-GHBP. To achieve this aim, an already existing plasmid that contained GHBP with a W104A amino acid change (i.e. pSecTag GH-GHBP-W104A-Hist) was digested by restriction enzymes to separate a GHBP fragment that contained the W104A amino acid change (See Appendix E for full nucleic acid and amino acid sequences). The fragment was ligated into the first construct, GCSF-GHBP that had been digested with the same restriction enzymes. The whole process and results are described in the following paragraphs.

3.5.1. Digestion of pSecTag GH-GHBP-W104A-Hist

pSecTag GH-GHBP-W104A-Hist plasmid was digested using AvrII and EcoRV-HF restriction enzymes in order to isolate a DNA fragment containing the W104A amino acid change (~280 bp). The digestion reaction was set up as follows:

Constituent	**Single digest with AvrII**	**Single digest with EcoRV-HF**	**Control reaction (buffer only)**
acBSA (1mg/ml)	1.5 µl	1.5 µl	1.5 µl
Cut smart buffer (10x)	1.5 µl	1.5 µl	1 µl
Plasmid (~2.5 µg) (0.374 µg/ µl)	6.5 µl	6.5 µl	1 µl
Nuclease-free water	4 µl	4 µl	7 µl
AvrII (10U/ µl)	1.5 µl	-	-
EcoRV-HF (10U/ µl)	-	1.5 µl	-
Total volume	15 µl	15 µl	15 µl

As can be observed in the setting up of the digestion reaction, a High Fidelity (HF) version of EcoRV restriction enzyme was used to ensure 100% activity in Cut Smart buffer as AvrII/EcoRV were not compatible. The reactions were left at 37°C for 2 hours. After that, a fifth of each single digest was pipetted into two sterile Eppendorf tubes; the remaining single digests were pooled together to give 24 µl in total. Then, 2 µl of BSA (1mg/ml), 2 µl of Cut smart buffer, 14 µl of nuclease-free water, 1 µl of AvrII, and 1 µl of EcoRV-HF were added to give 44 µl in total. The reactions were left at 37°C for 1 hour. All reaction mixtures were run on 1.2 % agarose gel alongside 100bp ladder as standard. As shown in Figure 17 below, a 280 bp fragment was isolated. The fragment was excised from the gel, and cleaned up using the GenElute kit, and quantified using the nanodrop at A260nm. 50, and 25 µl of the elution solution produced 10, and 7.6 ng/ µl, respectively.

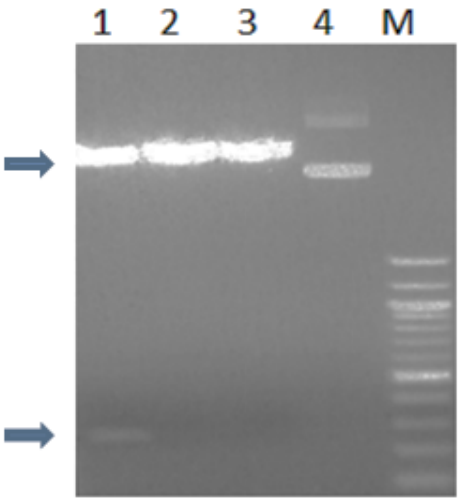


Figure 17. Double digest of pSecTag GH-GHBP-W104A-Hist.

Lane 1: The upper band represents pSecTagGH-GHBP-Hist, and the lower small band (~280 bp) represents a successful double digest by AvrII and EcoRV-HF restriction enzymes.
Lane2: The band represents pSecTagGH-GHBP-W104A-Hist as a result of a single digest using Not1.
Lane3: The band represents pSecTag GH-GHBP-W104A-Hist as a result of a single digest using Nhe1.
Lane 4: Undigested pSecTagGH-GHBP-W104A-Hist
Lane M: 100bp ladder**:** Molecular weight marker: Bands starting at the bottom: 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1200, 1517 bp.

As can be observed in Figure 17, the double digest of pSecTag GH-GHBP-W104A-Hist using AvrII and EcoRV-HF restriction enzymes produced 2 bands in lane 1. The upper band is the digested pSecTag GH-GHBP-Hist plasmid and the lower band is the 280 bp fragment (containing the W104A change). The result indicates that the double digest was successful. In addition, the 280 bp fragment was not visible in both single digest lanes as expected. The single digests have worked and both enzymes have efficiently linearized the plasmid.

3.5.2. Digestion of GCSF-GHBP

After successfully constructing the GCSF-GHBP, this was digested using AvrII and EcoRV-HF restriction enzymes in order to ligate to the 280 bp AvrII/EcoRV fragment (containing the W104A change). The digestion reaction was set up as follows:

Constituent	Single digest with AvrII	Single digest with EcoRV-HF	Control reaction (buffer only)
acBSA (1mg/ml)	1 µl	1 µl	1 µl
Cut smart buffer	1 µl	1 µl	1 µl
Plasmid (~.5 µg) (550 ng/ µl)	1 µl	1 µl	1 µl
Nuclease-free water	6 µl	6 µl	7 µl
AvrII (10U/ µl)	1 µl	-	-
EcoRV-HF (10U/ µl)	-	1 µl	-
Total volume	10 µl	10 µl	10 µl

The reactions were left at 37°C for 2 hours. After that, a fifth of each single digest was pipetted into two sterile Eppendorf tubes; the remaining single digests were pooled together to give 16 µl in total. Then, 2 µl of BSA (1mg/ml), 2 µl of Cut smart buffer, 13 µl of Nuclease-free water, 1 µl of AvrII, and 1 µl of EcoRV-HF were added to give 35 µl in total. The reactions were left at 37°C for 1 hour. All reaction mixtures were run on 1% agarose gel alongside 1kb ladder as standard. As shown in figure 18 below, the digested plasmid was isolated. It was cleaned up using the GenElute kit and quantified using the nanodrop at A260nm. 50, and 25 µl of the elution solution were produced 9.2, and 5.7 ng/ µl, respectively.

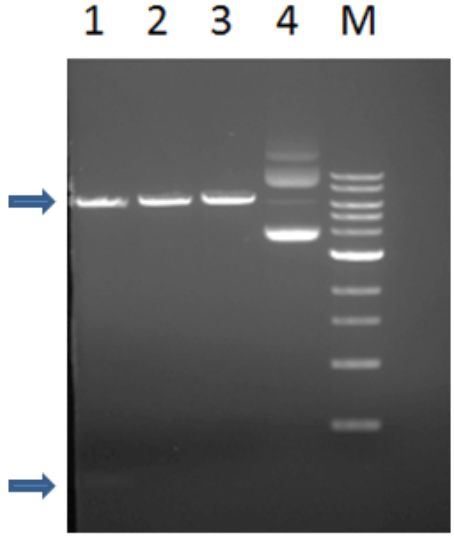


Figure 18. Double digest of GCSF-GHBP using AvrII and EcoRV-HF restriction enzymes.

Lane 1: The upper band represents the digested GCSF-GHBP, and the lower small band represents the AvrII/EcoRV fragment as a result of a successful double digest by both restriction enzymes.
Lane2: The band represents GCSF-GHBP as results of single digest using AvrII.
Lane3: The band represents GCSF-GHBP as a result of single digest using EcoRV-HF.
Lane 4: Undigested GCSF-GHBP
Lane M: 1kb DNA Ladder: Molecular weight marker: Bands starting at the bottom: 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, 5.0, 6.0, 8.0, 10.0 Kb.

As can be seen in Figure 18, the double digest of GCSF-GHBP using AvrII and EcoRV-HF produced 2 bands in lane 1. The upper band is the digested pSecTag GCSF-GHBP-Hist plasmid and the lower band is the AvrII/EcoRV fragment. The size of AvrII/EcoRV fragment is difficult to assess due to the incorrect ladder used but it is less than 0.5 kb, therefore, we can assume that it is the 280 bp insert.

3.5.3. CIAP treatment of digested GCSF-GHBP

Before ligating, the digested GCSF-GHBP plasmid was treated with calf intestinal alkaline phosphatase (CIAP) in order to remove the 5' phosphate group. This was necessary because the AvrII digest produced a 5' recessed sticky end, while EcoRV-HF produced a blunt end. These 2 ends potentially could re-ligate together and produce false positives or hinder the ligation process. CIAP treatment catalysed the removal of 5´-phosphate groups from DNA strands so that the resulting DNA cannot re-ligate. The reaction was set up as described in section 2.2.3.5. The treated GCSF-GHBP plasmid (~ 0.7 kb) was isolated by 1% agarose gel (Figure 19) and cleaned up using the GenElute kit. 50, and 25 µl of the elution solution produced 14.9, and 12.1 ng/ µl, respectively by using the nanodrop at A260nm.

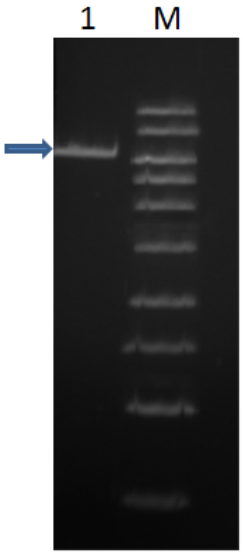


Figure 19. Digested of GCSF-GHBP after treatment with CIAP.

Lane 1: The band represents digested pSecTagGCSF-GHBP-Hist after treatment with CIAP
Lane M: 1kb DNA Ladder: Molecular weight marker: Bands starting at the bottom: 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, 5.0, 6.0, 8.0, 10.0 Kb.

3.5.4. Ligation of 280 bp fragment to GCSF-GHBP CIAP treated plasmid

The ratio of plasmid to insert was set up at 1:3 (molar) in a total volume of 10 µl. The amount of the 280 bp fragment to use was calculated as described in section 2.2.3.6. The ligation reaction was set up as follows:

Constituent	Ligation	Control 1	Control 2
pSecTag link, vector (20 ng) (14.9 ng/ µl)	1.35 µl	1.35 µl	1.35 µl
280bp fragment (10 ng/ µl)	3 µl	-	-
10X Ligase Buffer	1 µl	1 µl	1 µl
T4 DNA ligase (10U/ul)	1 µl	1 µl	-
Nuclease-free water to a final volume of	10 µl	10 µl	10 µl

The reactions were left at 37°C for 3 hours prior to transforming into *E.coli* chemically competent cells (Section 2.2.3.7). The ligation plate contained ~500 colonies, while the control plates contained no colonies. Plasmid preparations were made from 4 isolated colonies which were selected from the ligation plate and grown overnight at 37°C in LB media containing carbenicillin (100 µg/ml). The plasmid was purified using a Qiagen mini plasmid kit, and DNA eluted using 50 µl and 25 µl of the elution buffer (EB) for each colony. Sequencing confirmed the nucleotide sequences of all 4 colonies using BGH-R primer. One of the four colonies was selected to confirm the nucleotide sequences using the GCSF-F primer. Full nucleotide and amino acid sequences of GCSF-W104-GHBP are shown in Appendix F.

In summary: Construction of GCSF-W104-GHBP was successfully achieved and confirmed by sequencing.

3.6. Mammalian expression and analysis

Following the construction of GCSF-GHBP and GCSF-W104-GHBP, both plasmids were transfected into a CHO cell line (Both transiently and stably; section 2.2.4) in order to confirm protein expression. Serum free media was harvested for analysis using western blotting (Section 2.2.5.2) and ELISA (Section 2.2.5.4).

3.6.1. Analysis of transient transfections

As a first assessment of protein expression, GCSF-GHBP and GCSF-W104-GHBP were transiently transfected into CHO Flp-In cells, alongside a positive control (4A1) as described in section 2.2.4.2.

3.6.1.1. Western blot analysis

For western blotting, a 10% SDS-PAGE gel was prepared as described in section 2.2.5.1. Then, 10µl of each media sample including a negative control (media only) were analysed under non-reducing conditions. Western blotting membranes were

probed with anti GCSF antibody and detected with HRP labelled secondary antibody as described in section 2.2.5.2. Probed membranes were exposed to light sensitive film for 2 minutes and developed for 15 -20 seconds prior to fixation. Western blot results are presented in Figure 20.

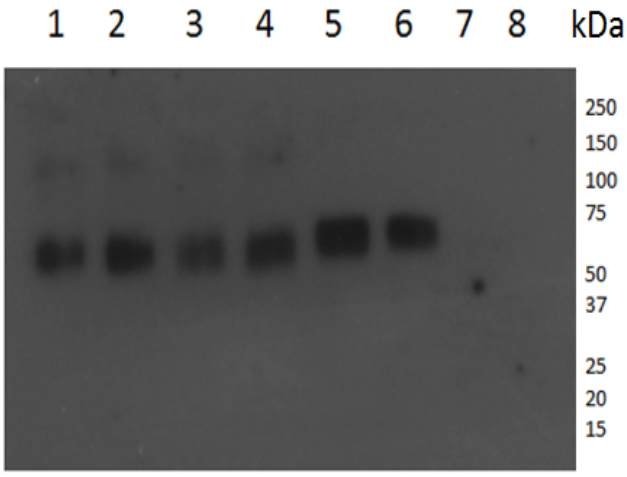


Figure 20. Western blot (non- reduced) of media from transiently transfected CHO Flp-In cells expressing GCSF-GHBP, and GCSF-W104-GHBP.

Lanes 1&2: GCSF-GHBP (~50-70 kDa)
Lanes 3&4: GCSF-W104-GHBP (~50-70 kDa)
Lanes 5&6: positive control (pSecTag GCSF-4A1) (~75 kDa)
Lane 7&8: Negative control (media only)
* All samples run a long side precision plus protein standard

Western blot successfully detected both constructs as can be observed in Figure 20, indicating that transient transfection had worked. All constructs were intact, and run at the correct molecular sizes. Slight formation of higher order structures (~100-150 kDa) (potentially dimers) was observed. The negative control confirmed that the staining was not nonspecific, while the positive control (~75 kDa) confirmed that the gel was transferred successfully.

3.6.1.2. GCSF ELISA analysis

ELISA was performed as described in section 2.2.5.4. The concentration of all constructs is shown in the following table:

Construct	Concentration (ng/ml)
4A1 (positive control)	34.03
Media only (negative control)	0
GCSF-W104-GHBP	438
GCSF-GHBP	204

In summary, all constructs were positive for GCSF and backed up western blot analysis. No GCSF was detected in the negative control sample.

3.6.2. Analysis of stable cell line expression

After successfully detecting transient protein expression for GCSF-GHBP, and GCSF-W104-GHBP, media samples from stably transfected cells, which had previously been adapted to Hyclone SFM4CHO Utility media (Section 2.2.1.4), were also tested using western blot and ELISA.

3.6.2.1. Western blot analysis

10% SDS-PAGE gel was prepared, and 10µl of each media sample were analysed. Films were exposure for 2-3 minutes. The films were developed for 15 -20 seconds prior fixation. Western blot results under non-reduced and reduced conditions are presented in Figure 21.

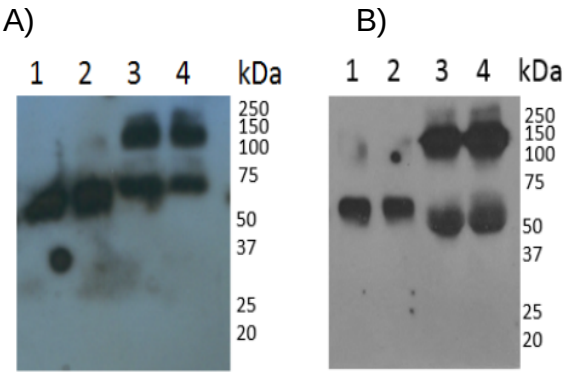


Figure 21. Western blotting (non – reduced [A], and reduced [B]) of media from stable CHO Flp-In cells expressing GCSF-GHBP, and GCSF-W104-GHBP.

Lane 1: Suspension adapted stable cell line expressing GCSF-GHBP
Lane 2: Suspension adapted stable cell line expressing GCSF-W104-GHBP
Lane 3: Adherent stable cell line expressing GCSF-GHBP
Lane 4: Adherent stable cell line expressing GCSF-W104-GHBP
* All samples run a long side precision plus protein standards.

Western blot successfully detected both GCSF constructs as can be observed in Figure 21. In addition, both suspension constructs were running slightly higher than the transiently expressed constructs. The upper bands suggest dimer formation as they are approximately double the molecular weight (~100-150 kDa) of monomer forms. But suspension cells appear to have lower amounts of dimer formation. Using up to 0.25 mM final DTT in sample preparation did not make any significant change in dimer formation (Figure 21B).

3.6.2.2 GCSF ELISA analysis

The concentrations of fusion constructs from media of both adherent and suspension adapted stable CHO Flp-In cell lines are shown in the following table:

Construct	Growth condition	Concentration (ng/ml)
GCSF-W104-GHBP	Adherent	392
	Suspension	437
GCSF-GHBP	Adherent	216
	Suspension	330

In summary, all constructs were positive for GCSF and backed up western blot analysis. Both transient and stable cell line expression gave detectable levels of protein of the correct molecular weight. Therefore stable cell line development and suspension adaptation to Hyclone SFM4CHO Utility media was a success. The adapted stable cells were be taken forward for protein expression and purification.

3.7. Discussion

In this chapter, GCSF-GHBP, and GCSF-W104-GHBP were successfully constructed. The expression of these constructs was enabled using a mammalian expression system, to promote the most relevant glycosylation pattern of the fusion protein, based on the Invitrogen CHO Flp-In system with a modified pSecTag-V5/FRT-Hist vector. The CHO Flp-In system was selected as it enabled easy, rapid and efficient generation of stable clones into specific sites within the host genome for high expression. Furthermore, it allows isogenic generation of the stable cell lines, and CHO cells possess a glycosylation mechanism similar to those in human cells (Damiani et al., 2009). Additionally, CHO cells are considered the most common mammalian cell line used in therapeutic protein mass production (Wurm, 2004). They are able to produce ~ 3-10 grammes per litre of recombinant protein (Wurm and Hacker, 2011). The adherent stable cell lines were adapted to serum-free suspension media in Hyclone SFM4CHO Utility media to allow for higher density cell growth and high productivity of our proteins. Expression of the constructs was tested using ELISA and Western blotting. Both techniques detected the constructs in both transient and stable cell lines. As judged by SDS-PAGE, followed by western blotting, the molecules appeared to be intact with no observed degraded products. Although monomers separate at ~ 50-70 kDa which is larger than the calculated molecular weight of ~ 46 kDa, this is

more than likely because both GHBP and GCSF are glycosylated which increases the observed molecular weight. GCSF has one *O*-linked glycosylation site; whereas GHBP has 6 potential *N*-linked glycosylation sites. Media from suspension adapted stable cell lines, when analysed by SDS-PAGE followed by western blotting showed that detected proteins have a slight decrease in molecular weight when compared to media samples from adherent stable cell lines. This is possibly due to differences in glycosylation patterns caused by differing culture conditions. This theory is backed up by a study carried out on rhGMCSF expression from a CHO cell line, grown as suspension and adherent cultures (Forno et al., 2004). GMCSF contains two potential *N*-glycosylation sites as well as a total of four potential *O*-glycosylation sites. Using SDS/PAGE, they demonstrated a significant molecular mass micro-heterogeneity, of rhGMCSF preparations, with three defined molecular mass areas, corresponding to the *O*-glycosylated forms with none, one or two *N*-glycosylation site occupancy. The rhGMCSF preparation from suspension culture apparently contained a lower amount of forms with two *N*-glycosylation sites and a higher amount of forms with one *N*-glycosylation site when compared to the product from adherent growing cells. Similarly, many other studies investigating the expression of human GMCSF from mammalian cells have also indicated a large difference in molecular mass forms of the protein (Moonen et al., 1987, Cebon et al., 1990, Okamoto et al., 1991, Forno et al., 2004). In this study, western blotting also detected higher orders structures (i.e., dimeric aggregation) of both constructs which were generally more prevalent in the stable cell line samples, and in the adherent stable cell lines, specifically. Although SDS-PAGE was run under reducing conditions using 0.25 mM final DTT, the formation of these higher order structure was still present. Similar observations were also noticed by Cox et al, as their GCSF fusion protein (i.e. GCSF/IgG-Fc) also showed the formation of a higher order band, however, SDS-PAGE was run under non-reducing conditions. To try to explain the reasons behind these higher order structures of GCSF, numerous studies have been done (Krishnan et al., 2002, Chi et al., 2003, Raso et al., 2005, Ribarska et al., 2008), however, there is a debate around calling these structures dimers or aggregates. Most studies agree that native monomeric rhGCSF reversibly forms a dimer under physiological conditions (i.e. pH 7.0 at 37 °C) (Chi et al., 2003, Raso et al., 2005, Ribarska et al., 2008) and that this dimeric species does not participate in the irreversible aggregation process (Chi et al., 2003). Reversible aggregates are linked covalently and caused by chemical reactions, e.g.,

disulphide exchange, β -elimination, or transamidation, whereas irreversible aggregates are linked non-covalently due to interactions between the exposed hydrophobic residues of denatured protein molecules (Chi et al., 2003, Ribarska et al., 2008). To differentiate between covalently and non-covalently linked aggregates, Ribarska et al, assessed the formation of aggregates in Lenograstim (glycosylated GCSF), under physiological conditions (i.e. PBS, pH 6.9 at 37 °C) comparing samples that had been incubated for 5 days or left untreated. SDS-PAGE was performed under reducing (using 2-mercaptoethanol), and non-reducing conditions. The investigators found that a distinct single band of MW~20 kDa assignable to monomer appeared when the untreated sample was analysed by SDS-PAGE under both conditions. In contrast, the treated sample showed a single distinct band corresponding to monomer under reducing conditions, but under non-reducing conditions, two bands were observed: one distinct band assignable to monomer (MW~ 20kDa) and another faint band of MW~40 kDa assignable to a higher molecular weight aggregate. The absence of higher molecular weight bands under reducing conditions for the treated sample indicates that the bonds must be of disulphide type if any soluble aggregates are attached covalently, since GCSF contains two disulphide bonds and a free cysteine (Cys) residue at amino acid position 17 (Cromwell et al., 2006, Gabrielson et al., 2007, Ribarska et al., 2008). Unpaired Cys 17 in GCSF has been implicated as a significant factor in aggregate formation (Imai et al., 1990, Arakawa et al., 1993, Raso et al., 2005). Raso et al. assessed the aggregate formation in a mutant GCSF protein that lacked Cys 17 (C17A GCSF). They demonstrated that the aggregation kinetics of C17A GCSF was slower than the wild-type protein at the same concentration. In addition, the aggregates observed in C17A GCSF could be broken with SDS compared to those formed in wild-type GCSF which could only be broken by SDS and a reducing agent, indicating that these were formed via disulphide bonds.

However, in this study, the presence of these higher order structures, even under reducing conditions is still ill-understood and appears not to be driven by disulphide bond formation via Cys 17, but perhaps by other irreversible aggregation mechanisms Most importantly the constructs are fusion proteins and thus also contain a GHBP moiety which might give further complexity to the results.

To try to inhibit the formation of these higher order structures in GCSF, Ishikawa et al, mutated cysteine to several other amino acids, such as alanine (Ala), serine (Ser), glycine (Gly), histidine (His), tyrosine (Tyr), arginine (Arg), and proline (Pro), to elucidate the role of the Cys-17 in the formation of disulphide bonds and GCSF stability. The investigators found that all mutant proteins, with the exception of a Pro 17-rhGCSF, retained biological activity by promoting the growth of mouse BM cells in vitro. Ala 17-rhGCSF, not only had the disulphide bond scrambling but it also improved the heat stability of rhGCSF more than 5 times compared to rhGCSF (Ishikawa et al., 1992). Furthermore, Krishnan and co-workers claimed that GCSF aggregation could be inhibited by adding sucrose. They assumed that a minor fluctuation of GCSF structure resulted in a transition from the compact native state to an expanded native-like state that was responsible for aggregation (Krishnan et al., 2002). Moreover, GCSF is resistant to aggregation at low pH and the best example for this is the liquid formulation of Filgrastim (Herman et al., 1996). Filgrastim at pH 4.0 is stable for over two years at 2-8°C in solution, however, it aggregates at elevated temperatures. This compact state shows a high degree of α-helicity and being in acid may lock it in a state highly resistant to self-association (Raso et al., 2005). Further analysis and characterisation of the proteins were carried out, after purifying using IMAC.

Chapter 4. Large-scale production and purification of GCSF-GHBP & GCSF-W104-GHBP

4.1. Summary

In the previous chapter, both constructs containing a *C*-terminal histidine tag were expressed stably in the CHO cell line. In this chapter, large-scale expression and purification of these proteins were carried out in order to evaluate theirs *in vitro* and *in vivo* activities. The results demonstrated that the use of His-tag/IMAC-based purification strategy is an appropriate method for purifying GCSF constructs. A total of 13.95 mg GCSF-W104-GHBP at 0.45 mg/ml, and 4.46 mg of GCSF-GHBP at 0.75 mg/ml were purified. Both proteins were considered to be of high purity (> 90% pure) and high integrity as judged by SDS-PAGE followed by coomassie staining and western blotting. Sufficient quantities of both proteins were produced for both PK and PD studies.

4.2. Introduction

The characterisation of the function, structure, and interactions of proteins is vital to our understanding of virtually all fundamental biological processes. Protein purification is a series of processes that are designed to isolate a single protein type, free of all other biomolecules, from a complex biological source. Before performing functional and structural studies on a protein of interest, a successful purification strategy is crucial (Walls et al., 2011). Currently, His-tag/IMAC-based strategy is the most widely used method in the area of recombinant protein purification, and it offers several key advantages (Walls and Loughran, 2011, Costa et al., 2014). The His-tag has a small size, 4 to 10 Histidine residues (Chaga, 2001, Manjasetty et al., 2008, Costa et al., 2014), which means that it does not generally interfere with the biochemical activities of the partner protein (Sharma et al., 1991, Passafiume et al., 1998, Costa et al., 2014) or with most downstream applications (Chaga, 2001, Costa et al., 2014). In addition, it can be used under native or denaturing conditions, and target proteins can be eluted under mild conditions by imidazole competition or low pH (Kimple and Sondek, 2004, Li, 2010, Kimple et al., 2013). Moreover, after purification of His-tagged proteins, they are readily detected by Western blotting using anti-His antibodies (Walls et al., 2011). Additionally, His-tagged proteins have a high selective affinity for Ni^{2+} and several other metal ions that can be immobilised using IMAC system. Following the successful cloning and expression of GCSF-GHBP and GCSF-W104-GHBP, which were tagged at the *C*-terminal with 6 Histidine residues, it was important to purify these proteins,

using a Ni^{2+} charged IMAC column, in order to assess theirs *in vitro* and *in vivo* activities.

4.3. Aims

The aims of this chapter are:

1- Large-scale expression and production of GCSF-GHBP and GCSF-W104-GHBP.
2- Purification using IMAC followed by dialysis.

4.4. Expression and purification of GCSF-W104-GHBP

4.4.1. Growth and expression in roller bottle culture

CHO cells stably expressing the protein of interest were grown in Hyclone SFM4CHO Utility media in T75 flasks. After two or three passages, the cells were seeded at a density of ~0.25 x 10^6 cells/ml and transferred to a non-vented roller bottle (RB) containing approximately 100 ml of Hyclone SFM4CHO Utility media. The cell density was checked regularly every 2 days, and once it reached around 1 x 10^6 cells/ml, it was diluted four times to ~0.25 x 10^6 cells/ml. When the cell density again reached ~ 1 x 10^6 cells/ml, it was diluted to ~0.25 x 10^6 cells/ml (Day 0) and expanded into five roller bottles (Codes: 1 to 5) with a total volume of 2400 ml. In early stages of expansion, media samples were tested to confirm protein expression (Figure 22).

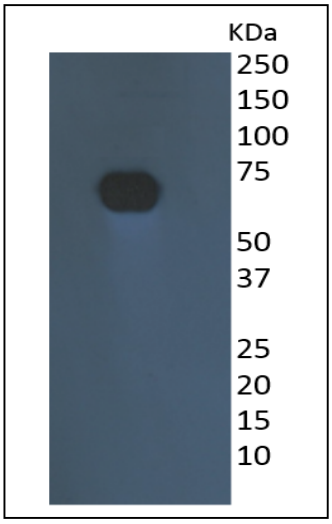


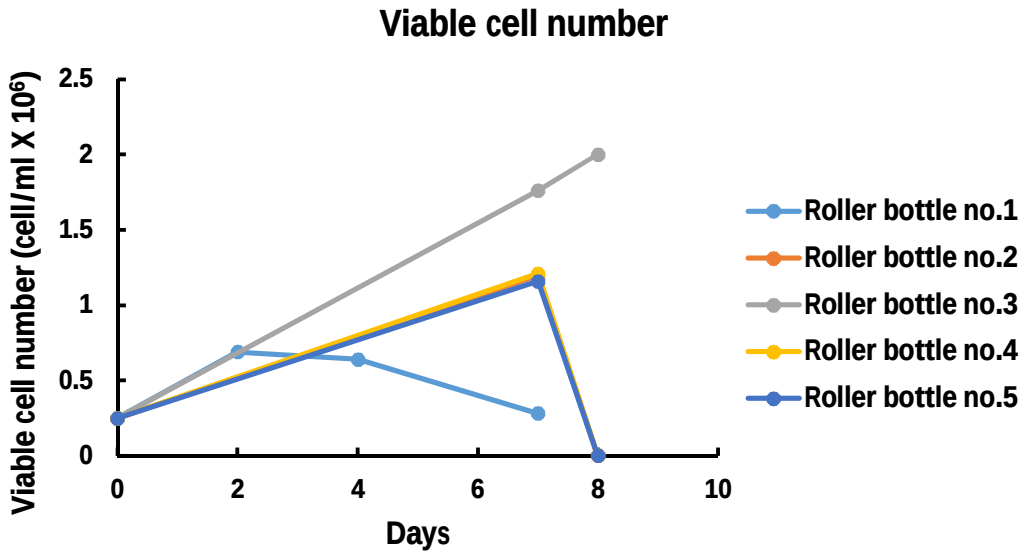
Figure 22. Western blot (non-reduced).
Media samples from CHO stable cell lines expressing GCSF-W104-GHBP (~50-70 kDa)
* All samples run a long side precision plus protein standard

As observed in Figure 22, western blotting successfully detected GCSF-W104-GHBP, indicating that the cells were still maintained protein expression. The construct was intact and separated at the expected molecular sizes.

The cells were then grown in a CO_2-free incubator at 37°C until viability dropped to approximately 30% and then harvested. Media samples were taken from RB no.1, every two or three days to check cell number and viability (Figure 23), and to test protein expression and integrity by western blotting (Figure 24). On day 7, media from all 5 RBs were checked and different viable cell densities and % viabilities were noticed. Only the culture media from RB no.1 was harvested on day 7 because its viability was less than 30 %. The remaining RBs were harvested on day 8 with varying viabilities (i.e.0 % for RBs no. 2, 4, & 5, whereas 88% for RB no.3) (Figure 23).

The culture media from all RBs was centrifuged using JLA-16.250, for 30 minutes at 18,000 g, 4°C to pellet the cell debris. Benzamidine hydrochloride (serine protease inhibitor) was added to the 2.5L culture media at a final concentration of 10mM.

A)



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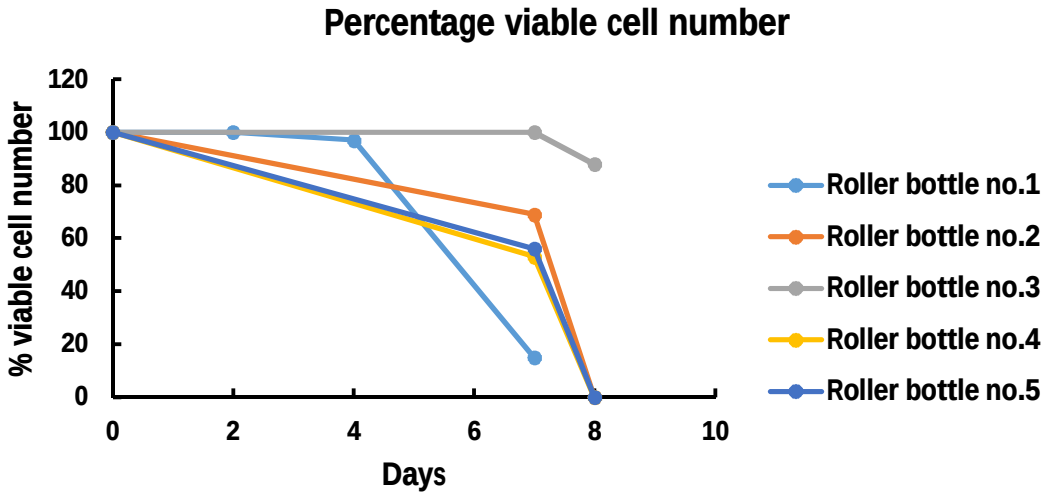


Figure 23. Roller bottle expression of GCSF-W104-GHBP.

A) Viable cell number

B) Percentage viable cell number

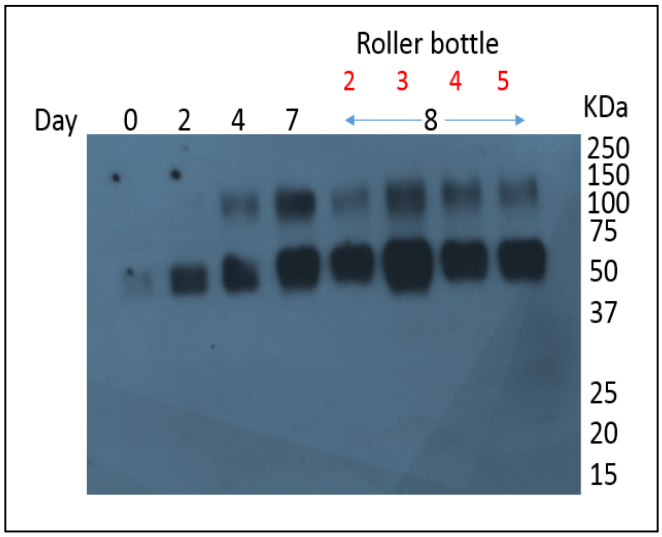


Figure 24. Western blot (non-reduced) of roller bottle media samples (GCSF-W104-GHBP).

Stable CHO Flp-In cells expressing GCSF-W104-GHBP were grown in roller bottle cultures, and samples were taken every 2 to 3 days for 8 days. 8µl of samples from culture medium were analysed by western blotting.
* All samples run a long side precision plus protein standard

The western blot results (Figure 24) show a consistent increase in protein expression of GCSF-W104-GHBP during the period of protein expression. Importantly, no sign of degradation was observed during all days of protein expression, although the presence of high molecular weight forms (~150 kDa) is noted in samples from days 4-8.

4.4.2. Concentration and purification

Using a Vivaflow 200 concentrator, media samples were concentrated approximately 8-fold to 300 ml, prior to purification. Both concentrated sample and filtrate were analysed by SDS-PAGE followed by western blotting, to check the proteins integrity (Figure 25).

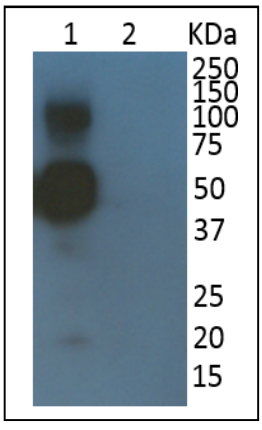


Figure 25. Western blot (non-reduced) of concentrated media samples of GCSF-W104-GHBP.

Lane 1: Concentrated samples
Lane 2: Filtrate
* All samples run a long side precision plus protein standards

As observed in Figure 25, western blotting of GCSF-W104-GHBP concentrated samples showed a major band running between 50-75 kDa in addition to a higher molecular weight band at ~ 100-150 kDa. There is also evidence of very faint lower

molecular weight bands at ~ 37 & 20 kDa. These lower molecular weight bands may have resulted from heat-induced protein degradation as the samples were heated at 95˚C for 5 minutes (More details will be discussed in the next chapter). No target protein was detected in the filtrate sample by western blotting, indicating a successful concentration step. Prior to purification, concentrated media samples were diluted 1:1 with equilibration buffer, as previously described in section 2.2.6. After diluting the concentrated media, target protein was purified using a 1ml Ni^{2+} charged IMAC column, and bound protein eluted using a gradient elution from 0-500 mM imidazole buffer, collecting 2 ml fractions. Importantly the purification process was carried out at 4˚C to prevent protein degradation. Eluted protein samples were measured by Bradford assay (Table 16) and total protein content calculated. Samples were analysed by SDS-PAGE followed by western blotting, and coomassie staining to check protein purity and integrity (Figure 26).

Table 16. Protein concentrations of IMAC purified GCSF-W104-GHBP elution samples.

Elution (E) sample	Protein (µg/ml)	Total Protein (mg)
E1	17.29	0.01
E2	28.42	0.02
E3	17.29	0.01
E4	131.16	0.13
E5	171.40	0.17
E6	277.56	0.27
E7	528.42	0.52
E8	714.21	0.71
E9	782.70	0.78
E10	655.13	0.65
E11	497.60	0.49
E12	308.39	0.30
E13	118.32	0.11
E14	42.97	0.04
E15	19.86	0.01

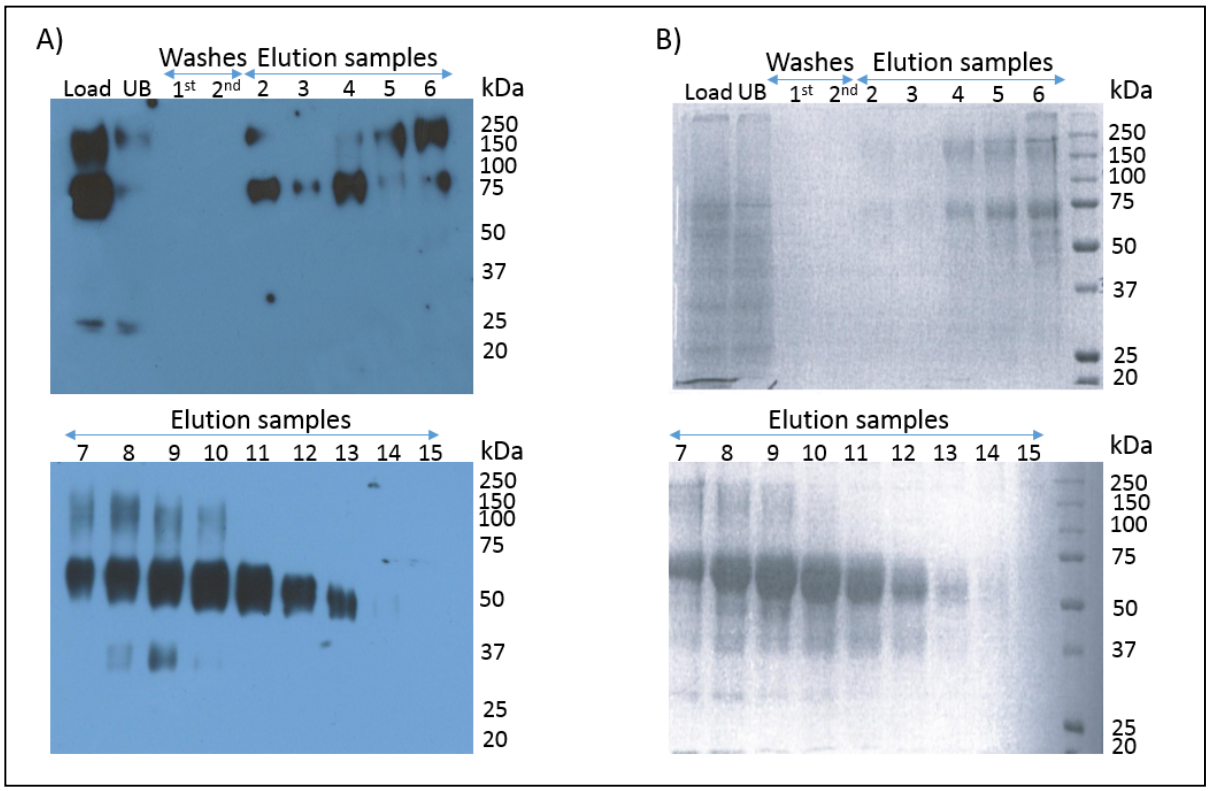


Figure 26. SDS-PAGE analysis of samples from IMAC purification of GCSF-W104-GHBP.

A) Western blot (non-reduced), 100ng loaded per lane
B) Coomassie staining (non-reduced), 5µg loaded per lane
UB: Unbound fraction
Wash 1: Sodium phosphate buffer+ 10mM imidazole (pH=7.4)
Wash 2: Sodium acetate buffer (pH=6)
2-15: Elution fractions
* All samples run a long side precision plus protein standards

Only eluted fractions which had a detectable level of protein (Table 16), were run on SDS-PAGE gels. As observed above in Figure 26 A, the majority of GCSF-W104-GHBP protein bound to the IMAC column with very little detected in the unbound fraction. Negligible amounts of protein were observed in the washes suggesting a successful binding of proteins to IMAC column. The majority of protein has separated at the expected molecular size between 50-75 kDa.

In western blot, lower molecular weight bands at ~20 kDa were detected in both load and unbound samples and at ~37 kDa for elution fractions no. 8 and 9. Whereas low molecular weight bands at ~30-37 kDa were detected in elution fractions 7 -12 after coomassie staining (Figure 26 B). All these lower molecular weight bands were shown to be the result of heat-induced protein degradation upon preparation of samples for

SDS-PAGE. Furthermore, in both western blot and coomassie staining, high molecular weight bands at ~ 100-150 kDa were detected for load samples and elution fractions 4 -10. There is also a shift in molecular weights across the elutions indicating potentially different glycosylation forms of the protein. All elution fractions which had a protein content > 0.1mg (i.e. from no. 4 to no. 13), were pooled together and dialysed as previously described in section 2.2.7, to remove any excess imidazole or other salts. The dialysed sample was finally centrifuged at ~ 20k x g for 20 minutes to pellet debris. Pre- and post-dialysed samples, as well as the post spin pellet, were then measured by Bradford assay (Table 18) and analysed by western blotting and coomassie staining to confirm the purity and integrity (Figure 27). In both blots, no bands were detected in pellet samples indicating a successful dialysis process. Only around 8.2% of the protein was lost during dialysis (Table 18), and a total of 13.95 mg of GCSF-W104-GHBP protein was collected after dialysis in a total volume of 31 ml at a concentration of 0.45mg/ml.

Table 18. Concentrations of pre- and post-dialysed GCSF-W104-GHBP samples.

Sample	Protein (µg/ml)	Total Protein (mg)	Percentage (%)
Pre-dialysis	495.8	15.19	100
Post-dialysis	450	13.95	91.8
Pellet	1	0.001	0.006

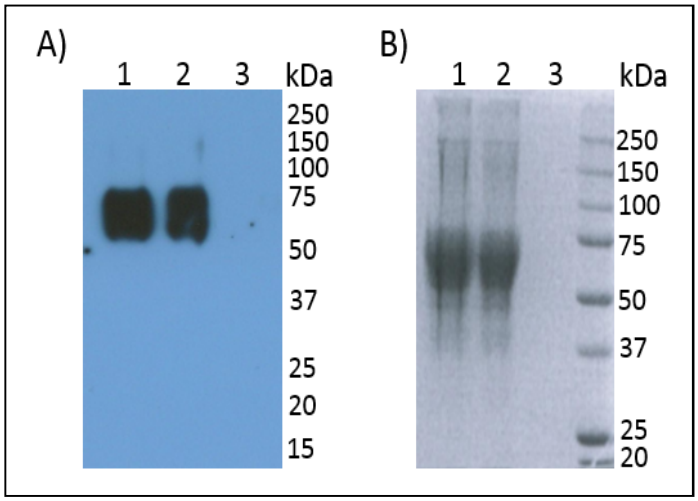


Figure 27. SDS-PAGE analysis of pre- and post-dialysed GCSF-W104-GHBP samples.

A) Western blot (non-reduced), 100 ng loaded per lane
B) Coomassie staining (non-reduced), 5µg loaded per lane
Lane 1: Pre-dialysis
Lane 2: Post-dialysis
Lane 3: Pellet
* All samples run a long side precision plus protein standards

4.5. Expression and purification of GCSF-GHBP

4.5.1. Growth and expression in roller bottle cultures

CHO cells stably expressing the protein of interest were grown in Hyclone SFM4CHO Utility media in T75 flasks. After two or three passages, the cells were seeded at a density of ~0.25 x 10^6 cells/ml cells/ml and transferred to a non-vented RB containing approximately 100 ml of Hyclone SFM4CHO Utility media. The cell density was checked regularly every 2 days, and once it reached around 1 x 10^6 cells/ml, it was diluted four times to ~0.25 x 10^6 cells/ml. When the cell density reach ~ 2 x 10^6 cells/ml, it was diluted to ~0.4 x 10^6 cells/ml (Day 0) and expanded into four RBs, (Codes: 1 to 4) with a total volume of 2000 ml. In the early stage of expansion, media samples were tested to confirm protein expression (Figure 28).

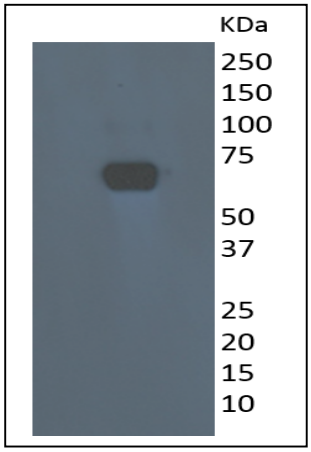


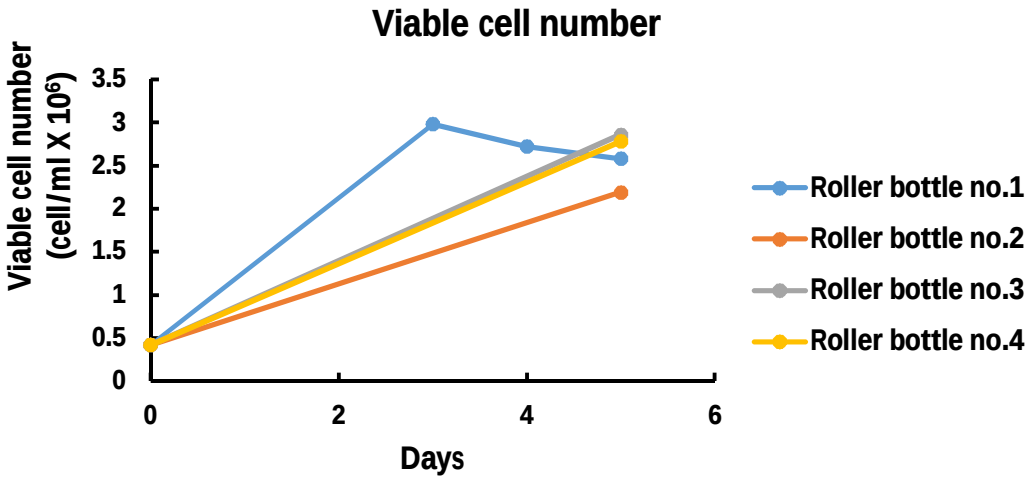
Figure 28. Western blot (non-reduced).

Media sample from a CHO stable cell line expressing GCSF-GHBP (~50-70 kDa)
* All samples run a long side precision plus protein standard

Western blotting successfully detected GCSF-GHBP, indicating that the cells still maintained protein expression. The construct was intact and separated at the expected molecular sizes.

The cells were then grown in a CO_2-free incubator at 37°C until viability dropped to approximately 30% and then harvested. Media samples were taken from RB no.1, every two or three days to check cell number and viability (Figure 29), and to test protein expression and integrity by western blotting (Figure 30). On day 5, % viabilities were between 82-90 %, for all 4 RBs (Figure 29). At this point, to avoid a sudden drop in cell viability, the culture media from all RBs was harvested, as done previously with GCSF-W104-GHBP, and benzamidine hydrochloride was added to a final concentration of 10mM.

A)



B)

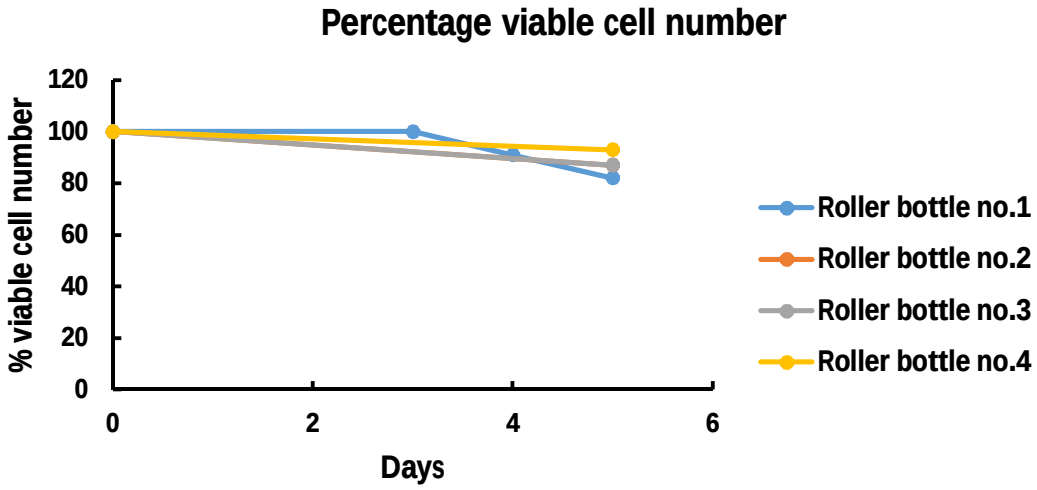


Figure 29. Roller bottle expression of GCSF-GHBP.

A) Viable cell number
B) Percentage viable cell number

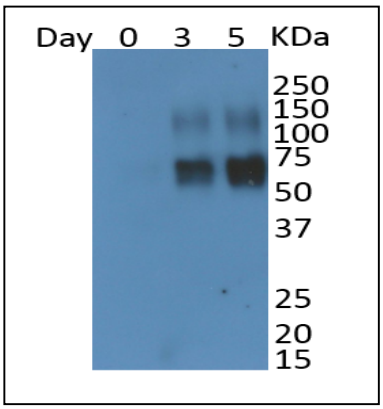


Figure 30. Western blot (non-reduced) of roller bottle media samples (GCSF-GHBP).

Stable CHO Flp-In cells expressing GCSF-GHBP were grown in roller bottle cultures, and samples were taken every 2 to 3 days for 5 days. 8µl of samples from culture medium were analysed by western blotting.
* All samples run a long side precision plus protein standard

As shown in Figure 30, the western blotting results of RB media samples revealed a consistent increase in the protein expression of GCSF-GHBP up to 5 days. No sign of degradation was observed but the formation of high molecular weight forms is noted in day 3-5 samples at ~150 kDa.

4.5.2. Concentration and purification

Media samples were concentrated approximately 10-fold to 200 ml, prior to purification. Concentrated sample and filtrate were analysed by SDS-PAGE followed by western blotting, to check the protein purity and integrity (Figure 31).

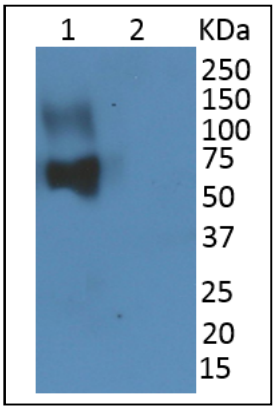


Figure 31. Western blot (non-reduced) of concentrated media samples of GCSF-GHBP.

Lane 1: Concentrated samples
Lane 2: Filtrate
* All samples run a long side precision plus protein standards

As observed in Figure 31, western blotting of GCSF-GHBP media sample showed a main band at 50-75 kDa along with a faint high molecular weight band at ~ 100-150 kDa. No target protein was detected in the filtrated samples, indicating a successful concentration step. Prior to purification, concentrated media samples were diluted 1:1 with equilibration buffer, as previously described in section 2.2.6, and purified using a 1ml Ni^{2+} charged IMAC column. Bound protein was eluted using a step gradient elution of 100 and 500 mM imidazole, collecting ~2 ml fractions. As performed previously with GCSF-W104-GHBP protein, purifications were carried out at 4˚C to

prevent protein degradation. Eluted protein samples were measured by Bradford assay (Table 19), and analysed using SDS-PAGE followed by western blotting and coomassie staining to check the proteins purity and integrity (Figure 32).

Table 19. Protein concentrations of IMAC purified GCSF-GHBP samples.

Sample	Imidazole concentration (mM)	Protein (µg/ml)	Total Protein (mg)
Load	-	293.83	0.29
Unbound	-	261.30	0.26
1ST Wash pH 7.4	-	3.59	0.003
2ND Wash pH 6.0	-	0	0
E1	100	0	0
E2		627.73	0.62
E3		251.02	0.25
E4		95.20	0.09
E5	500	58.39	0.05
E6		891.43	0.89
E7		181.67	0.18
E8		22.43	0.02

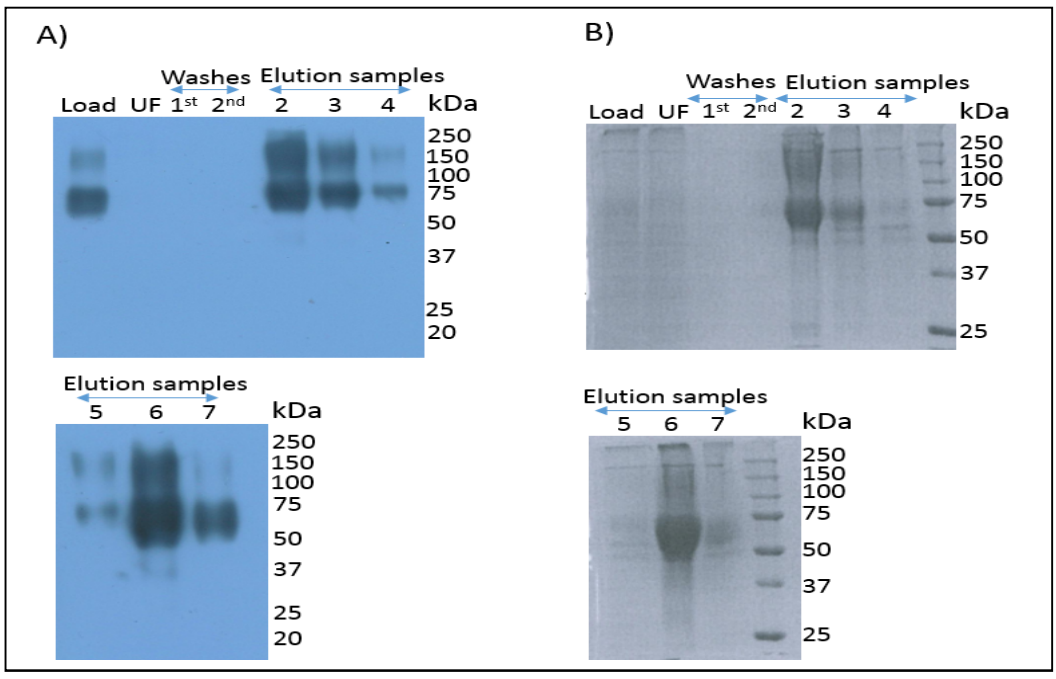


Figure 32. SDS-PAGE analysis of samples from IMAC purification of GCSF-GHBP.

A) Western blot (non-reduced), 100ng loaded per lane
B) Coomassie staining (non-reduced), 5µg loaded per lane
UB: Unbound fraction
2-4: Elution fractions of 100 mM imidazole
5-7: Elution fractions of 500 mM imidazole
* All samples run a long side precision plus protein standards

Only eluted fractions which had a detectable level of protein (i.e. > 0.1 mg) (Table 18), were run on SDS-PAGE gels. As observed in Figure 32 A, the majority of GCSF-GHBP protein bound to the IMAC column with negligible amounts observed in the unbound fraction and washes. There is a slight formation of a lower molecular weight band at ~37 kDa in elution fraction no.6. In western blot and coomassie staining, higher molecular weight bands at ~ 100-150 kDa were detected for load and elution fractions. The main bands present in elution fractions were the protein of interest at 50-75 kDa. Elution fractions which had a protein amount > 0.6 mg (i.e. no. 2 and no. 6), were pooled together and dialysed as previously described in section 2.2.7. The dialysed sample was finally centrifuged at ~ 20k x g for 20 minutes to pellet debris. Pre- and post-dialysed samples, as well as the post spin pellet, were then measured by Bradford assay (Table 20) and analysed by western blot and coomassie staining to confirm the purity and integrity (Figure 33). No signs of degradation were detected in both pre and post dialysed samples, with the main band being the protein of interest at 50-75 kDa, with a faint detection of HMW bands at ~100-150 kDa. No protein was detected in pellet samples indicating a successful dialysis process. Only around 6.8% of the protein was lost during dialysis (Table 20), and a total of 4.46 mg of GCSF-GHBP protein was collected after dialysis in a total volume of 5.9 ml of 0.765mg/ml.

Table 20. Concentrations of pre- and post-dialysed GCSF-GHBP samples.

Sample	Protein (µg/ml)	Total Protein (mg)	Percentage (%)
Pre-dialysis	820.37	0.820	100
Post-dialysis	765.16	0.765	93.2
Pellet	25	0.025	0.03

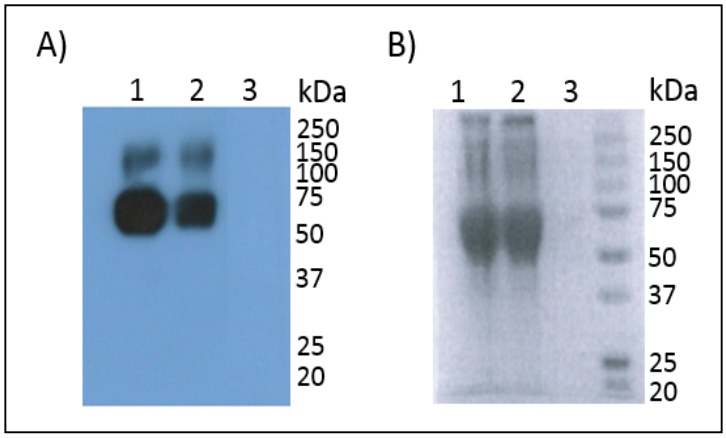


Figure 33. SDS-PAGE analysis of pre- and post-dialysed GCSF-GHBP samples.

A) Western blot (non-reduced), 100ng loaded per lane
B) Coomassie staining (non-reduced), 5µg loaded per lane
Lane 1: Pre-dialysis
Lane 2: Post-dialysis
Lane 3: Pellet
* All samples run a long side precision plus protein standards

4.6. Discussion

In the previous chapter, it was successfully showed that both constructs containing a C-terminal histidine tag, could be stably expressed in a CHO cell line. The next step was large-scale expression and purification of these proteins in order to evaluate their *in vitro* and *in vivo* activities. During the expression and production processes of GCSF- W104-GHBP, different viabilities were noticed among the five RBs on day 7, ranging from 15% to 100% although there were seeded at the same cell densities. Furthermore, the next day a dramatic decline in % viability was observed (i.e. three RBs were 0 % and one RB was 88%). This may have been due to media nutrients being used up especially at the end of the culture period or inefficient aeration in the non-vented RBs due to too much media present (Total volume used ~500ml) leading to poor control of pH. Thus, when we started to express GCSF-GHBP, our plan was to harvest the cells earlier and at higher viabilities.

Prolonging cell viabilities have been highlighted by several studies to control cell growth and to limit apoptotic cell death as extending cell lifetimes by inhibiting or slowing the onset of cell death can lead to more productive cell culture systems for biotechnology applications. Particularly in our lab, Dr Ian Wilkinson found that using vented RBs during large scale expression of GH or by using lower volumes of media in combination with non-vented RBs i.e. 100-200 ml helped to solve the problems associated with variable cell viabilities between RBs (Data not published). In addition, two strategies that have been evaluated to limit cell death and extend cell growth are genetic engineering through alteration of intracellular biochemistry, and manipulation of the extracellular environment through advancements in batch media and fed-batch compositions, including nutritional or chemical methods (Arden and Betenbaugh, 2004). Genetic engineering by using anti-apoptotic proteins have proved highly effective techniques in delaying and inhibiting apoptosis in cell cultures. Different studies demonstrated that overexpression of anti-apoptosis genes (i.e. Bcl-2 and Bcl_{xL}) that inhibit the release of pro-apoptotic molecules from the mitochondria, were able to inhibit apoptotic cell death in different mammalian culture systems such as baby hamster kidney (BHK) and CHO cells (Mastrangelo et al., 2000, Laken and Leonard, 2001). Furthermore, the expression of X-linked inhibitor of apoptosis (XIAP), has been shown to increase viabilities of CHO and human embryonic kidney (HEK)

293 cells in culture (Sauerwald et al., 2003). Additionally, co-expression of p27, a cyclin-dependent kinase inhibitor protein (which induces cell cycle arrest at the G1/S phase border), with secreted alkaline phosphatase (SEAP) in a CHO cell line showed that growth arrest resulted in an increase in specific productivity by 15-fold (Fussenegger et al., 1998, Kaufmann et al., 2001). Alteration of media has also proven successful in prolonging cell viability of mammalian cultures through supplementation with nutrients, anti-apoptotic chemicals or peptides (Andersen and Krummen, 2002, Arden and Betenbaugh, 2004). An example of this is from a study completed by Zanghi et al, that showed by adding suramin, a polysulfated naphthyl urea, protects CHO cells in serum-free culture from apoptosis during the exponential growth phase (Zanghi et al., 2000). Moreover, adding insulin-like growth factor-I (IGF-I) and transferrin to CHO cell cultures resulted in the increased maintenance of cell growth in serum-free media (Sunstrom et al., 2000). Adding valproic acid, a histone deacetylase inhibitor, to CHO cell cultures (Backliwal et al., 2008b, Yang et al., 2014) and HEK 293 (Backliwal et al., 2008a) has also been shown to improve recombinant protein expression. In addition, feeding CHO cell cultures with glutamine (Sanfeliu and Stephanopoulos, 1999), threonine, asparagine, glycine or glycine betaine restored growth of cells exposed to environmental stresses, such as nutrient starvation, hyperosmolarity, and elevated partial pressure of CO_2 (deZengotita et al., 2002). Another important factor to extend cell viability and productivity is a temperature shift. Kaufmann et al reported that protein production at a lower temperature of 30°C led to a significant increase in the degree of sialylation, and protein productivity of tetracycline-dependent transactivator in a CHO cell line. Also, Yoon et al demonstrated that lowering the culture temperature to 33°C resulted in a 2.5-fold increase of erythropoietin from a CHO cell line compared to growing cells at higher temperatures (i.e. 37°C).

Before purification, media were concentrated and analysed by SDS-PAGE followed by western blotting, to check the proteins integrity. Both constructs showed the development of higher order structures, the potential reasons for this and their possible causes have already been discussed in the previous chapter. However, GCSF-W104-GHBP showed two lower molecular weight bands at 37 and 20 kDa, which we believe resulted from heating the sample for 5 minutes at 95°C instead of 15 minutes at 65°C, as no signs of degradation were noticed when samples were treated at 65°C. More

details regarding the temperature stability of these constructs are presented in the next chapter.

Purification was carried out at 4°C to prevent potential protein degradation using a Ni^{2+} charged IMAC column. In this system, elution of bound protein was facilitated by the addition of high concentrations of imidazole, which is an analogue of histidine. Some of the eluted fractions of both constructs, containing a high amount of protein, revealed lower bands at 37 kDa. Again, this was shown to be due to heating samples for 5 minutes at 95˚C instead of 65˚C. For GCSF-W104-GHBP eluted fractions, particularly, there was a shift in molecular weights across the elutions indicating potentially different glycosylation forms of the protein. Western blots and coomassie staining of both purified proteins revealed a main band with a molecular weight of between 50-75 kDa with no signs of degradation, indicating successful purification processes. Final purified proteins were measured by Bradford assay and resulted in a total of 13.95 mg (0.45 mg/ml), and 4.46 mg (0.75 mg/ml), for GCSF-W104-GHBP, and GCSF-GHBP, respectively, which were sufficient for both PK and PD studies. In addition, the post-dialysed purified proteins were considered to be > 90% pure as assessed by Bradford assay, and SDS-PAGE followed by western blotting. Further analysis of both proteins to assess their *in vitro* activity and temperature stability were carried out in the next chapter.

Chapter 5. *In vitro* bioactivity and temperature stability

5.1. Summary

Purification of both GCSF proteins, GCSF-GHBP and GCSF-W104-GHBP is achievable, as evidenced by the previous chapter. Prior to proceeding to an *in vivo* study, it was important to assess their *in vitro* biological activity and the AML-193 proliferation assay was used for this purpose. The results showed that for both proteins the *in vitro* bioactivity was higher than native rhGCSF, with EC_{50}'s of ~0.02nM compared to 0.05nM for rhGCSF, respectively. Additionally, the short- and long-term stability under different temperature conditions of both proteins was assessed. The data presented in this study have demonstrated that both proteins can be kept either at room temperature or -4°C, for up to 8 days, whereas at -80°C for up to 3 months, without any visible degradation as judged by western blotting and coomassie staining.

5.2. Introduction

Following protein purification, it is important to assess molecules biological activity prior to proceeding to an *in vivo* study. Bioactivity is assessed using an *in vitro* assay involving the proliferation of AML-193 cells in the presence of both molecules. Generally, AML (acute myeloid leukaemia) is a heterogeneous clonal disorder of haematopoietic progenitor cells (HPC's) which lose the ability to respond to normal regulators of proliferation and to differentiate normally (Estey and Döhner, 2006). Specifically, AML-193 is one of cell lines which were identified from cells of childhood acute leukaemic patients. In addition, among these cells, AML-193 and two others are the only AML cell lines that depend on the presence of GMCSF to grow (i.e. GMCSF-dependent cell lines) (Valtieri et al., 1991). Other cytokines including interleukin-3 and GCSF can also support short- and long-term growth of these cells and acts synergistically with GMCSF in inducing proliferation of the cells. Based on these data, we assumed that our constructs would be able to induce cell proliferation in a manner comparable to the rhGCSF. Briefly, the AML-193 cells were plated in 96 well plates and serial dilution of rhGCSF/ test constructs were added to the plate. The plate then incubated for 3 days at 37^{o}C /5% CO_2, followed by adding MTS reagent to estimate the proliferation of AML-193 cells. MTS reagent is composed of a mix of a novel tetrazolium compound and an electron coupling reagent. It is bio-reduced by the cells into aqueous, soluble formazan product. The formation of formazan is accomplished by dehydrogenase enzymes found in metabolically active cells (Cory et

al., 1991, Riss, 1992). The absorbance of the formazan at 490nm was recorded using a microplate reader and is directly proportional to the number of living cells in culture. The full method is described in section 2.2.8.4.

Optimisation of proteins thermal stability and storage over time is also important as proteins may lose their biological activity and become unstable through a variety of chemical or physical mechanisms, even at cold temperatures (Carpenter et al., 2002, Simpson, 2010). Thus, the best storage conditions can vary and depend on the protein. The stability of the purified proteins was assessed at three different temperatures (RT, 4°C and -80°C freeze-thaw (F/T) cycles) over an 8 day period and longer term stability studied over 3 months at -80°C. Samples were analysed by SDS and Native PAGE. The effect of heating protein samples at 65°C for 15 minutes versus 95°C for 5 minutes prior to SDS-PAGE analysis, was also assessed.

5.3. Aims

The aims of this chapter are:

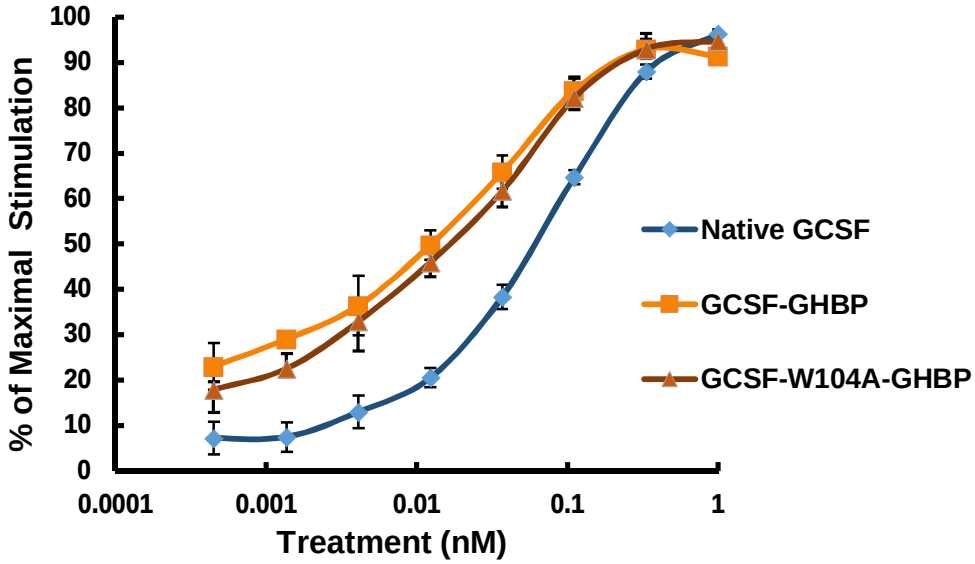
1. Assessment of the biological activity of GCSF-GHBP and GCSF- W104-GHBP using the AML-193 cell line.

2. Assessment of both short- and long-term stability under different temperature conditions.

5.4. *In-vitro* biological activity of GCSF-GHBP and GCSF- W104-GHBP

The results in Figure 34, show that rhGCSF, GCSF-GHBP, and GCSF- W104-GHBP were all able to stimulate the proliferation of the AML-193 cell line. Both proteins demonstrated significantly improved biological activity when compared to rhGCSF with both standard curves shifted to the left (Figure 34 A). Based on the respective dose-response curves (logarithmic, 4-parametrical) the values of EC50 (the concentration which causes 50% of maximal response) for each protein were calculated. The values of the EC50 of both constructs were significantly lower compared to rhGCSF (Figure 34 B).

A)



B)

Construct	EC50 (nM)	SEM
rhGCSF	0.056 (n =12)	0.009
GCSF-GHBP	0.024 (n =6)	0.004
GCSF- W104A-GHBP	0.020 (n =6)	0.006

Figure 34. Proliferation of AML-193 cells.

AML-193 cells were stimulated with GCSF-GHBP, GCSF-W104-GHBP, and rhGCSF. **A)** The percentages of maximal AML-193 cells stimulation, were plotted against the GCSF concentrations (GCSF-GHBP, GCSF-W104- GHBP, and rhGCSF). Each point represents the mean value of triplicate wells, while error bars represent the standard error of the mean. **B)** EC50 values calculated for GCSF-GHBP, GCSF-W104-GHBP, and rhGCSF using Excel and GraphPad Prism.

5.5. Assessment of short-term stability

The method as described previously in section 2.2.9.1. Day 0 samples, which were the untreated control samples were made up with 10µL Laemmli buffer, heated for 15 minutes on a heating block at 65°C, and then stored at -20°C. The remaining samples were incubated at room temperature, 4°C and -80°C. At each subsequent time point of 1, 4 and 8 days, 10 µL of sample was removed and treated the same as the day 0 sample. These were called samples 'A'. For western blot analysis, 1.5µL (~225ng) of each sample "A" was pipetted into new Eppendorfs containing 18.5µL of (1x) Laemmli

buffer, creating samples “B” (~17ng/µL). The remaining “A” samples (~4µg) were used for coomassie staining analysis. Samples were analysed by SDS-PAGE gels are shown in Figures 35 and 36.

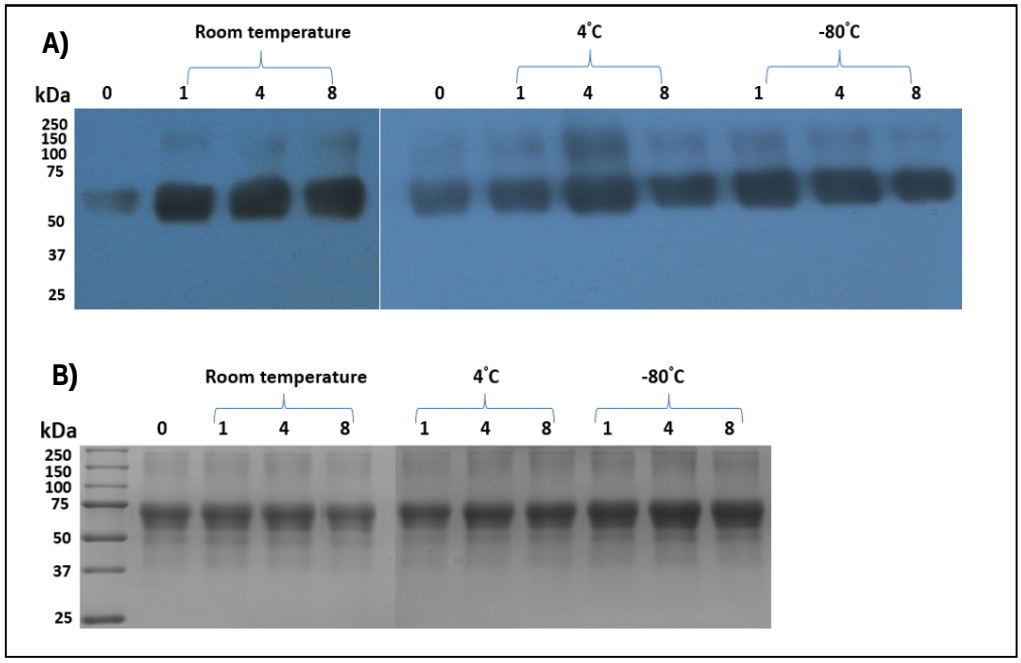


Figure 35. Short term stability of GCSF-GHBP over 8 days.

A) Western blot (non-reduced), 100ng loaded per lane
B) Coomassie staining (non-reduced), 4µg loaded per lane
0, 1, 4 and 8: Days of the experiment
* All samples run a long side precision plus protein standards

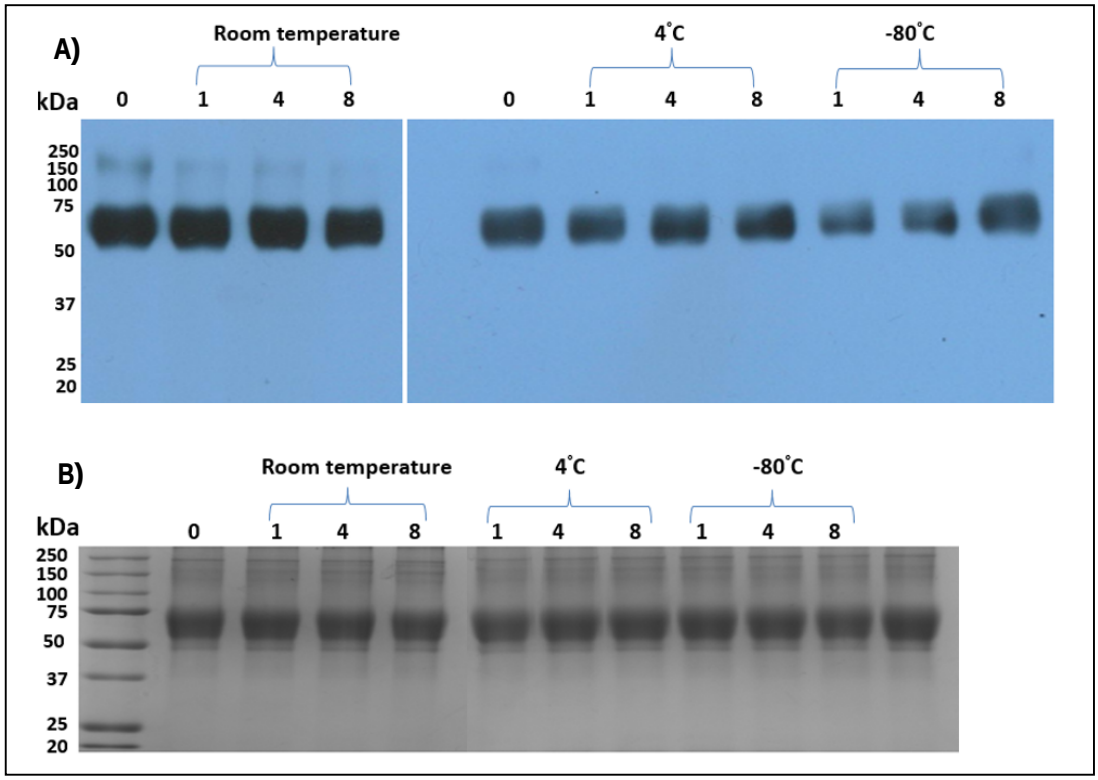


Figure 36. Short term stability of GCSF-W104-GHBP over 8 days.

A) Western blot (non-reduced), 100ng loaded per lane
B) Coomassie staining (non-reduced), 4µg loaded per lane
0, 1, 4 and 8: Days of the experiment
* All samples run a long side precision plus protein standards

As shown above, western blotting and coomassie staining for GCSF-GHBP (Figure 35) and GCSF-W104-GHBP (Figure 36) revealed a main band present in all samples at 50-75 kDa, presumed to be the monomeric protein with no visible degradation of protein samples under all conditions studied (room temperature, 4°C, and -80°C freeze/thaw), over the 8 days period, which implies a high short-term stability of the proteins. However, the formation of a variable degree of high molecular weight bands at ~150 kDa is noted across the experiment and is present in both test and control samples for both proteins.

The native-page analysis was also used to assess the stability of the proteins but only on day 8 samples. Briefly, 10µL (~5µg) of each sample incubated at room temperature, 4˚C and -80°C were taken along with 10µL of the untreated day 8 (UD8) sample which acted as an untreated control and pipetted into labelled Eppendorfs

containing 10µL of (5x) Native-PAGE loading buffer creating samples "a" (~0.225µg/µl). For western blotting analysis, 1µL of each sample "a" (~337ng) was pipetted into a new Eppendorf containing 18.5µL of (1x) Native-PAGE loading buffer, creating samples "b" (~17ng/µL). The remaining samples from "a" were used for analysis by coomassie staining (~4µg). Samples were analysed by Native-PAGE gels are shown in Figure 37.

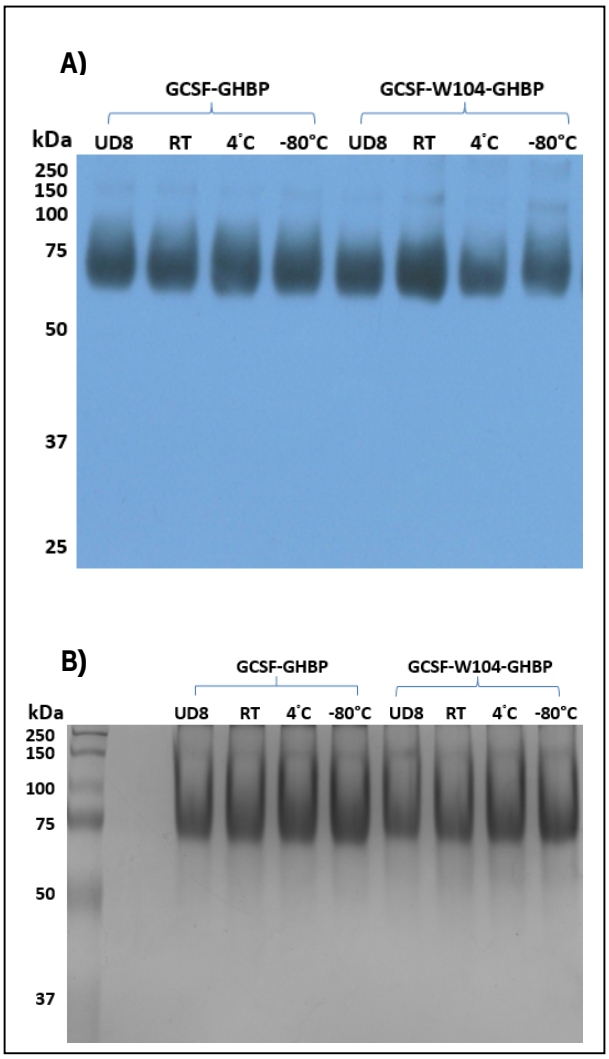


Figure 37. Native-PAGE analysis of GCSF-GHBP and GCSF-W104-GHBP on day 8.

A) Western blot, 100ng loaded per lane
B) Coomassie staining), 4µg loaded per lane
UD8: untreated day 8
RT: room temperature
* All samples run a long side precision plus protein standards

Native-PAGE gels on day 8, followed by western blotting (Figure 37 A), and coomassie staining (Figure 37 B) showed a main band present in all samples, presumed to be the monomeric protein. There is a slight indication of a slower moving band that is possibly the dimer. The banding pattern does not change across all treatments giving further support for the short term stability of our molecules.

5.6. Assessment of long-term stability

The stability of protein samples was also assessed over a longer period of time (3 months incubation at room temperature, 4˚C, and -80˚C). The samples were only analysed by SDS-PAGE followed by coomassie staining (Figure 38). 10 µl of each sample was pipetted into Eppendorfs containing 10 µl of Laemmli buffer and heated for 15 min at 65˚C. A total of 17 µl of each sample were analysed (~4µg of each protein), in addition, to the control sample (U3M), which had been stored at -80˚C for 3 months.

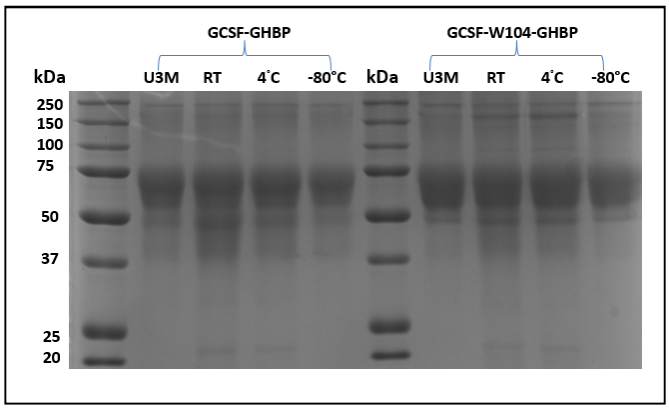


Figure 38. SDS-PAGE analysis of GCSF-GHBP and GCSF-W104-GHBP over 3 months.

Coomassie staining (non-reduced), 4µg loaded per lane
U3M: untreated 3 months
RT: room temperature
* All samples run a long side precision plus protein standards

All samples tested show a major band at 50-75 kDa, presumed to be the intact monomeric protein. However, coomassie staining of GCSF-GHBP and GCSF-W104-GHBP samples at both room temperature and 4°C reveals a very faint lower molecular weight band at ~20 kDa, along with a general increase in band intensity between 37-50 kDa. In contrast, there is no visible degradation at -80°C in all samples over the same time period, which implies a high long-term stability of the proteins at -80°C.

5.7. Assessment of heating protein samples at different temperature and time

The effect of heating protein samples at 65°C for 15 minutes versus 95°C for 5 minutes was also assessed in this chapter using SDS-PAGE followed by western blotting. Previously it was thought that perhaps heating samples at 95°C for 5 minutes was responsible for the appearance of lower molecular weight bands. As shown below in Figure 39, no signs of protein degradation were noticed for both GCSF-GHBP and GCSF-W104-GHBP samples when heated at 65°C for 15 minutes. On the other hand, lower molecular weight bands at ~ 37-40 kDa were observed for both protein samples

when heated at 95°C for 5 minutes, indicating that preparation of protein samples at 95°C for SDS-PAGE analysis leads to protein instability.

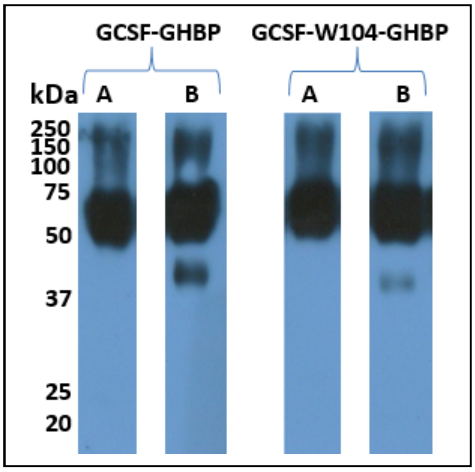


Figure 39. Western blotting (non-reduced) for heating protein samples at different temperature and time.

A) Heating protein samples at 65°C for 15 minutes (~100ng loaded per lane)
B) Heating protein samples at 95°C for 5 minutes (~100ng loaded per lane)
* All samples run alongside precision plus protein standards

5.8. Discussion

The first aim of this chapter was to assess the *in vitro* bioactivity for the fusion proteins before proceeding to *in vivo* study. The results show that the AML-193 proliferation assay is valid *in vitro* model for measuring GCSF bioactivity and that both GCSF-GHBP and GCSF-W104-GHBP clearly exhibit agonistic activity. Moreover, the *in vitro* bioactivity for GCSF-GHBP and GCSF-W104-GHBP in the AML193 assay is a higher than its corresponding un-fused native protein with EC50 of 0.02nM compared to 0.05nM, respectively. However, the reason for this increased activity is unclear as yet. All these results together demonstrate that human GCSF can be fused to GHBP without significantly affecting *in vitro* biological activity. Unfortunately, it was difficult to compare the *in vitro* activity of these molecules to other GCSF molecules as in some cases the *in vivo* studies were carried out without initial testing of *in vitro* activity (Zhao et al., 2013), or different cell lines were used for the analysis (Halpern et al., 2002, Cox et al., 2004, Wadhwa et al., 2015), or that a different AML-193 proliferation assay method was used (Thomas et al., 1995, Abegg et al., 2002) (Thomas et al 1995, Abegg et al 2002). Although in Abegg et al study, the in vitro activity of Filgrastim and Leridistim (a chimeric dual GCSF and IL-3 receptor agonist), was assessed using AML-193 proliferation assay with EC50 of 0.007 and 0.002 nM, respectively, cells were incubated after 72 hours with methyl- 3H thymidine for 16-24 hours, and then harvested onto a glass fiber filter mat for measurement of radioactivity with a beta counter. This is different from this study's method as after the cells were incubated for

72 hours, cell titre reagent was added and the plate readings were taken after 40, 80, and 120 minutes at 490nm.

The conformational structure of proteins plays an important role in the therapeutic efficacy of protein-based medicines, however, proteins are flexible structures, complex, and sensitive to external environmental conditions (Frokjaer and Otzen, 2005). To study the stability of GCSF-GHBP and GCSF-W104-GHBP proteins, different storage conditions were used (i.e. RT, 4°C and -80°C freeze-thaw (F/T) cycles). The data presented in this study, from western blotting and coomassie staining, have demonstrated that both proteins can be kept either at RT or -4°C, for up to 8 days, whereas at -80°C for up to 3 months, without any degradation. On the basis of these findings, scientists would not be concerned about exposure of GCSF-W104-GHBP to RT conditions for a single period of 8 days. Furthermore, these data provide practical advantages for patients receiving GCSF for short periods as it does not require refrigerated storage after dispensing from the pharmacy as long as it is used within 8 days. Putting into consideration that further analysis is required and needs to be assessed carefully before proceeding to clinical trial including; size exclusion high-performance liquid chromatography (SEC-HPLC) to assess the formation of dimers and higher order aggregates under physiological and stress conditions (Ribarska et al., 2008), different buffer formulation and concentration to assess protein stability, and buffer co-solvents. The co-solvents can facilitate proper protein folding and solubility by either destabilising aggregates or enhance native protein stability (Bondos and Bicknell, 2003).

The present study also gathered data demonstrating that our proteins stability is unlikely to be affected by freeze/thaw cycling for up to 8 days reflecting that frequent thermal variations do not affect the protein stability which helps in protein storage and transport. In this chapter, the effect of heating protein samples at 65°C for 15 minutes versus 95°C for 5 minutes, during protein preparation was also assessed. Lower degraded bands at ~ 37-40 kDa were observed when the protein samples being heated at 95°C for 5 minutes, indicating that the preparation of samples for SDS-PAGE is flawed if samples are heated to 95°C. In the next chapter, *in vivo* activity of GCSF-W104-GHBP was assessed using a mouse model.

Chapter 6. PK and PD studies

6.1. Summary

After showing that GCSF-W104-GHBP had higher *in vitro* bioactivity in comparison to rhGCSF combined with high stability, it was important to study the PK and PD profiles of this protein using a mouse model. Results showed that GCSF-W104-GHBP displayed a reduced rate of clearance compared to Filgrastim (rhGCSF). In addition, the maximum plasma concentration (C_{max}) for Filgrastim was 16.4 nM with a time of maximum concentration (T_{max}) of approximately 2 hours. Whereas GCSF-W104-GHBP showed a delayed T_{max} of approximately 12 hours with a C_{max} of 35.4 nM. The results from the PD study showed that a single dose of GCSF-W104-GHBP produced a prolonged haematopoietic activity in mice with an increase in the number of blood neutrophils for up to 72 hours, and HPC's for up to 48 hours, compared to 24 hours for Filgrastim.

6.2. Introduction

Studying and characterising the relationship between PK (concentration vs. time) and PD (effect vs. time) is a crucial tool in the discovery and development of new drugs. In other words, PK describes administrated drugs action in the body over a period of time, including the processes of absorption (movement of drug from the site of administration into circulation; bioavailability), distribution (pattern of scatter of specified amount of drug among various locations in the body; volume of distribution), metabolism (enzyme catalysed chemical transformation of drugs within living organism) and excretion (exertion of drug metabolites outside the body; clearance & half-life) (ADME); while PD is related to the biochemical and physiological effects of administrated drugs on the body.

PK studies play an important role in the pre-clinical and clinical development of therapeutic proteins. Thus, the PK evaluation of these proteins should be done as accurately as possible. A number of factors may affect the pharmacokinetics and pharmacodynamics of a therapeutic protein such as changes in sequence or structure of protein molecules (glycosylation or PEGylating), antibody formation, and immunogenicity, time of drug administration, site and rate of drug delivery. Furthermore, the presence of endogenous proteins during the administration of exogenous proteins can influence the stimulation or feedback mechanism of these

proteins, which may result in difficulties in estimation of PK parameters. The clearance of therapeutic proteins, in particular, can be affected by several mechanisms including immune-mediated clearance, which may result in non-constant clearance (Mahmood and Green, 2005). Therefore, the assessment the PK and PD profiles of therapeutic proteins using relevant non-human species such as mice, rats, and monkeys, can really help in predicting its effects in humans.

After we successfully proved, in the previous chapter, that GCSF-W104-GHBP had a better *in vitro* bioactivity in comparison to rhGCSF, with high stability, it was important to study its PK and PD profiles using a mouse model. As it mentioned previously, rhGCSF has proven successful in the treatment of neutropenia and the mobilisation of stem cells in the context of bone marrow transplantation. Commercially available rhGCSF products fall under the category of either short-acting (i.e., Filgrastim, Lenograstim) wherein the structural homology and PK properties of the molecule are essentially similar with those of endogenous (native) GCSF, or long-acting (i.e., Pegfilgrastim, Lipegfilgrastim), wherein chemical modification through PEGylating has been applied to prolong PK activity through delayed renal clearance. As the use of the short-acting GCSF product has been limited by the need for frequent (daily) dosing, the shift towards the use of the long-acting product has predominated over recent years. There has been significant competitive development for follow-on GCSF molecules which have the potential to match or improve on the PK and efficacy profile of Pegfilgrastim. In this project, protein fusion technology (ProFuse^{TM}) was applied to develop a new class of long-acting GCSF molecules that address not only the pharmacokinetic and efficacy challenges set by long-acting GCSF products but also to provide scope for more competitive manufacturing and cost-of-goods advantages over PEGylating-based technologies.

6.3. Aims

The aims of this chapter are:

1. Studying the PK profiles of GCSF-W104-GHBP in normal mice after a single S.C injection.

2- Studying the PD profiles of GCSF-W104-GHBP in blood and BM of normal mice after a single S.C injection.

3- Comparing the results of PK and PD of treated mice with those receiving Filgrastim.

6.4. Comparing the effect of mouse serum and plasma on the GCSF Elisa assay

Elisa was used in this part of the current project to assess GCSF concentrations in a mouse model after a single administration of either GCSF-W104-GHBP or Filgrastim. Thus, it was important first to choose which bio-fluids (i.e. serum or plasma) will be used to determine the concentration. In order to do that, it was important to assess if there was any important differences or interference between using serum or plasma on the sensitivity of the Elisa. Hence, 2% (v/v) of mouse serum and plasma was used in the initial trial Elisa experiments (Figure 40)

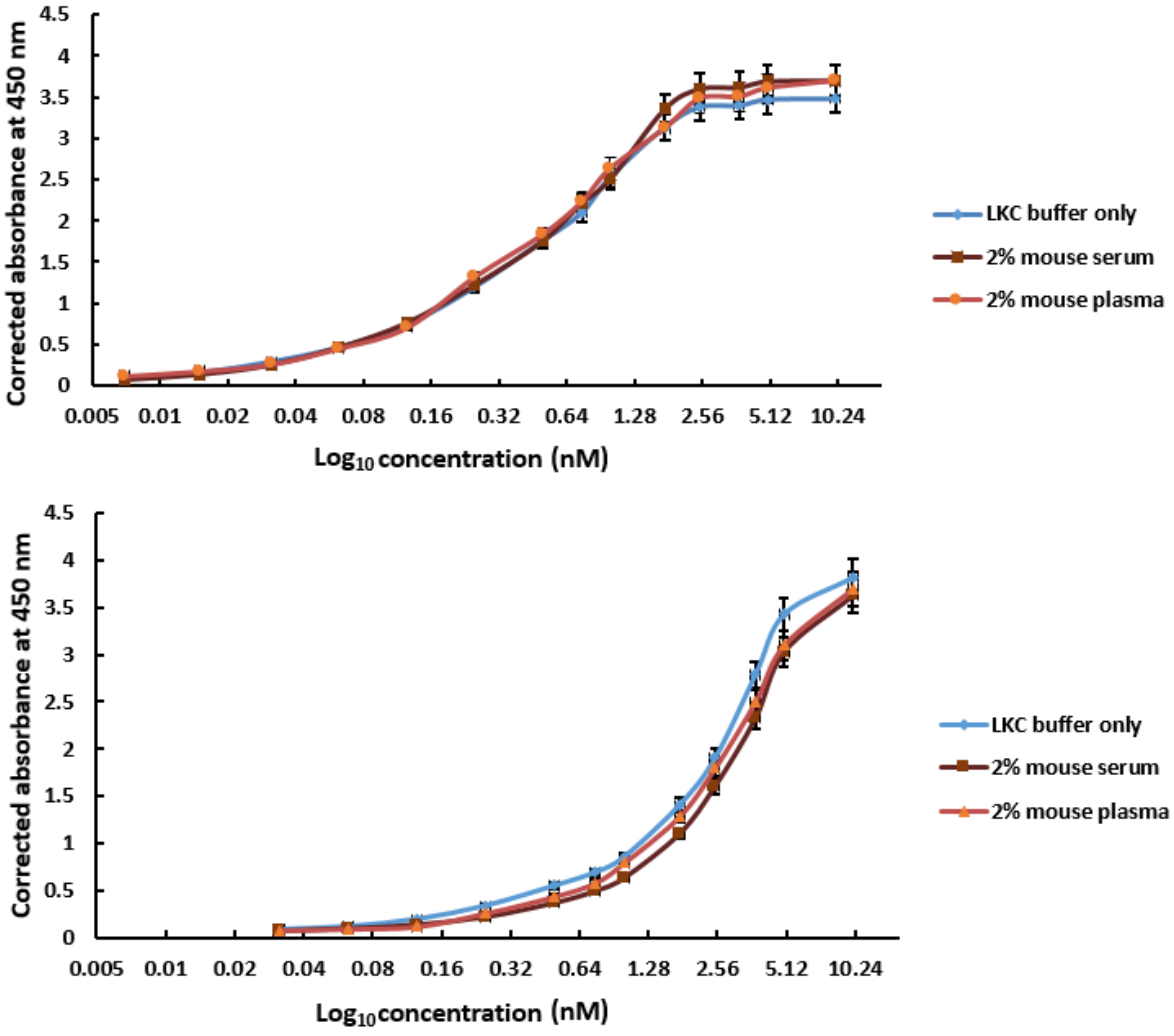


Figure 40. Effect of 2% of mouse serum and plasma on the sensitivity of Elisa assay.

Top panel: A comparison between Filgrastim diluted in 2% mouse serum, 2% mouse plasma and LKC buffer only. **Bottom panel:** A comparison between GCSF-W104-GHBP diluted in 2% mouse serum, 2% mouse plasma and LKC buffer only. Each point represents the mean value of triplicate wells, while error bars represent the standard error of the mean.

As shown in Figure 40, up to 2% of mouse serum and plasma had no effect on both Filgrastim and GCSF-W104-GHBP standard curves indicating that cross-reactivity of anti-human GCSF antibody with endogenous proteins in mouse serum or plasma was low. Therefore, either mouse serum or plasma can be collected to measure the concentrations of Filgrastim and GCSF-W104-GHBP, and PK samples can be diluted to contain a maximum of 2% mouse serum or plasma. In this study, the PK study was performed using mouse plasma as the whole blood was collected in EDTA tubes in order to do flow-cytometry and coulter counter analysis for the PD study. The limit of detection of the assay in mouse plasma was 0.007 nM for Filgrastim and 0.06 nM for GCSF-W104-GHBP.

6.5. PK studies

The PK profile of GCSF-W104-GHBP was evaluated in normal, male BDF1 mice to assess the longevity of exposure in comparison to Filgrastim. As described previously in the method section 2.2.10, the mice received a single SC injection in the mid-scapular region of either Filgrastim at a dose of 0.25 mg/kg (13nmol/kg) or GCSF-W104-GHBP at a dose of 0.61 mg/kg (13nmol/kg). Control mice were injected SC with 5% dextrose. A total of 4 mice (1 from control group, 1 from Filgrastim group, and 2 from GCSF-W104-GHBP group) were sacrificed per time point (2, 6, 12, 24, 36, 48, 72, and 96 hours) after injection as well as 2 mice, from the control group, at 0 hours. Blood was collected in EDTA tubes and centrifuged to obtain plasma. The plasma concentrations of Filgrastim and GCSF-W104-GHBP were determined using a human GCSF ELISA kit in the presence of 2% mouse plasma. Analysis of the pattern of protein peak concentrations and clearance is presented in Figure 41.

From the Elisa data, GCSF-W104-GHBP has a Cmax (peak concentration) of 35.4 nM and Tmax of around 12 hours compared to a Cmax of 16.4 nM and Tmax of ~2 hours for Filgrastim. GCSF-W104-GHBP was still detected in plasma samples up to 36 hours post-injection compared to 12 hours for Filgrastim. The Area Under the Curve (AUC) for Filgrastim was 69.18 nM.hr, whereas for GCSF-W104-GHBP was 785.9 nM.hr, using the linear trapezoidal rule in GraphPad Prism.

In summary, GCSF-W104-GHBP seems to have a much better delay in clearance and a long time of absorbance compared to Filgrastim.

Time (hour)	Control	Filgrastim	GCSF-W104-GHBP		
			1st mouse	2nd mouse	Mean
0	0	-	-	-	-
2	0	16.4	5.89	12.9	9.39
6	0	2.3	26.6	17.8	22.2
12	0	0.9	28	42.9	35.4
24	0	0	17.1	30.4	23.7
36	0	0	0.5	6.09	3.3
48	0	0	0.1	0	0.1
72	0	0	0	0	0
96	0	0	0	0	0

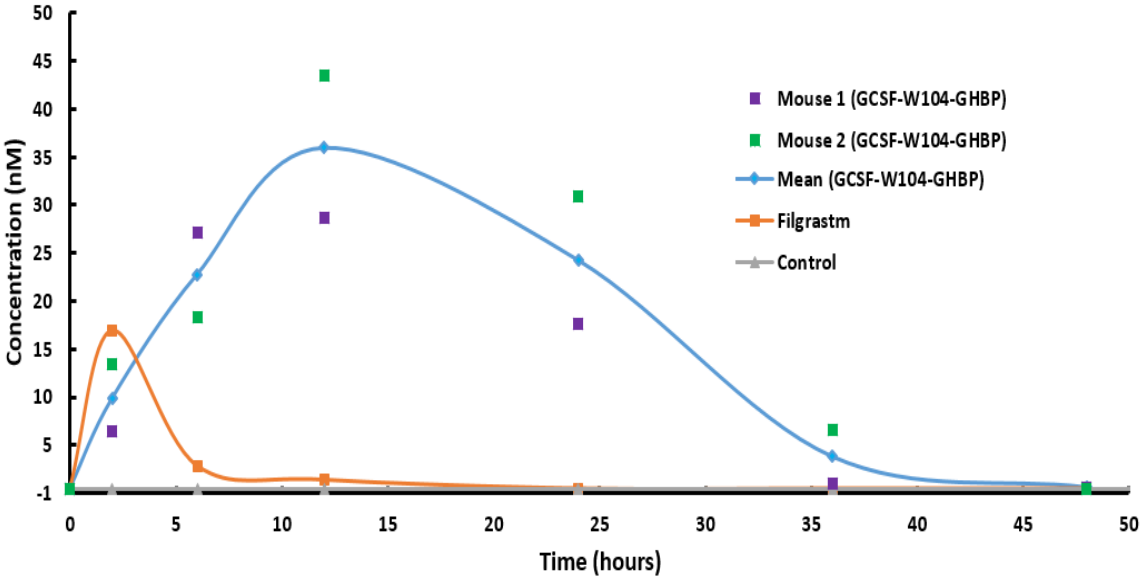


Figure 41. Pharmacokinetic analysis**.**

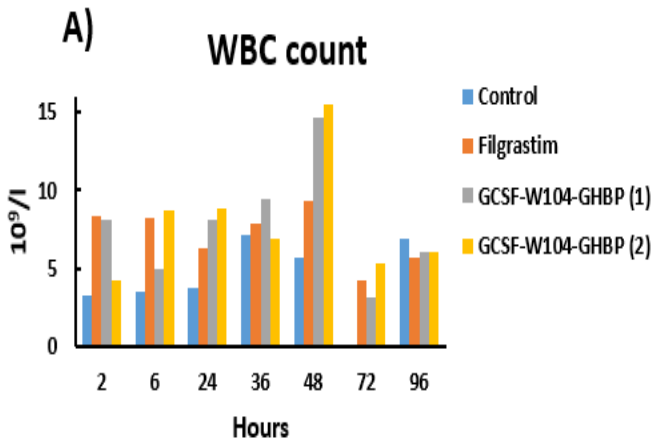
A total of 16 mice were injected SC with 0.61 mg/kg (13nmol/kg) of GCSF-W104-GHBP, 8 mice were injected with 0.25 mg/kg (13nmol/kg) of Filgrastim, and 10 mice were injected with 5% dextrose (control group). **TOP:** Plasma samples from 4 mice (1 from control group, 1 from Filgrastim group, and 2 from GCSF-W104-GHBP group) were collected at 2, 6, 12, 24, 36, 48, 72, and 96 hours post injection as well as plasma samples of 2 mice from the control group, were also collected at 0 hours. The samples were analysed by ELISA to determine the protein concentrations (nM) in the plasma and clearance rate. The results for each mouse at each individual sampling time points are tabulated. **BOTTOM:** The plasma concentrations were plotted against the time of sampling.

6.6. PD studies

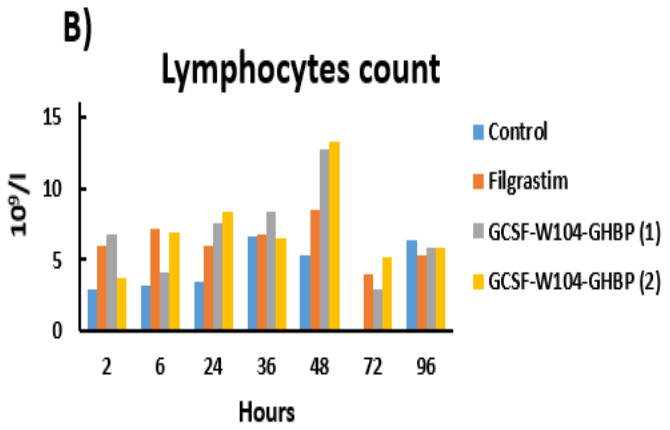
As described previously (Section 2.2.10.4), the number of neutrophils and haematopoietic progenitor cells in blood and BM mice was evaluated daily for 5 consecutive days, after single SC injections of Filgrastim (0.25 mg/kg), GCSF-W104-GHBP (0.61 mg/kg) or 5% dextrose (control group).

6.6.1. Complete blood count (CBC) using an automated haematology analyser (XN10 Sysmex)

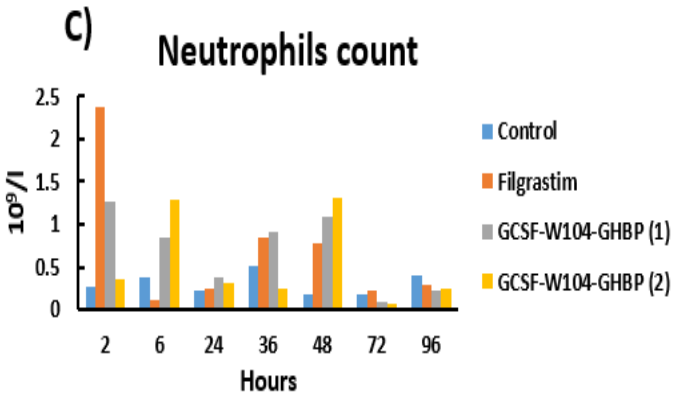
Blood samples from 4 mice (1 from the control group, 1 from the Filgrastim group, and 2 from the GCSF-W104-GHBP group) were collected at 2, 6, 24, 36, 48, 72, and 96 hours post injection. CBC was performed on these samples using an automated haematology analyser (Figure 42).



Time (hour)	Control	Filgrastim	GCSF-W104-GHBP		
			1st mouse	2nd mouse	Mean
2	3.25	8.38	8.05	4.22	6.13
6	3.53	8.27	4.99	8.74	6.86
24	3.73	6.23	8.04	8.82	8.43
36	7.13	7.91	9.48	6.9	8.19
48	5.63	9.33	14.62	15.5	15.06
72	-	4.25	3.15	5.36	4.25
96	6.91	5.64	6.02	6.07	6.04



Time (hour)	Control	Filgrastim	GCSF-W104-GHBP		
			1st mouse	2nd mouse	Mean
2	2.86	5.97	6.72	3.67	5.19
6	3.15	7.12	4.05	6.93	5.49
24	3.48	5.94	7.61	8.42	8.01
36	6.6	6.81	8.34	6.44	7.39
48	5.34	8.48	12.78	13.23	13
72	-	4	2.96	5.2	4.08
96	6.37	5.32	5.77	5.77	5.77



Time (hour)	Control	Filgrastim	GCSF-W104-GHBP		
			1st mouse	2nd mouse	Mean
2	0.27	2.38	1.26	0.36	0.81
6	0.37	0.12	0.84	1.29	1.06
24	0.23	0.24	0.37	0.32	0.34
36	0.51	0.85	0.92	0.24	0.58
48	0.18	0.77	1.09	1.3	1.19
72	-	0.23	0.09	0.07	0.08
96	0.4	0.3	0.22	0.24	0.23

Figure 42. WBC, Lymphocytes and neutrophils counts using XN10 Sysmex.

A total of 16 mice were injected SC with 0.61 mg/kg (13nmol/kg) of GCSF-W104-GHBP, 8 mice were injected with 0.25 mg/kg (13nmol/kg) of Filgrastim, and 10 mice were injected with 5% dextrose (control group). Approximately 200 µl of blood was taken from each of 4 mice (1 from control group, 1 from Filgrastim group, and 2 from GCSF-W104-GHBP group), at 2, 6, 24, 36, 48, 72, and 96 hours post injection. The blood was analysed using an automated haematology analyser. **A)** WBC mobilisation. **B)** Peripheral lymphocytes mobilisation. **C)** Peripheral neutrophils mobilisation. Missing samples shaded in red.

As shown above in Figure 42, WBCs increased at 2 hours and were maintained at steady levels until a peak maximum at 48 hours for all samples above that of control then returned to previous levels at 72-96 hours (Figure 42 A). This increase in WBC's could be mostly attributed to the increasing levels of lymphocytes over the same time period (Figure 42 B). The maximum mobilisation of peripheral neutrophils for Filgrastim group occurred at 2 hours and then rapidly tailed off at 6 hours. At 36 hours, the neutrophils count was increased again compared to control group and returned to normal levels at 72 hours. The picture for test molecule (i.e. GCSF-W104-GHBP) was a little less clear with differences seen between the 2 mouse values. However, depending on the means of two mice, the peripheral neutrophils started to mobilise at 2 hours and peaked at 6 hours before declining at 24 hours post injection. They started to increase again gradually at 36 hours, to reach the maximum mobilisation at 48 hours post-injection and then returned to their normal level at 72 hours.

6.6.2. Neutrophils count using flow cytometry

Flow cytometry was also used to count mice neutrophils in blood and BM samples, using FITC- conjugated Gr-1 (Ly-6G/Ly-6C), and PeCy5- conjugated MAC-1 (CD11b) antibody markers (Full methods are described in section 2.2.10.4). Both CD11b and Ly-6G/Ly-6C are expressed on monocytes and neutrophils. In addition, Ly-6G/Ly-6C is also expressed on lymphocytes. Therefore, we followed a strategy to exclude monocytes and lymphocytes from the analysis as described in Figure 43. Briefly, after staining the cells with a cocktail of antibody markers described above, the cell population of Ly-6G^{hi} and CD11b^{hi} were neutrophils, Ly-6C^{hi} and CD11b^{low} cells were monocytes, and Ly-6C^{hi} cells were lymphocytes.

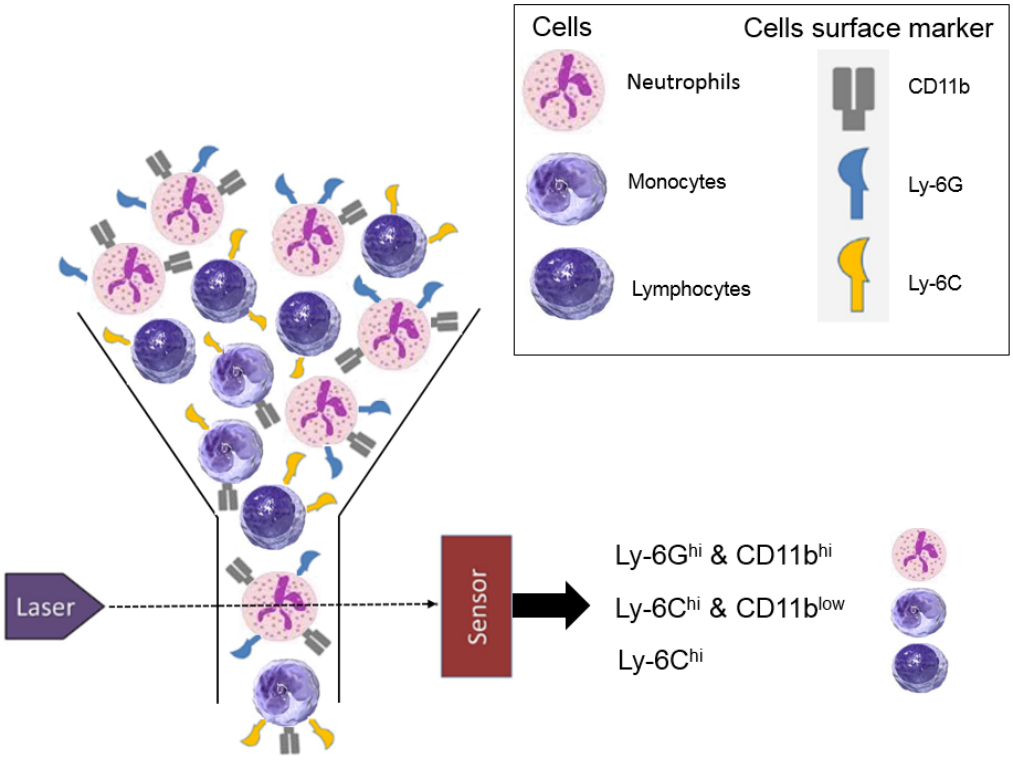


Figure 43. Schematic diagram describing the isolation and flow cytometry analysis of haemolysed mouse blood samples for quantitation of neutrophils, monocytes and lymphocytes.

After staining haemolysed mouse blood samples with Ly-6G/Ly-6C and CD11b antibody markers, the sample was analysed by flow-cytometry. Cells expressing Ly-6G^{hi} & CD11b^{hi} were neutrophils, cells expressing Ly-6C^{hi} and CD11b^{low} were monocytes, whereas cells expressing Ly-6C^{hi} were lymphocytes.

An example of the gating strategy used for counting neutrophils in normal mouse blood is presented in Figure 44 A-F. The percentage of neutrophils counts in blood and BM samples are presented in Figures 45 & 46, respectively, and calculated as the number of neutrophils divided by the number of total WBC's and multiplied by 100.

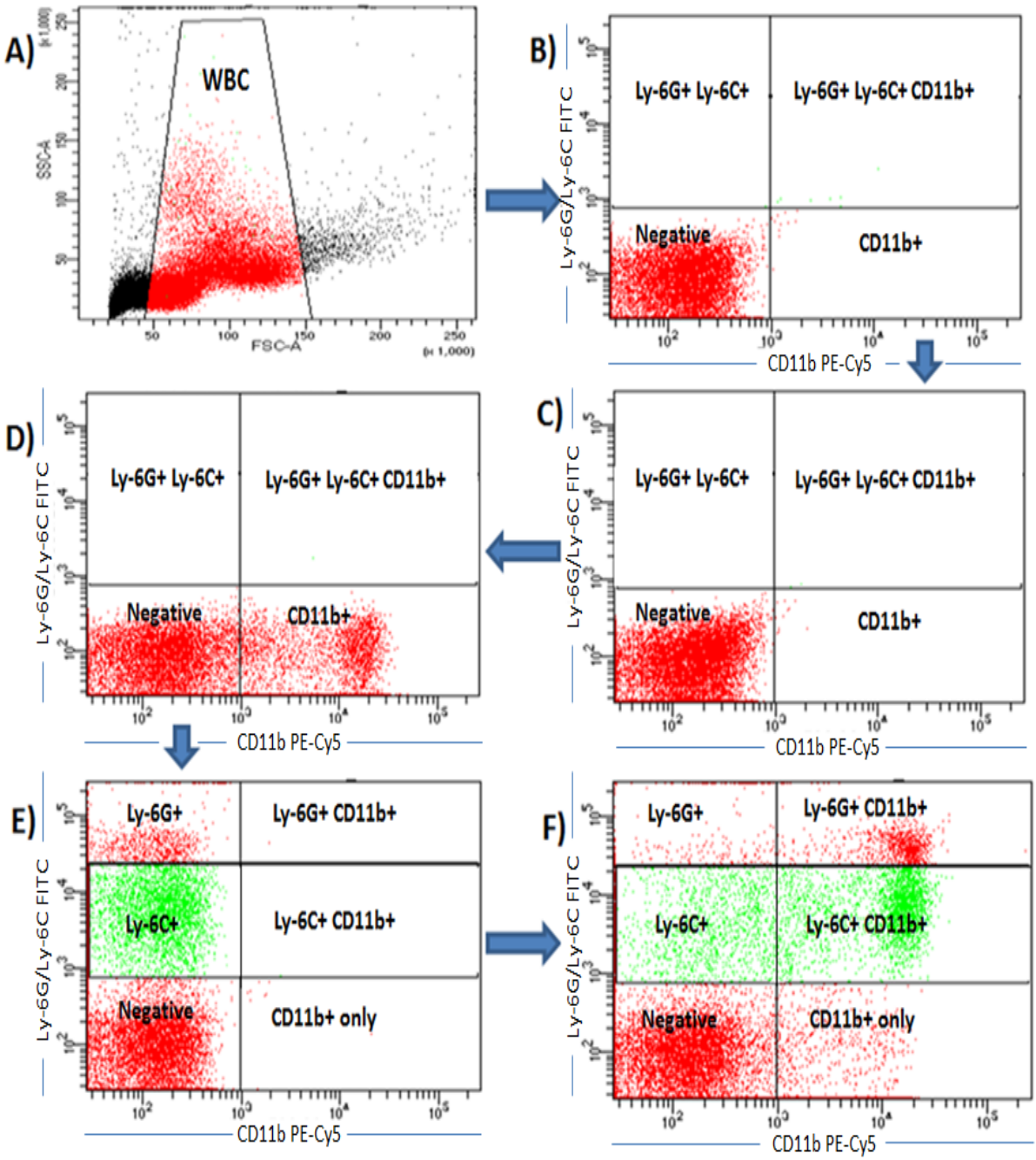


Figure 44. Gating strategy for counting neutrophils in haemolysed normal mouse blood.

A) All WBC populations (i.e., Lymphocytes, monocytes and neutrophils) were gated on a forward scatter (FSC)/side scatter (SSC) plot. **B)** Unstained cells (containing no antibodies), acts a negative control/ background auto fluorescence control. **C)** Cocktail of antibody isotypes (containing FITC- and PE-Cy5- rat IgG2b), to determine the background caused by non-specific antibody binding. **D)** Single staining for CD11b used to set gate. **E)** Single staining for Ly-6G/Ly-6C used to set gate. **F)** Cocktail of antibody markers. Monocytes (Ly-6C+ CD11b+) and lymphocytes (Ly-6C+) stained as dim populations at the middle level of staining, whereas neutrophils stained as a bright population (Ly-6G+ CD11b+) in the upper right corner of the plot. The same strategy was using to gate neutrophils in BM samples.

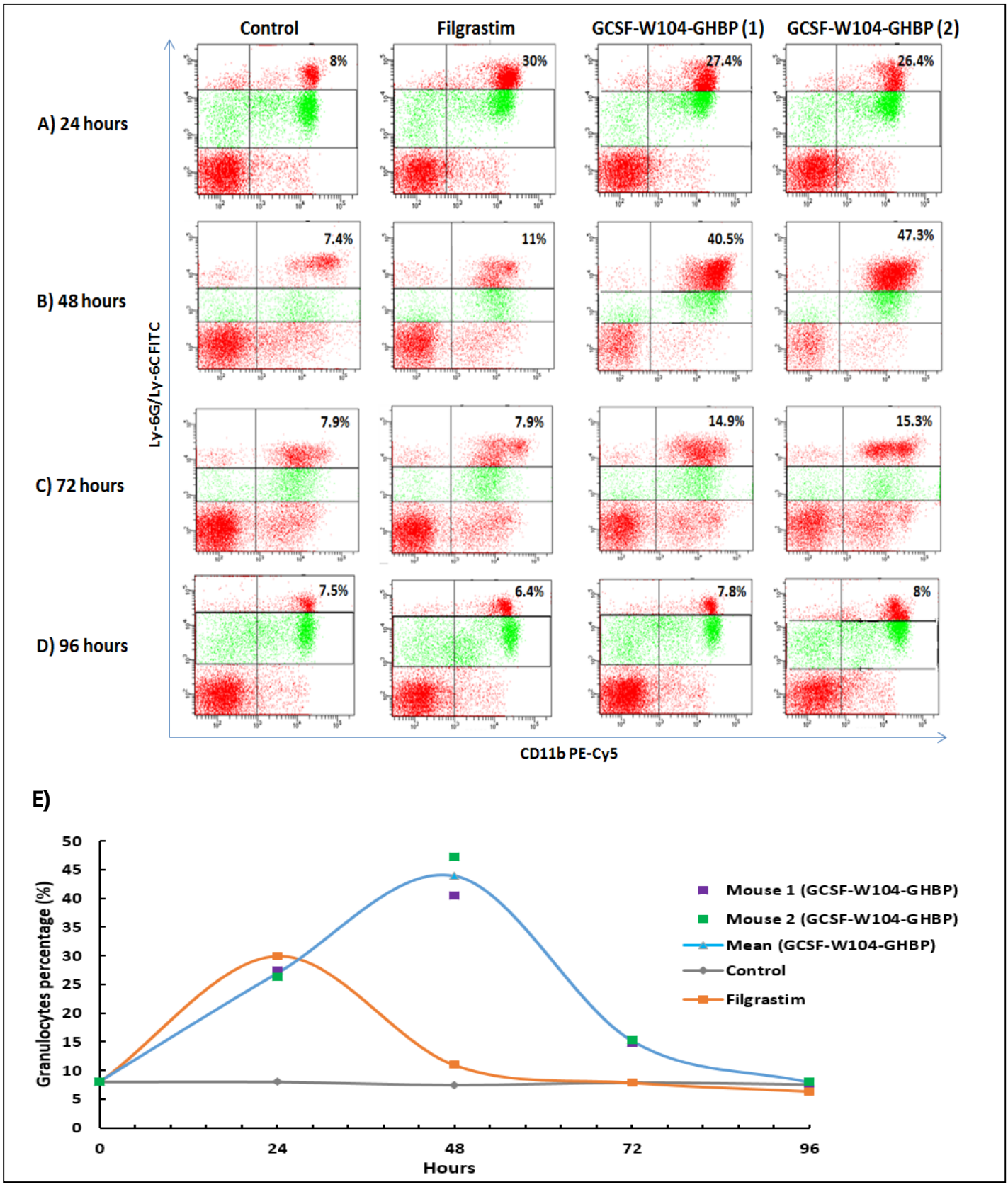


Figure 45. Mobilisation of neutrophils in normal mouse blood.

A total of 16 mice were injected SC with 0.61 mg/kg (13nmol/kg) of GCSF-W104-GHBP, 8 mice were injected with 0.25 mg/kg (13nmol/kg), and 10 mice were injected with 5% dextrose (control group). Blood was collected from 4 mice (1 from control group, 1 from Filgrastim group, and 2 from GCSF-W104-GHBP group) at 24 (A), 48 (B), 72 (C), and 96 (D) hours post injection. The blood was haemolysed and then analysed using a flow-cytometry. Neutrophils were stained using Ly-6G/Ly-6C and CD11b antibodies, and presented on upper right corner of each scatter plot. The percentage of neutrophils was plotted against the time of sampling for each individual mouse **(E)**.

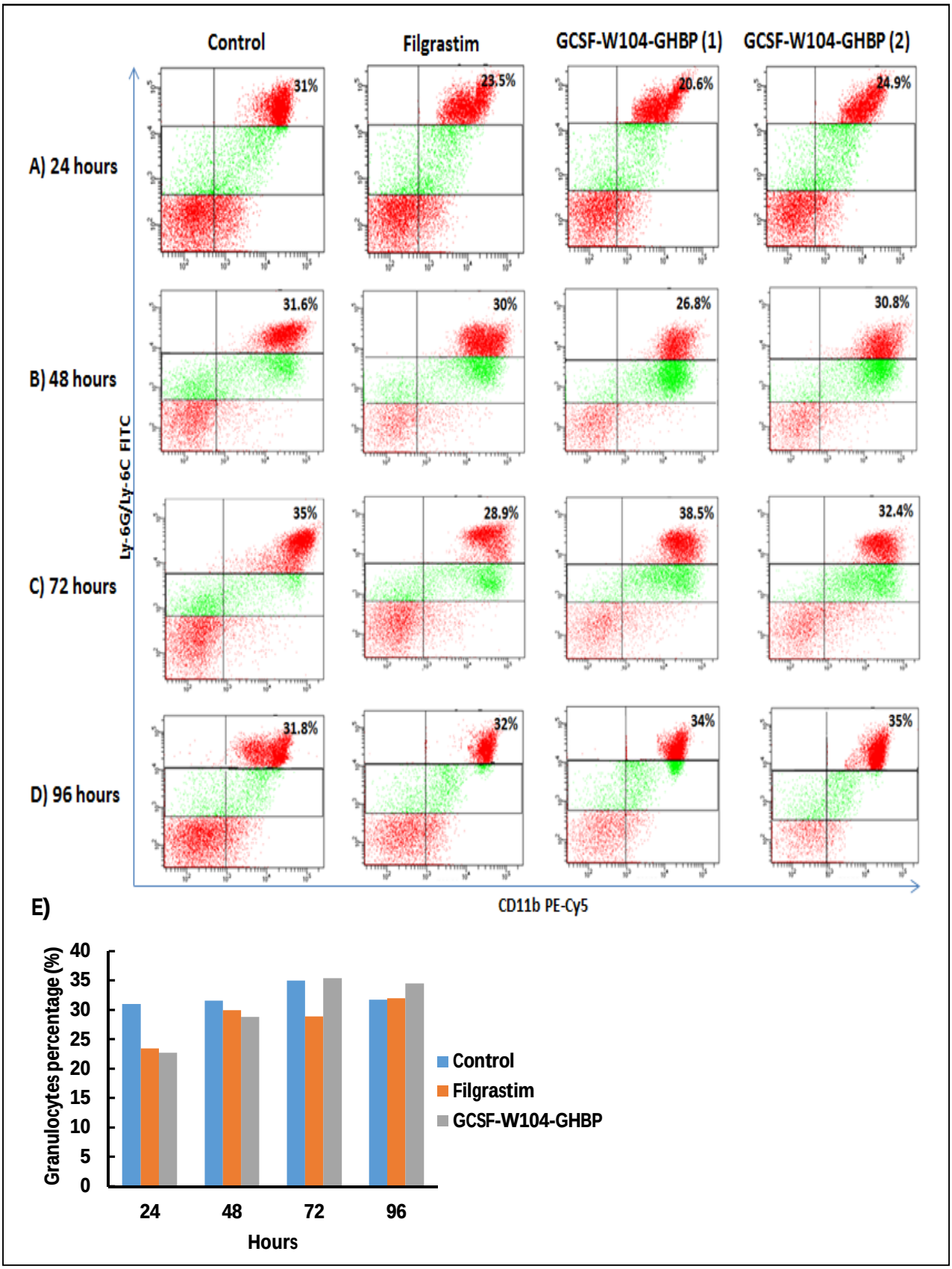


Figure 46. Mobilisation of neutrophils from mouse BM.

BM samples were collected from 4 mouse femurs: 1 from control group, 1 from Filgrastim group, and 2 from GCSF-W104-GHBP group (labelled 1 & 2) at 24 (A), 48 (B), 72 (C), and 96 (D) hours post injection. The BM was haemolysed and analysed using flow-cytometry. Granulocytes were stained using Ly-6G/Ly6C and CD11b antibodies, and presented in the upper right corner of each scatter plot. The percentage of neutrophils was plotted against the time of sampling for each individual mouse **(E)**. To simplify the data, the percentage of neutrophil in the GCSF-W104-GHBP treated group was plotted as the mean of 2 mice at each time points.

As shown above in Figure 45, the maximum mobilisation of neutrophils in the blood occurred at 48 hours after a single administration of GCSF-W104-GHBP (0.61 mg/kg) with a 5.7-fold increase when compared with the mean of control mice. Neutrophils returned to normal levels at 96 hours. The maximum mobilisation of neutrophils in the blood occurred at 24 hours after a single administration of Filgrastim (0.25 mg/kg) with a 3.8-fold increase when compared with the mean control mice. Neutrophils returned to normal levels at 48 hours. In the BM (Figure 46), after administration of single doses Filgrastim and GCSF-W104-GHBP, neutrophil counts slightly decreased at 24 hours when compared to control mice and returned to the normal level at 48 hours for both treated groups.

6.6.3. HPC counts using flow cytometry

Flow cytometry was also used in this experiment to count mouse HPC's in both blood and BM using PE- conjugated CD117 (c-Kit) (Full methods were described in section 2.2.10.4). An example for gating strategy for counting HPC's in normal mouse BM is presented in Figure 47. Percentage of HPC counts in blood and BM samples are presented in Figures 48 & 49, respectively, and calculated as the number of HPC's divided by the number of total cells and multiplied by 100.

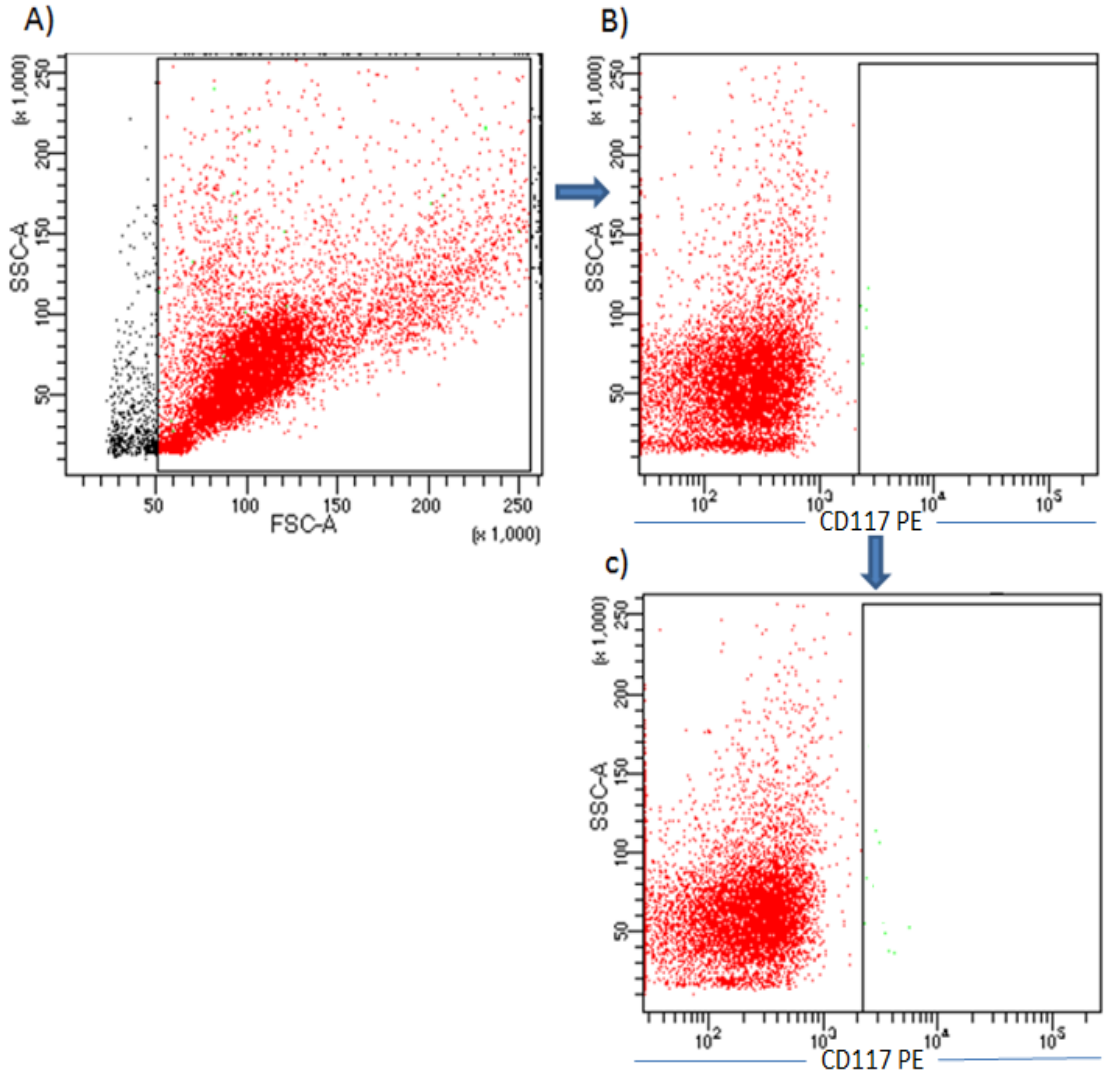
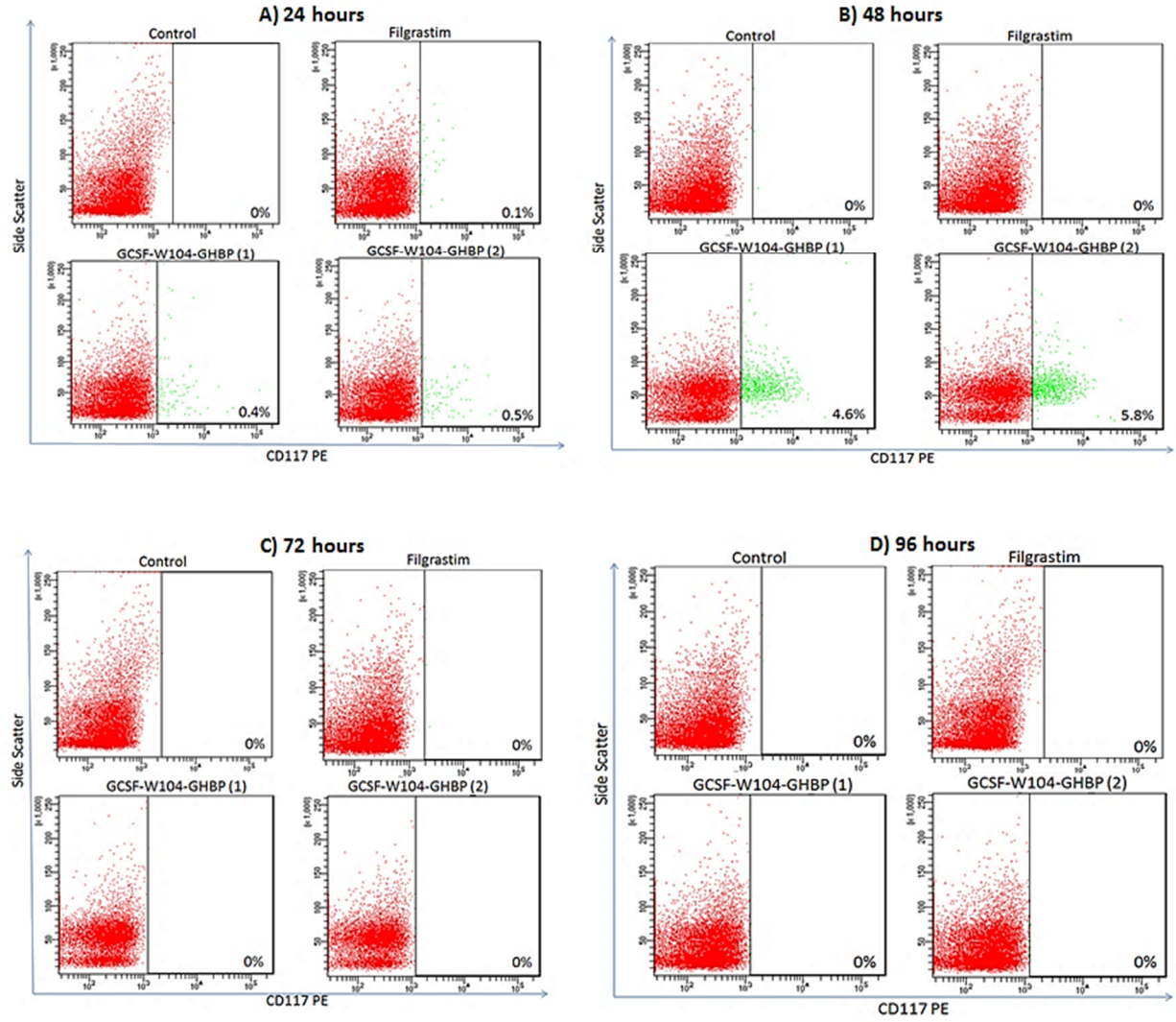


Figure 47. Gating strategy for counting HPC's in normal mouse BM.

A) All BM cells were gated on a forward scatter (FSC)/side scatter (SSC) plot. **B)** For unstained tube (Containing no antibodies), BM cells were copied to a CD117- scatterplot and used as a negative control/ background auto fluorescence control. **C)** Then, for isotype control (Containing PE rat IgG2b), BM cells were copied to a CD117- scatterplot to determine the background caused by nonspecific antibody binding. **D)** The same strategy was using to gate HPC's in blood samples.



E)

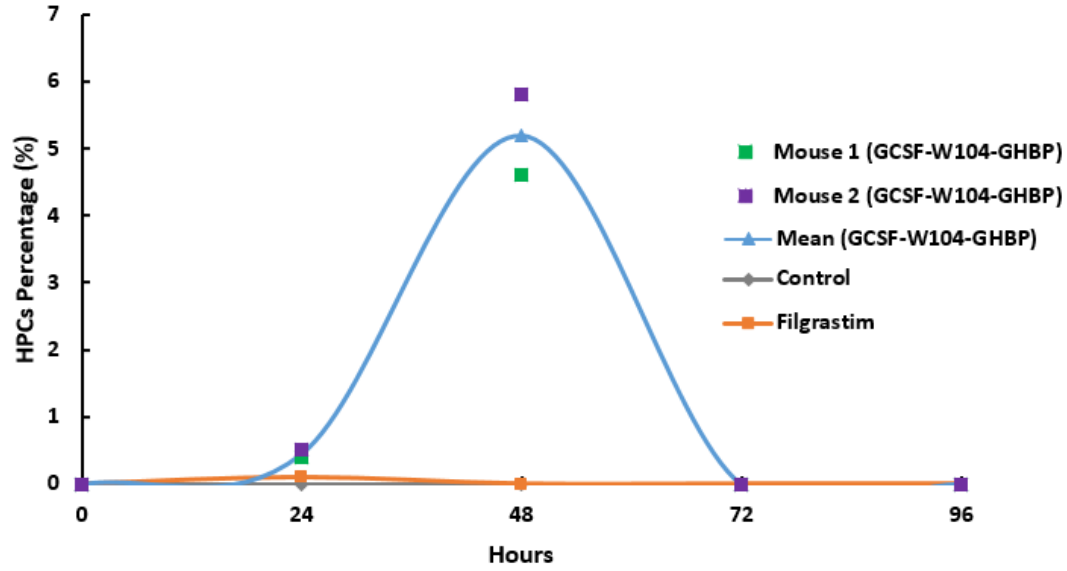
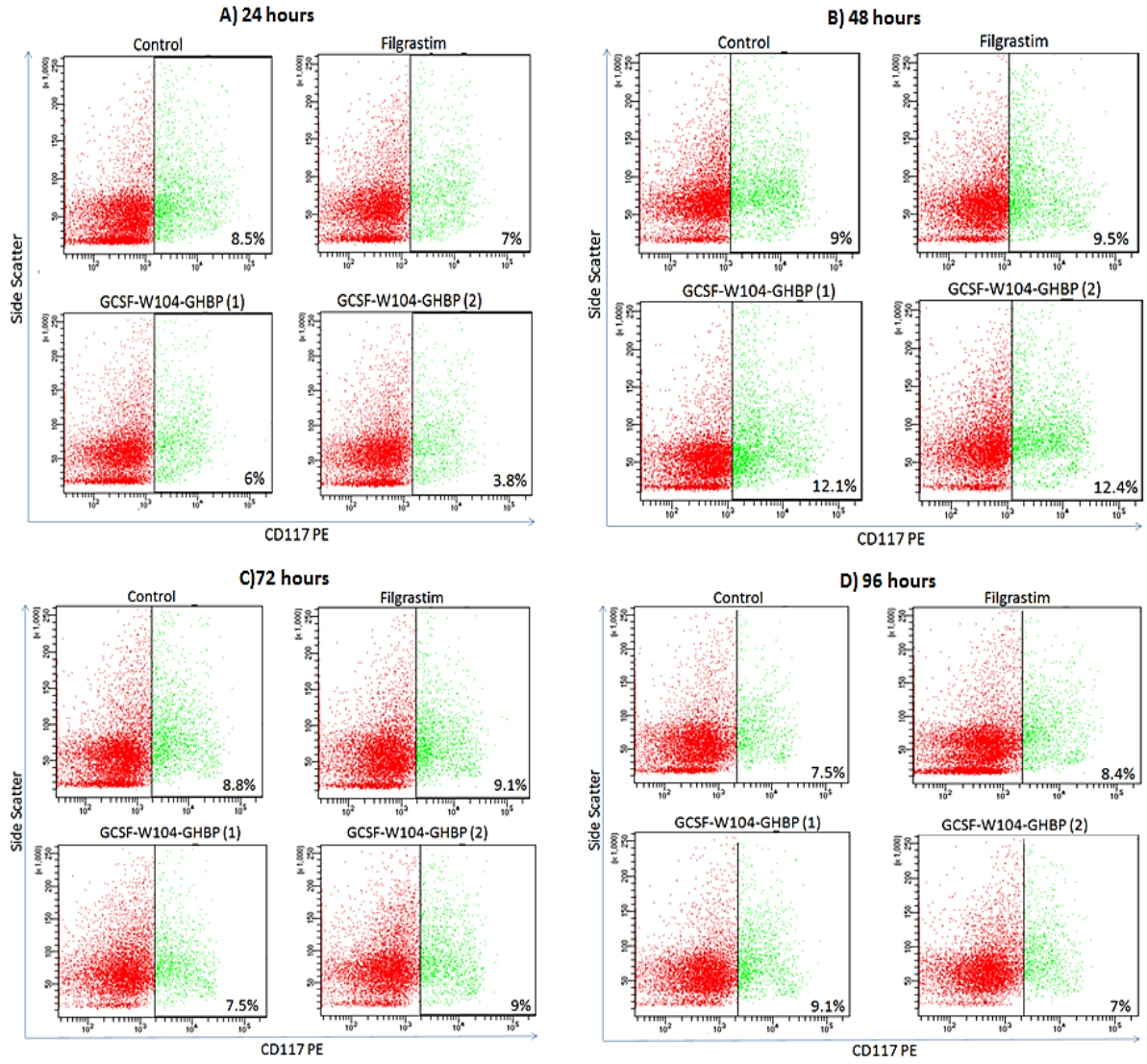


Figure 48. Mobilisation of HPC's in mouse blood.

A total of 16 mice were injected SC with 0.61 mg/kg (13nmol/kg) of GCSF-W104-GHBP, 8 mice were injected with 0.25 mg/kg (13nmol/kg) of Filgrastim, and 10 mice were injected with 5% dextrose (control group). Blood was collected from 4 mice (1 from control group, 1 from Filgrastim group, and 2 from GCSF-W104-GHBP group) at 24 **(A)**, 48 **(B)**, 72**(C)**, and 96 **(D)** hours post injection. The blood was haemolysed first and then analysed using a flow cytometry. HPC's were counting using CD117 antibody, and presented on right side of each plot. HPC's percentage was plotted against the time of sampling for each individual mouse **(E)**.



E)

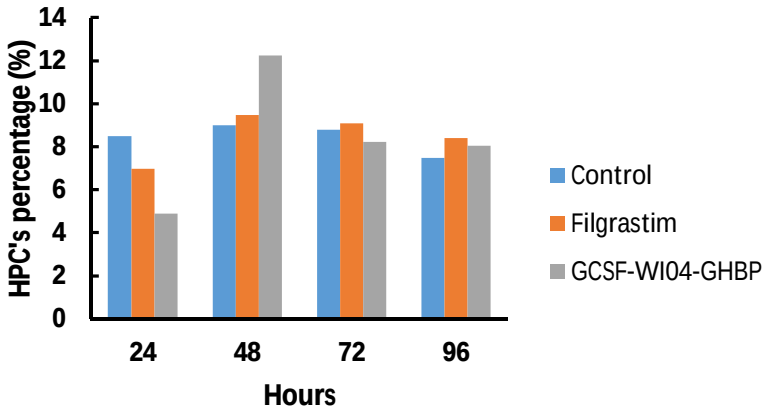


Figure 49. Mobilisation of HPC's in mice BM.

BM samples were collected from 4 mice femurs (1 from control group, 1 from Filgrastim group, and 2 from GCSF-W104-GHBP group) at 24 **(A)**, 48 **(B)**, 72 **(C)**, and 96 **(D)** hours post injection. BM cells were first haemolysed and then analysed using FACS. HPC's were counted using CD117 antibody, and presented on the right side of each plot in green. Percentage of HPC's were plotted against the time of sampling for each individual mouse **(E)**. To simplify the data, the percentage HPC's for the GCSF-W104-GHBP group was plotted as the mean of 2 mice at each time points.

As shown in Figure 48, a single SC administration of GCSF-W104-GHBP also resulted in an increase in HPC's (c-Kit$^+$ cells) at various stages of differentiation. The maximum mobilisation of HPC's in the blood occurred at 48 hours for GCSF-W104-GHBP with a 5.2-fold increase when compared with control mice. HPC's returned to normal levels at 72 hours. The maximum mobilisation of HPC's in the blood occurred at 24 hours for Filgrastim with a modest 0.1-fold increase when compared with control mice. HPC's returned to normal levels at 48 hours. In the BM (Figure 49), after administration of single dose of Filgrastim and GCSF-W104-GHBP, HPC's counts decreased at 24 hours when compared to controls and for the Filgrastim group returned to normal levels at 48 hours, whilst for GCSF-W104-GHBP group, they peaked at 48 hours, and returned to normal levels at 72 hours.

6.7. Discussion

In the previous chapter, the GCSF-W104-GHBP molecule showed better *in vitro* biological activity compared to the rhGCSF and demonstrated high stability at RT, 4°C, and multiple F/T cycles. In order to confirm GCSF-W104-GHBP fusion protein maintains bioactivity when used in vivo, the PK and PD profiles were analysed, using a specific pathogen-free BDF1 mouse model. As mentioned previously in section 2.2.10.1, the main reason for choosing this specific strain of mouse was because they have been shown to have a robust response to GCSF as reported by Molineux et al, 1999, and Lord et al, 2001. PK analysis was performed on mouse plasma samples using a GCSF Elisa. Prior to the experiment, the effect of both 2% mouse serum and plasma on the ELISA was assessed. Both matrices had no effect or interference on both the Filgrastim and GCSF-W104-GHBP standard curves. Comparatively, a methodology paper by Yu et al analysed the concentrations of 163 metabolites in both EDTA plasma and serum collected from 377 individuals. The paper reported that reproducibility was good in both plasma and serum, and better in plasma. Furthermore, either matrix would generate similar results in biological and clinical studies as long as the same blood preparation procedure is used (Yu et al., 2011). However, these metabolites were analysed using metabolomics kit which is different than our method (i.e. ELISA). Also, they used human blood which might give different results than mice blood. Another study conducted by Joyce et al showed that human IgG monoclonal antibody collected from mouse serum and EDTA-treated plasma gave

similar concentrations using a Gyrolab immunoassay platform (Joyce et al., 2014). Here again, a different method was used to estimate drug concentration.

We performed our PK study analysis of samples using mouse plasma so that whole blood could be collected in EDTA tubes for both flow-cytometry and coulter counter analysis for the PD study.

In the preliminary PK analysis, the maximum plasma concentration (C_{max}) for Filgrastim was 16.4 nM with a time of maximum concentration (T_{max}) of approximately 2 hours. Whereas GCSF-W104-GHBP showed delayed T_{max} of approximately 12 hours with a C_{max} of 35.4 nM. From the literature, SC administration of Filgrastim in healthy BDF1 mice has a half-life was 2.5 hours and T_{max} 0.25 hours (Halpern et al., 2002). The longer T_{max} in this study compared to the literature is because this study did not obtain earlier time points so the later T_{max} reflects the first sampling time point. The results from this study demonstrate that GCSF-W104-GHBP has a prolonged T_{max} which could reflect both delayed absorption and delayed clearance after SC administration. One explanation could be that after SC administration of a recombinant protein drug, it enters into the systemic circulation via either the blood capillaries or lymphatic system depending on its molecular weight (Mahmood and Green, 2005). The lymphatic system becomes the main pathway for absorption when the MW of proteins is > 16,000 Da (Supersaxo et al., 1990, McLennan et al., 2005). Moreover, the time of maximum concentration of therapeutic proteins increases with increasing MW (Kota et al., 2007). In other words, the larger the drug, the slower the absorption rate. Accordingly, both Filgrastim and GCSF-W104-GHBP would be absorbed by the lymphatic system, as both MW > 16,000 Da. Additionally, GCSF-W104-GHBP has a higher MW (46,900 Da) compared to Filgrastim (19,000 Da), thus, its T_{max} is expected to be longer.
The results of this study suggested that the clearance of GCSF-W104-GHBP was approximately 3 times slower than that of Filgrastim, as it was still detected at 36 hours compared to12 hours for Filgrastim.

As described previously in section 1.6, the two main independent processes to eliminate GCSF from the body are non-specific elimination via renal filtration, and specific degradation via neutrophil and GCSF receptor-mediated clearance. If the

hydrodynamic size of a GCSF conjugate was above the threshold for renal clearance, then neutrophil and GCSF receptor-mediated clearances would be the predominant ways to clear the conjugate from the body. Pegfilgrastim has a MW of 39,000 Da and is eliminated mainly by neutrophil-mediated clearance (Scholz et al., 2009). The role of the liver in the elimination of rhGCSF seems to be insignificant as the PK for Filgrastim after SC administration was found to be similar between healthy volunteers and hepatic patients (Roskos, 2012). Therefore, from our data, it is fair to assume that GCSF-W104-GHBP having a MW of 46,900 Da will also be mainly eliminated from the body by a neutrophil-receptor mediated process.

Table 21 (below) compares the PK results of our fusion protein with those obtained from the literature for other long-acting GCSF molecules, which were using a mouse model.

Table 21. PK results of some long-acting GCSF products injected SC to mice.

Drug	Dose	$t_{1/2}$ (h)	T_{max} (h)	C_{max} (ng/ml)	AUC (h.ng/ml)	References
GCSF-HAS*	1.25mg/kg	5.68	16	7339	196250	(Halpern et al., 2002)
	0.25mg/kg	5.58	6	901	20513	
GCSF-3DHSA**	0.58mg/kg	3.42	2.09	2314	16339	(Zhao et al., 2013)
GCSF-W104-GHBP	0.6 mg/kg	-	12	1663	36858	This study

*HAS: Human serum albumin, 3DHSA: domain III of Human serum albumin

As shown in Table 21, a single injection of GCSF-HAS at a dose of 1.25 mg/kg, has a T_{max} of 16 hours and C_{max} of 7339 ng/ml compared with a T_{max} of 12 hours and C_{max} of 1,663 ng/ml for our GCSF fusion at a dose of 0.6 mg/kg. While a single injection of GCSF-3DHSA at a dose of 0.58 mg/kg, has a T_{max} of only 2 hours and C_{max} of 2,314 ng/ml compared with our GCSF fusion at the most similar dose (i.e. 0.6 mg/kg). These results indicating that GCSF-W104-GHBP molecule has a much better PK profile compared to GCSF-HAS and GCSF-3DHSA. Available PK data from the literature for Pegfilgrastim and Lipegfilgrastim were obtained after IV injection into rats, and SC injection into monkeys, respectively (Arvedson et al., 2015), therefore no comparison with data from this study was made.

We next determined the preliminary PD for Filgrastim and GCSF-W104-GHBP using the same normal mice. Blood samples were collected via cardiac bleeding and

analysed using an automated haematology analyser to obtain WBC and its differentials at 2, 6, 24, 36, 48, 72, 69 hours after administration. For the control group, total WBC and lymphocytes counts were slightly similar to those obtained by Nemzek et al., whilst being higher than those obtained from Doeing et al. Neutrophil counts in our study were significantly lower than values obtained by Nemzek et al. and Doeing et al. (Table 22).

Table 22. A comparison of normal mice WBC, lymphocytes, and neutrophils counts obtained from various studies.

	This study	**(Nemzek et al., 2001)**	**(Doeing et al., 2003)**
WBC	$5.19 \pm 1.9 \times 10^9$/L	$4 \pm 1 \times 10^9$/L	$2.7 \pm 0.5 \times 10^9$/L
Lymphocytes	$4.73 \pm 1.8 \times 10^9$/L $90.3 \pm 2.3\%$	3×10^9/L	74 ± 3 %
Neutrophils	0.27 ± 0.09 10^9/L $5.8 \pm 2.5\%$	1×10^9/L	24 ± 3 %

One possible explanation of these differences is the procedure used in blood analysis. Nemzek et al. used an automated, animal coulter counter, Doeing et al. performed the analysis manually using a haemocytometer, whilst in our study the cell counts were done using the XN10 analyser (Sysmex). This analyser was set up for only human cell determination and not for animals. A study that backs up this theory was completed by Tabata et al. who performed a parallel evaluation on three automated haematology analysers, which were calibrated with commercially available material based on human blood products, using animal blood samples from three healthy different species (monkeys, dogs, and rats). Each analyser gave different results for at least one blood parameter. The investigators explained these biases as being due to inherent physical differences between the analytical methods and/or the calibration techniques (Tabata et al., 1998). Therefore, in our case, it is possible that the total WBC, lymphocyte, and neutrophils counts might be overestimated or underestimated. Another reason for these differences seen in cell counts may due to the different anaesthetics used, as some studies have shown that total WBC counts in mice could be affected by the type of anaesthetic used (Diehl et al., 2001). In this study, isoflurane was used whereas Nemzek et al used xylazine and ketamine and Doeing et al used methoxyflurane. Another theory that may lead to an increase in total WBC count is the stress induced by the procedure used to sample blood. Doeing et al. found significant differences in WBC counts collected from saphenous, foot, and tail veins

compared to cardiac bleeding, as these areas were rubbed, massaged, and pressure applied to in order to find a well visible vein beneath the skin. In this study, although cardiac puncture is considered an easy and quick procedure to draw blood, we found difficulties in locating the heart left ventricle, which required a great deal of manipulation. Such manipulations may have caused local release of WBC's from the marginal pool into the blood stream and thus affecting the final analysis. Another possible explanation is gender variation. We used only male mice, while Nemzek et al. used female mice. Doeing et al. observed significant differences in differential WBC counts between female and male blood samples obtained by cardiac puncture, while there were no significant differences between the total WBC counts of male and female blood samples. The percentage of neutrophils in blood collected via cardiac puncture in female mice was significantly lower than those in male mice. The reason for this is those female steroid hormones, 17 beta-estradiol and progesterone can modulate the expression of adhesion molecules on human peripheral blood WBC's, thereby, affecting their circulation and activation. Moreover, in women, infiltration of neutrophils into the uterus leads to a consistent fall in the neutrophil count at menstruation. Last but not least, strain differences can also influence the results of blood parameters. In this study, BDF1 was used, whilst Nemzek et al. used BALB/c and Doeing et al. used C57BL/6. Our theory is backed up by a study that measured blood parameters for 16 commonly used strains of inbred mice (Kile et al., 2003). The investigators demonstrated that a high degree of strain-related variation exists in the peripheral blood cell values. Therefore these explained differences in methodology and study design could be one reason why we see discrepancies in blood counts between the studies.

For Filgrastim group, the total WBC counts peaked at 2 hours and then started to fall down, but still remained higher than the control group. At 48 hours, the second peak of total WBC's occurred before falling down. While, for GCSF-W104-GHBP, the total WBC counts rose steadily to reach a maximum count at 48 hours before falling down. Surprisingly, the main cells that were responsible for this increase in total WBC counts were the lymphocytes and not the neutrophils as we first thought. These data implied that GCSF is increasing the number of lymphocytes (or could it be the experimental design: cardiac puncture/ anaesthetic /stress, etc.). A few studies have been completed that back up our own observations and report a relationship between the

dose of GCSF administrated to normal human subjects (Gürman et al., 2001) or neutropenic patients (Böhme M1, 1994), and the extent of increase in peripheral blood lymphocytes. However, most studies agree that there is no relationship between GCSF administration and increasing lymphocytes in both humans (Dreger et al., 1994, Höglund M1, 1996, Stroncek et al., 1996b, Stroncek et al., 1996a) and mice (Okabe et al., 1990, Ohdo et al., 1998). Neutrophil counts were also obtained from the automated haematology analyser. From this data, we found that both Filgrastim and GCSF-W104-GHBP had to 2 peaks of increasing neutrophil counts compared to control groups. The 1st peak of both treated groups was at 2 hours, and the 2nd peak for Filgrastim was at 36 hours compared to 48 hours for GCSF-W104-GHBP, which might indicate a prolonged mode of action for our construct. A number of studies have shown an initial transient decrease in the number of peripheral neutrophils immediately (i.e. within one hour) after SC or IV administration of Filgrastim (Bronchud et al., 1988, Morstyn G, 1988), followed consistently by a rapid and significant increase to a greater than normal count within 4 to 6 hours. Welte et al (1996) also found there was a second neutrophil peak between 24 and 48 hours, which is similar to our observations. The exact mechanism of the early neutrophil depression was thought to be due to margination of neutrophils to endothelial cells. Because the increase in neutrophil counts occurs within 6 hours, it is possible that mature neutrophil demargination or prolongation of circulating neutrophil survival may contribute to the earliest observed neutrophil increase (Morstyn G, 1988, Welte et al., 1996). The occurrence of the observed 2nd peak (shown in this study and the study by Welte et al) is suggested to be a result of increased progenitor production and the eventual maturing and release of those cells into circulation (Loving et al., 2013).

In contrast to our results for Filgrastim, after healthy (Halpern et al 2002), or splenectomised (Zhao et al 2001), BDF1 mice received a single SC injection of Filgrastim (0.25 mg/kg), both WBC and neutrophils peaked at 24 hours and returned to their normal levels at 48 hours.

Neutrophils count was also assessed in peripheral blood and BM samples by flow-cytometry using Ly-6G/Ly-6C (Clone: RB6-8C5), and CD11b antibodies. RB6-8C5 binds to Ly-6G, which is present on neutrophils, and to Ly6C, which is expressed on subpopulations of lymphocytes and monocytes (BD Pharmingen). CD11b marker is

expressed on neutrophils, monocytes, and activated lymphocytes (ProSci). Thus, both markers were not specific for neutrophils. To overcome this problem, we followed a gating strategy for measuring neutrophil depletion, which was described by Hasenberg et al., with some modifications as we did not use a CD45 marker (Hasenberg et al., 2015). The investigators used a CD45 marker to exclusively analyse WBC's, while we did the analysis on lysed RBC samples. In addition to this strategy, we relied on evidence that after staining the samples with Ly-6G/Ly-6C antibody, the cells bright population corresponded to Ly-6G expressing neutrophils, whereas, the cells dim population corresponded to Ly-6C- expressing lymphocytes and monocytes (BD Pharmingen, 2011). Finally, we believed that the cells that expressed Ly-6G and CD11b were neutrophils as recorded by Jönsson et al (Jonsson et al., 2011). After flow-cytometry analysis of both blood and BM neutrophils, we found that GCSF-W104-GHBP produced a prolonged haematopoietic activity in mice with an increase in the number of peripheral blood neutrophils for up to 72 hours after a single dose of GCSF-W104-GHBP versus 24 hours after a single dose of Filgrastim. In the BM, neutrophil counts slightly decreased at 24 hours and returned to their normal level at 48 hours for both treated groups. This initial decrease in neutrophil counts at 24 hours is expected since treatment with GCSF mobilises the neutrophils from BM into the circulation, levels are then returned back to their normal levels at 48 hours. HPC's were also assessed in peripheral blood and BM samples with flow-cytometry using a CD117 marker. In BM, CD117 is expressed on haematopoietic progenitor cells, progenitors committed to erythroid and/or myeloid lineages, and precursors of B and T lymphocytes (BD Pharmingen, 2011). In the current study, we observed that a single administration of GCSF-W104-GHBP also resulted in an increase in HPC's (c-Kit+) at various stage of differentiation. The maximum mobilisation of HPC's in the blood occurred at 48 hours for GCSF-W104-GHBP compared to 24 hours for Filgrastim. Our data for Filgrastim is similar to those obtained by Halpern et al, as they found that in healthy BDF1 mice after receiving a single administration of Filgrastim, both peripheral neutrophils and HPC's mobilised at 48 hours and returned to their normal levels at 72 hours. In the BM, HPC's counts decreased at 24 hours and returned to normal levels at 48 hours for Filgrastim group, while for the GCSF-W104-GHBP group, they slightly peaked at 48 hours, and returned to normal levels at 72 hours. Unsurprisingly, we observed the percentage of $CD117^{+}$ cells in the BM were less than 10%. This is because according to Jindal et al,

approximately 59.75% of BM cells that express CD117 are a lineage negative cell population while 9.8% are lineage positive cells.

Table 22 (below) compares the PD results of GCSF-W104-GHBP with those obtained from the literature for other long-acting GCSF molecules, which were using a mouse model.

Table 22. PD results of some long acting GCSF products injected SC to mice

		WBC count		Neutrophils count		Peripheral HPC count		
Drug	**Dose**	**Maximum peak**	**Returning to normal level**	**Maximum peak**	**Returning to normal level**	**Maximum peak (day)**	**Returning to normal level**	**References**
Pegfilgrastim	1mg/kg	Day 4	Day 6-7	Day 4	Day 6-7	-	-	Lord et al 2001
GCSF-HAS*	1.25mg/kg	-	-	Day 2	Day 4	Day 2	Day 4	Halpern et al, 2002
	0.25mg/kg	-	-	Day 2	Day 3	Day 2	Day 3	
	5 mg/kg	-	-	Day 4	Day 5	Day 4	Day 5	
GCSF-W104-GHBP	0.6 mg/kg	Day 2	Day 3	Day 3	Day 4	Day 2	Day 3	Our study

*HAS: Human serum albumin

As shown in Table 22, a single SC injection of Pegfilgrastim at a dose of 1 mg/kg has a longer PD action (i.e. mobilisation of peripheral WBC's and neutrophils) compared with GCSF-W104-GHBP and GCSF-HAS, however, it might due to dose differences. While a single SC injection of our fusion GCSF protein at a dose of 0.6 mg/kg has a longer PD action (i.e. mobilisation of peripheral neutrophils and HPC's) compared with GCSF-HAS at a double dose (1.25 mg/kg). Available PD data from the literature for Lipegfilgrastim were obtained after SC injection into neutropaenic rats, (Arvedson et al., 2015), therefore no comparison with data from this study was made.

In summary, a single dose of GCSF-W104-GHBP produced a prolonged haematopoietic activity in mice with an increase in the number of blood neutrophils for up to 72 hours, and HPC's for up to 48 hours, compared to 24 hours for Filgrastim. The results of GCSF-W104-GHBP demonstrated that W104-GHBP as a fusion partner increased the bioactivity of GCSF. From the present work, it is expected that further preclinical and animal experiments are necessary to prove the efficacy and safety of GCSF-W104-GHBP. In addition, detailed PK analyses are required compared with

Pegfilgrastim and would further advance the alternative clinical application of this compound in neutropaenia.

Chapter 7. General discussion

Granulocyte colony-stimulating factor (GCSF) is a hormone which has the primary function of stimulating the production of neutrophils. The human recombinant form of granulocyte colony-stimulating factor (rhGCSF) has proven successful in the treatment of neutropenia and the mobilisation of stem cells in the context of bone marrow transplantation. Commercially available rhGCSF products fall under the category of either short-acting (i.e., Filgrastim, Lenograstim) wherein the structural homology and pharmacokinetic properties of the molecule are essentially similar with those of endogenous (native) GCSF, or long-acting (i.e., Pegfilgrastim, Lipegfilgrastim), wherein chemical modification through PEGylating has been applied to prolong pharmacokinetic activity through delayed hepatic and renal clearance. As the use of the short-acting G-CSF product has been limited by the need for frequent (daily) dosing, the shift towards the use of the long-acting product has predominated over recent years.

With the market size for GCSF continuing to grow, there has been significant competition for the development of second-generation long-acting GCSF products that can match or improve on the pharmacokinetics and efficacy profile of Pegfilgrastim. In order to make a significant impact on the market penetration, it is anticipated that new generation GCSF products would need to provide key differentiating features which can compete directly with the long-acting GCSF, and set new standards in clinical performance, including: superior PK profile, greater reproducible and responsiveness of treatment for chemotherapy-induced neutropaenia and reduced cost of manufacture specifically against PEGylated products that require post-expression chemical modification and multi-step purification. Using Asterion's ProFuse™ technology, our group has already designed, cloned, and expressed a GCSF protein fusion construct by linking full-length GCSF to its extracellular receptor domain via a flexible glycine-serine linker (code name: 4A1). The pharmacokinetic performance of 4A1 showed longevity of action, and excellent delayed clearance properties over commercially available GCSF products in a mouse model (unpublished data). This technology has been applied to hGH and demonstrated improved PK profile and enhanced *in vivo* biological activity. Because the use of fusion proteins that link ligand to an extracellular receptor has proved fruitful in previous work, it was decided to expand this concept and use ProFuse™ technology to link full-length GCSF to inactive GHBP via a flexible glycine-serine linker. The inactive GHBP

includes an amino acid change at tryptophan-104 (A tryptophan to alanine substitution). This change prevents GH binding to GHBP in the circulation. In this project, we have shown that it is possible to construct and express GCSF-GHBP and GCSF-W104-GHBP, in a CHO Flp-In system. The CHO Flp-In system was selected as it enabled easy, rapid and efficient generation of stable clones into specific sites within the host genome for high expression. Also, it allows isogenic generation of the stable cell lines, and CHO cells possess a glycosylation mechanism similar to those in human cells (Damiani et al., 2009). Expression of our constructs was tested using ELISA and Western blotting. Both techniques detected the constructs in both transient and stable cell lines. As judged by SDS-PAGE, followed by western blotting, the molecules appeared to be intact with no observed degraded products. Monomers separate at ~ 50-70 kDa which is larger than the calculated molecular weight of ~ 46 kDa, this is more than likely due to both GHBP and GCSF being glycosylated which increases the observed molecular weight. Western blotting also detected higher orders structures (i.e., dimeric aggregation) of both constructs although SDS-PAGE was run under reducing conditions using 0.25 mM final DTT. Most studies agree that native monomeric rhGCSF reversibly forms a dimer under physiological conditions (i.e. pH 7.0 at 37 °C) (Chi et al., 2003, Raso et al., 2005, Ribarska et al., 2008) and that this dimeric species does not participate in the irreversible aggregation process (Chi et al., 2003).

During the large-scale expression and production processes of GCSF-W104-GHBP, different viabilities were noticed among the five roller bottles on day 7, ranging from15% to 100% although there were seeded at the same cell densities. This may have been due to media nutrients being used up especially at the end of the culture period or inefficient aeration in the non-vented roller bottles due to too much media present (Total volume used ~500ml) leading to poor control of pH. Protein purification was carried out at 4°C to prevent potential protein degradation using a Ni^{2+} charged IMAC column. For GCSF-W104-GHBP eluted fractions, particularly, there was a shift in molecular weights across the elutions indicating potentially different glycosylation forms of the protein. Western blots and coomassie staining of both purified proteins revealed a main band with a molecular weight of between 50-75kDa with no signs of degradation, indicating successful purification processes. Final purified proteins were measured by Bradford assay and resulted in a total of 13.95 mg (0.45 mg/ml), and

4.46 mg (0.75 mg/ml), for GCSF-W104-GHBP, and GCSF-GHBP, respectively. In addition, the post-dialysed purified proteins were considered to be > 90% pure as assessed by Bradford assay, and SDS-PAGE followed by western blotting.

The *in vitro* bioactivity for GCSF-GHBP and GCSF- W104-GHBP in the AML193 assay is a higher than its corresponding un-fused native protein with EC_{50} values of 0.02 nM, compared to 0.05 nM, respectfully, indicating that human GCSF can be fused to GHBP without significantly affecting in vitro biologic activity of GCSF and may even enhance biological activity.

Protein stability tests demonstrated that our proteins can be kept either at RT or 4°C, for up to 8 days and at -80°C for up to 3 months, without any detectable degradation.

Using a specific pathogen-free BDF1 mouse model the PK and PD profile of GCSF-W104-GHBP and Filgrastim were analysed. GCSF-W104-GHBP had a higher maximal plasma concentration (C_{max}) of 35.4 nM compared to Filgrastim at 16.4 nM. GCSF-W104-GHBP also showed an approximate 6-fold increase in the time of maximal concentration (T_{max}) of 12 hrs compared to 2 hrs for Filgrastim. Results also suggested that the clearance of GCSF-W104-GHBP was approximately 3 times slower than that of Filgrastim, as it was still detected at 36 hours post-injection compared to 12 hrs for Filgrastim. In addition, these results indicating that our molecule has a much better PK profile compared to GCSF-HAS and GCSF-3DHSA. The results also showed that a single dose of GCSF-W104-GHBP produced a prolonged haematopoietic activity in mice with an increase in the number of blood neutrophils for up to 72 hours, and HPC's for up to 48 hours, compared to 24 hours for Filgrastim. Moreover, a single SC injection of GCSF-W104-GHBP at a dose of 0.6 mg/kg has a longer PD action (i.e. mobilisation of peripheral neutrophils and HPC's) compared with GCSF-HAS at a double dose (1.25 mg/kg).

The increase in the *in vitro* bioactivity combined with the *in vivo* delayed clearance and increased haematopoietic activity shows the potential of this technology to generate a long-acting GCSF molecule for the treatment of neutropaenia and mobilisation of stem cells.

Chapter 8. Future work and conclusion

8.1. Future work

While this thesis has demonstrated the potential of efficiently developing a long-acting GCSF molecule by linking it to GHBP, many opportunities for extending the scope of this thesis remain. This section presents some of these directions.

Site-directed mutagenesis of human GCSF gene

As highlighted in this study, western blotting detected higher orders structures (i.e., both dimeric and potential aggregation) of both constructs. Since GCSF contains two disulphide bonds and a free cysteine (Cys) residue at amino acid position 17 (Cromwell et al., 2006, Gabrielson et al., 2007, Ribarska et al., 2008), this unpaired Cys 17 in GCSF has been implicated as a significant factor in aggregate formation (Oh-eda et al., 1990, Arakawa et al., 1993, Raso et al., 2005). Therefore, it could be interesting to consider inhibiting the formation of these higher order structures by mutating cysteine-17 to other amino acids, such as alanine (Ala), serine (Ser), glycine (Gly), histidine (His), tyrosine (Tyr), or arginine (Arg), as done by Ishikawa et al., and then comparing these molecules to the original in terms of biological activity, stability as well as dimer and potential aggregate formation.

Other methods to inhibit GCSF aggregation

The addition of sucrose to the final formulation buffer can inhibit conformational fluctuations within the native state of proteins as well as increase the free energy of unfolding (Kim et al., 2003). Moreover, GCSF is resistant to aggregation at low pH as the compact state shows a high degree of α-helicity and being in acid may lock it in a state highly resistant to self-association (Raso et al., 2005). Therefore, the effect of lowering pH (i.e. 4.0) could be investigated. In addition to pH, other factors related to solution conditions can influence whether, or how quickly a protein in solution will aggregate, such as salt concentration, salt type, the type and amount of osmolytes present (Wang, 2005, Roberts, 2014), which all need to be assessed carefully.

Prolonging cell culture viability and limiting apoptotic cell death

During large scale production of GCSF-W104-GHBP, we faced some difficulties to maintain cell viabilities for longer time periods. It would be helpful to try one or two methods to extend cell lifetimes and inhibit or slow the onset of cell death, including

overexpression of anti-apoptosis genes (i.e. Bcl-2 and BclxL) (Mastrangelo et al., 2000, Laken and Leonard, 2001), expression of X-linked inhibitor of apoptosis (XIAP) (Sauerwald et al., 2003), co-expression of p27, a cyclin-dependent kinase inhibitor protein (Fussenegger et al., 1998, Kaufmann et al., 2001), media supplementation with suramin (Zanghi et al., 2000), insulin-like growth factor-I, transferrin (Sunstrom et al., 2000), valproic acid (Backliwal et al., 2008b, Yang et al., 2014), glutamine (Sanfeliu and Stephanopoulos, 1999), threonine, asparagine, glycine or glycine betaine (deZengotita et al., 2002). Another important factor to extend cell viability and productivity, which is simple to carry out and worth trying, is lowering the culture temperature (i.e. 30-33°C) (Kaufmann et al., 2001, Yoon et al., 2003). However, the easiest ways that we have found in the lab to maintain cell viabilities for a longer time and thus increase productivity are by adding valproic acid and/or lowering the temperature.

Histidine switching

Sarkar and his colleagues have successfully demonstrated rational cytokine design, using GCSF, for increased half-life and enhanced potency by histidine substitution. This method reduced receptor binding affinity in intracellular endosomal compartments by switching protonation states between the cell surface and endosomal pH, this, in turn, leads to increased recycling in the ligand-sorting process and an extended half-life in the extracellular medium (Sarkar et al., 2002).

Further analysis of *in vivo* study

From the present work, it is expected that further preclinical and animal experiments are necessary to prove the efficacy and safety of GCSF-W104-GHBP. Furthermore, detailed PK characterisations compared with Pegfilgrastim would further advance the alternative clinical application of this compound in neutropenia. For a PD study, in particular, using an automated haematology analyser that is set up for testing animal blood is crucial to obtain precise FBC. In addition, using specific mouse neutrophil markers such as Ly-6G to perform flow cytometry, will allow easier counting of neutrophils and provide more accurate results.

8.2. Conclusion

We successfully expressed GCSF-W104-GHBP fusion protein for the first time. The strategy established earlier in this work suggested the CHO cell line is an efficient host for GCSF-W104-GHBP expression. This study showed that a GCSF fusion with GHBP (GCSF-W104-GHBP) has the promise to extend its *in vivo* half-life beyond that of Filgrastim whilst retaining bioactivity. More importantly, this study provides experimental evidence for the use of GHBP to be applied as a fusion partner to extend the half-life of some other recombinant proteins and peptides in further research.

Appendix

(A)

4A1 nucleic acid sequence of nucleotides:

GCSF in red, upper case, GCSFR in blue, upper case, linker in black, lower case, and signal sequence in purple, lower case; * refers to the stop codon.

atggctggacctgccacccagagccccatgaagctgatggccctgcagctgctgctgtggcacagtgcactctggacagtgcag
gaagcc

ACCCCCCTGGGCCCTGCCAGCTCCCTGCCCCAGAGCTTCCTGCTCAAGTGCTTAGAGC
AAGTGAGGAAGATCCAGGGCGATGGCGCAGCGCTCCAGGAGAAGCTGTGTGCCACCT
ACAAGCTGTGCCACCCCGAGGAGCTGGTGCTGCTCGGACACTCTCTGGGCATCCCCT
GGGCTCCCCTGAGCAGCTGCCCCAGCCAGGCCCTGCAGCTGGCAGGCTGCTTGAGCC
AACTCCATAGCGGCCTTTTCCTCTACCAGGGGCTCCTGCAGGCCCTGGAAGGGATCTC
CCCCGAGTTGGGTCCCACCTTGGACACACTGCAGCTGGACGTCGCCGACTTTGCCACC
ACCATCTGGCAGCAGATGGAAGAACTGGGAATGGCCCCTGCCCTGCAGCCCACCCAG
GGTGCCATGCCGGCCTTCGCCTCTGCTTTCCAGCGCCGGGCAGGAGGGGTCCTGGTT
GCCTCCCATCTGCAGAGCTTCCTGGAGGTGTCGTACCGCGTTCTACGCCACCTTGCCC
AGCCC

ggtggcggaggtagtggtggcggaggtagcggtggcggaggttctggtggcggaggttccggtggcggaggtagtggtggcg
gaggtagt

GAGTGCGGGCACATCAGTGTCTCAGCCCCCATCGTCCACCTGGGGGATCCCATCACA
GCCTCCTGCATCATCAAGCAGAACTGCAGCCATCTGGACCCGGAGCCACAGATTCTGT
GGAGACTGGGAGCAGAGCTTCAGCCCGGGGGCAGGCAGCAGCGTCTGTCTGATGGG
ACCCAGGAATCTATCATCACCCTGCCCCACCTCAACCACACTCAGGCCTTTCTCTCCTG
CTGCCTGAACTGGGGCAACAGCCTGCAGATCCTGGACCAGGTTGAGCTGCGCGCAGG
CTACCCTCCAGCCATACCCCACAACCTCTCCTGCCTCATGAACCTCACAACCAGCAGC
CTCATCTGCCAGTGGGAGCCAGGACCTGAGACCCACCTACCCACCAGCTTCACTCTGA
AGAGTTTCAAGAGCCGGGGCAACTGTCAGACCCAAGGGGACTCCATCCTGGACTGCGT
GCCCAAGGACGGGCAGAGCCACTGCTGCATCCCACGCAAACACCTGCTGTTGTACCA
GAATATGGGCATCTGGGTGCAGGCAGAGAATGCGCTGGGGACCAGCATGTCCCCACA
ACTGTGTCTTGATCCCATGGATGTTGTGAAACTGGAGCCCCCCATGCTGCGGACCATG
GACCCCAGCCCTGAAGCGGCCCCTCCCCAGGCAGGCTGCCTACAGCTGTGCTGGGAG
CCATGGCAGCCAGGCCTGCACATAAATCAGAAGTGTGAGCTGCGCCACAAGCCGCAG
CGTGGAGAAGCCAGCTGGGCACTGGTGGGCCCCCTCCCCTTGGAGGCCCTTCAGTAT
GAGCTCTGCGGGCTCCTCCCAGCCACGGCCTACACCCTGCAGATACGCTGCATCCGC
TGGCCCCTGCCTGGCCACTGGAGCGACTGGAGCCCCAGCCTGGAGCTGAGAACTACC
GAA*

4A1 amino acid sequence of amino acids:

MAGPATQSPMKLMALQLLLWHSALWTVQEATPLGPASSLPQSFLLKCLEQVRKIQGDGAA
LQEKLCATYKLCHPEELVLLGHSLGIPWAPLSSCPSQALQLAGCLSQLHSGLFLYQGLLQAL
EGISPELGPTLDTLQLDVADFATTIWQQMEELGMAPALQPTQGAMPAFASAFQRRAGGVL
VASHLQSFLEVSYRVLRHLAQPGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSECGHI
SVSAPIVHLGDPITASCIIKQNCSHLDPEPQILWRLGAELQPGGRQQRLSDGTQESIITLPHL
NHTQAFLSCCLNWGNSLQILDQVELRAGYPPAIPHNLSCLMNLTTSSLICQWEPGPETHLPT
SFTLKSFKSRGNCQTQGDSILDCVPKDGQSHCCIPRKHLLLYQNMGIWVQAENALGTSMS
PQLCLDPMDVVKLEPPMLRTMDPSPEAAPPQAGCLQLCWEPWQPGLHINQKCELRHKPQ
RGEASWALVGPLPLEALQYELCGLLPATAYTLQIRCIRWPLPGHWSDWSPSLELRTTE*

(B)

4D1 nucleic acid sequence of nucleotides:

GCSF in red, upper case, GCSFR in blue, upper case, linker in black, lower case, and signal sequence in purple, lower case; * refers to the stop codon.

atggcaaggctgggaaactgcagcctgacttgggctgccctgatcatcctgctgctccccggaagtctggag

GAGTGCGGGCACATCAGTGTCTCAGCCCCCATCGTCCACCTGGGGGATCCCATCACA
GCCTCCTGCATCATCAAGCAGAACTGCAGCCATCTGGACCCGGAGCCACAGATTCTGT
GGAGACTGGGAGCAGAGCTTCAGCCCGGGGGCAGGCAGCAGCGTCTGTCTGATGGG
ACCCAGGAATCTATCATCACCCTGCCCCACCTCAACCACACTCAGGCCTTTCTCTCCTG
CTGCCTGAACTGGGGCAACAGCCTGCAGATCCTGGACCAGGTTGAGCTGCGCGCAGG
CTACCCTCCAGCCATACCCCACAACCTCTCCTGCCTCATGAACCTCACAACCAGCAGC
CTCATCTGCCAGTGGGAGCCAGGACCTGAGACCCACCTACCCACCAGCTTCACTCTGA
AGAGTTTCAAGAGCCGGGGCAACTGTCAGACCCAAGGGGACTCCATCCTGGACTGCGT
GCCCAAGGACGGGCAGAGCCACTGCTGCATCCCACGCAAACACCTGCTGTTGTACCA
GAATATGGGCATCTGGGTGCAGGCAGAGAATGCGCTGGGGACCAGCATGTCCCCACA
ACTGTGTCTTGATCCCATGGATGTTGTGAAACTGGAGCCCCCCATGCTGCGGACCATG
GACCCCAGCCCTGAAGCGGCCCCTCCCCAGGCAGGCTGCCTACAGCTGTGCTGGGAG
CCATGGCAGCCAGGCCTGCACATAAATCAGAAGTGTGAGCTGCGCCACAAGCCGCAG
CGTGGAGAAGCCAGCTGGGCACTGGTGGGCCCCCTCCCCTTGGAGGCCCTTCAGTAT
GAGCTCTGCGGGCTCCTCCCAGCCACGGCCTACACCCTGCAGATACGCTGCATCCGC
TGGCCCCTGCCTGGCCACTGGAGCGACTGGAGCCCCAGCCTGGAGCTGAGAACTACC
GAA

ggtggcggaggtagtggtggcggaggtagcggtggcggaggttctggtggcggaggttccggtggcggaggtagtggtggcg
gaggtagt

ACCCCCCTGGGCCCTGCCAGCTCCCTGCCCCAGAGCTTCCTGCTCAAGTGCTTAGAGC
AAGTGAGGAAGATCCAGGGCGATGGCGCAGCGCTCCAGGAGAAGCTGTGTGCCACCT
ACAAGCTGTGCCACCCCGAGGAGCTGGTGCTGCTCGGACACTCTCTGGGCATCCCCT
GGGCTCCCCTGAGCAGCTGCCCCAGCCAGGCCCTGCAGCTGGCAGGCTGCTTGAGCC
AACTCCATAGCGGCCTTTTCCTCTACCAGGGGCTCCTGCAGGCCCTGGAAGGGATCTC
CCCCGAGTTGGGTCCCACCTTGGACACACTGCAGCTGGACGTCGCCGACTTTGCCACC
ACCATCTGGCAGCAGATGGAAGAACTGGGAATGGCCCCTGCCCTGCAGCCCACCCAG
GGTGCCATGCCGGCCTTCGCCTCTGCTTTCCAGCGCCGGGCAGGAGGGGTCCTGGTT
GCCTCCCATCTGCAGAGCTTCCTGGAGGTGTCGTACCGCGTTCTACGCCACCTTGCCC
AGCCC*

4D1 amino acid sequence of amino acids:

MARLGNCSLTWAALIILLLPGSLEECGHISVSAPIVHLGDPITASCIIKQNCSHLDPEPQILWRL
GAELQPGGRQQRLSDGTQESIITLPHLNHTQAFLSCCLNWGNSLQILDQVELRAGYPPAIPH
NLSCLMNLTTSSLICQWEPGPETHLPTSFTLKSFKSRGNCQTQGDSILDCVPKDGQSHCCI
PRKHLLLYQNMGIWVQAENALGTSMSPQLCLDPMDVVKLEPPMLRTMDPSPEAAPPQAG
CLQLCWEPWQPGLHINQKCELRHKPQRGEASWALVGPLPLEALQYELCGLLPATAYTLQIR
CIRWPLPGHWSDWSPSLELRTTEGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSTPLG
PASSLPQSFLLKCLEQVRKIQGDGAALQEKLCATYKLCHPEELVLLGHSLGIPWAPLSSCPS
QALQLAGCLSQLHSGLFLYQGLLQALEGISPELGPTLDTLQLDVADFATTIWQQMEELGMA
PALQPTQGAMPAFASAFQRRAGGVLVASHLQSFLEVSYRVLRHLAQP*

(C)

pSecTag leptin-GHBP-His nucleic acid sequence of nucleotides:

Leptin in green, upper case, GHBP in pink, upper case, linker in black, lower case, and signal sequence in purple, lower case; * refers to the stop codon.

atgcattggggaaccctgtgcggattcttgtggctttggccctatcttttctatgtccaagct
GTGCCCATCCAAAAAGTCCAAGATGACACCAAAACCCTCATCAAGACAATTGTCACCAG
GATCAATGACATTTCACACACGCAGTCAGTCTCCTCCAAACAGAAAGTCACCGGTTTGG
ACTTCATTCCTGGGCTCCACCCCATCCTGACCTTATCCAAGATGGACCAGACACTGGCA
GTCTACCAACAGATCCTCACCAGTATGCCTTCCAGAAACGTGATCCAAATATCCAACGA
CCTGGAGAACCTCCGGGATCTTCTTCACGTGCTGGCCTTCTCTAAGAGCTGCCACTTG
CCCTGGGCCAGTGGCCTGGAGACCTTGGACAGCCTGGGGGGTGTCCTGGAAGCTTCA
GGCTACTCCACAGAGGTGGTGGCCCTGAGCAGGCTGCAGGGGTCTCTGCAGGACATG
CTGTGGCAGCTGGACCTCAGCCCTGGGTGC
*g*gcggccgcggtggcggaggtagtggtggcggaggtagcggtggcggaggttctggtggcggaggttccgaattc

TTTTCTGGAAGTGAGGCCACAGCAGCTATCCTTAGCAGAGCACCCTGGAGTCTGCAAA
GTGTTAATCCAGGCCTAAAGACAAATTCTTCTAAGGAGCCTAAATTCACCAAGTGCCGT
TCACCTGAGCGAGAGACTTTTTCATGCCACTGGACAGATGAGGTTCATCATGGTACAAA
GAACCTAGGACCCATACAGCTGTTCTATACCAGAAGGAACACTCAAGAATGGACTCAAG
AATGGAAAGAATGCCCTGATTATGTTTCTGCTGGGGAAAACAGCTGTTACTTTAATTCAT
CGTTTACCTCCATCTGGATACCTTATTGTATCAAGCTAACTAGCAATGGTGGTACAGTG
GATGAAAAGTGTTTCTCTGTTGATGAAATAGTGCAACCAGATCCACCCATTGCCCTCAA
CTGGACTTTACTGAACGTCAGTTTAACTGGGATTCATGCAGATATCCAAGTGAGATGGG
AAGCACCACGCAATGCAGATATTCAGAAAGGATGGATGGTTCTGGAGTATGAACTTCAA
TACAAAGAAGTAAATGAAACTAAATGGAAAATGATGGACCCTATATTGACAACATCAGTT
CCAGTGTACTCATTGAAAGTGGATAAGGAATATGAAGTACGCGTGAGATCCAAACAACG
AAACTCTGGAAATTATGGCGAGTTCAGTGAGGTGCTCTATGTAACACTTCCTCAGATGA
GCCAA*

pSecTag leptin-GHBP-His amino acid sequence of amino acids:
MHWGTLCGFLWLWPYLFYVQAVPIQK V Q D D T K T L I K T I V T R I N D I S H T Q S V S
S K Q K V T G L D F I P G L H P I L T L S K M D Q T L A V Y Q Q I L T S M P S R N V I Q I
S N D L E N L R D L L H V L A F S K S C H L P W A S G L E T L D S L G G V L E A S G Y S
T E V V A L S R L Q G S L Q D M L W Q L D L S P G C G G R G G G G S G G G G S G G
G G S G G G G S E F F S G S E A T A A I L S R A P W S L Q S V N P G L K T N S S K E P
K F T K C R S P E R E T F S C H W T D E V H H G T K N L G P I QLFYTRRNT Q E W T Q
E W K E C P D Y V S A G E N S C Y F N S S F T S I W I P Y C I K L T S N G G T V D E K C
F S V D E I V Q P D P P I A L N W T L L N V S L T G I H A D I Q V R W E A P R N A D I Q K
G W M V L E Y E L Q Y K E V N E T K W K M M D P I L T T S V P V Y S L K V D K E Y E V
R V R S K Q R N S G N Y G E F S E V L Y V T L P Q M SQKLFDTG*

(D)

pSecTag GCSF-GHBP-Hist nucleic acid sequence of nucleotides:

G-CSF in red, upper case, GHBP in pink, upper case, linker in black, lower case, and signal sequence in purple, lower case; * refers to the stop codon. The AvrII and EcoRV sites (CCTAGG & GATATC, respectively) are shown in bold and underlined.

atggctggacctgccacccagagccccatgaagctgatggccctgcagctgctgctgtggcacagtgcactctggacagtgcag
gaagcc
ACCCCCCTGGGCCCTGCCAGCTCCCTGCCCCAGAGCTTCCTGCTCAAGTGCTTAGAGC
AAGTGAGGAAGATCCAGGGCGATGGCGCAGCGCTCCAGGAGAAGCTGTGTGCCACCT
ACAAGCTGTGCCACCCCGAGGAGCTGGTGCTGCTCGGACACTCTCTGGGCATCCCCT
GGGCTCCCCTGAGCAGCTGCCCCAGCCAGGCCCTGCAGCTGGCAGGCTGCTTGAGCC
AACTCCATAGCGGCCTTTTCCTCTACCAGGGGCTCCTGCAGGCCCTGGAAGGGATCTC
CCCCGAGTTGGGTCCCACCTTGGACACACTGCAGCTGGACGTCGCCGACTTTGCCACC
ACCATCTGGCAGCAGATGGAAGAACTGGGAATGGCCCCTGCCCTGCAGCCCACCCAG
GGTGCCATGCCGGCCTTCGCCTCTGCTTTCCAGCGCCGGGCAGGAGGGGTCCTGGTT
GCCTCCCATCTGCAGAGCTTCCTGGAGGTGTCGTACCGCGTTCTACGCCACCTTGCCC
AGCCC
ggcggccgcggtggcggaggtagtggtggcggaggtagcggtggcggaggttctggtggcggaggttccgaattc

TTTTCTGGAAGTGAGGCCACAGCAGCTATCCTTAGCAGAGCACCCTGGAGTCTGCAAA
GTGTTAATCCAGGCCTAAAGACAAATTCTTCTAAGGAGCCTAAATTCACCAAGTGCCGT
TCACCTGAGCGAGAGACTTTTTCATGCCACTGGACAGATGAGGTTCATCATGGTACAAA
GAA**CCTAGG**ACCCATACAGCTGTTCTATACCAGAAGGAACACTCAAGAATGGACTCAAG
AATGGAAAGAATGCCCTGATTATGTTTCTGCTGGGGAAAACAGCTGTTACTTTAATTCAT
CGTTTACCTCCATCTGGATACCTTATTGTATCAAGCTAACTAGCAATGGTGGTACAGTG
GATGAAAAGTGTTTCTCTGTTGATGAAATAGTGCAACCAGATCCACCCATTGCCCTCAA
CTGGACTTTACTGAACGTCAGTTTAACTGGGATTCATGCA**GATATC**CAAGTGAGATGGG
AAGCACCACGCAATGCAGATATTCAGAAAGGATGGATGGTTCTGGAGTATGAACTTCAA
TACAAAGAAGTAAATGAAACTAAATGGAAAATGATGGACCCTATATTGACAACATCAGTT
CCAGTGTACTCATTGAAAGTGGATAAGGAATATGAAGTACGCGTGAGATCCAAACAACG
AAACTCTGGAAATTATGGCGAGTTCAGTGAGGTGCTCTATGTAACACTTCCTCAGATGA
GCCAA*

pSecTag GCSF-GHBP-Hist amino acid sequence of amino acids:

M A G P A T Q S P M K L M A L Q L L L W H S A L W T V Q E A T P L G P A S S L P Q S F
L L K C L E Q V R K I Q G D G A A L Q E K L C A T Y K L C H P E E L V L L G H S L G I P
W A P L S S C P S Q A L Q L A G C L S Q L H S G L F L Y Q G L L Q A L E G I S P E L G P
T L D T L Q L D V A D F A T T I W Q Q M E E L G M A P A L Q P T Q G A M P A F A S A F
Q R R A G G V L V A S H L Q S F L E V S Y R V L R H L A Q P G G R G G G G S G G G G
S G G G G S G G G G S E F F S G S E A T A A I L S R A P W S L Q S V N P G L K T N S S
K E P K F T K C R S P E R E T F S C H W T D E V H H G T K N L G P I Q L F Y T R R N T
Q E W T Q E W K E C P D Y V S A G E N S C Y F N S S F T S I W I P Y C I K L T S N G G
T V D E K C F S V D E I V Q P D P P I A L N W T L L N V S L T G I H A D I Q V R W E A P
R N A D I Q K G W M V L E Y E L Q Y K E V N E T K W K M M D P I L T T S V P V Y S L K V
D K E Y E V R V R S K Q R N S G N Y G E F S E V L Y V T L P Q M S Q K L F D T G*

(E)

pSecTag GH-GHBP-W104A-His nucleic acid sequence of nucleotides:

GH in green, upper case, GHBP in pink, upper case, linker in black, lower case, and signal sequence in purple, lower case; * refers to the stop codon. The AvrII and EcoRV sites (CCTAGG & GATATC, respectively) are shown in bold and underlined. W104A mutation in brown, lower case.

atgcattggggaaccctgtgcggattcttgtggctttggccctatcttttctatgtccaagct
GTGCCCATCCAAAAAGTCCAAGATGACACCAAAACCCTCATCAAGACAATTGTCACCAG
GATCAATGACATTTCACACACGCAGTCAGTCTCCTCCAAACAGAAAGTCACCGGTTTGG
ACTTCATTCCTGGGCTCCACCCCATCCTGACCTTATCCAAGATGGACCAGACACTGGCA
GTCTACCAACAGATCCTCACCAGTATGCCTTCCAGAAACGTGATCCAAATATCCAACGA
CCTGGAGAACCTCCGGGATCTTCTTCACGTGCTGGCCTTCTCTAAGAGCTGCCACTTG
CCCTGGGCCAGTGGCCTGGAGACCTTGGACAGCCTGGGGGGTGTCCTGGAAGCTTCA
GGCTACTCCACAGAGGTGGTGGCCCTGAGCAGGCTGCAGGGGTCTCTGCAGGACATG
CTGTGGCAGCTGGACCTCAGCCCTGGGTGC
ggcggccgcggtggcggaggtagtggtggcggaggtagcggtggcggaggttctggtggcggaggttccgaattc

TTTTCTGGAAGTGAGGCCACAGCAGCTATCCTTAGCAGAGCACCCTGGAGTCTGCAAA
GTGTTAATCCAGGCCTAAAGACAAATTCTTCTAAGGAGCCTAAATTCACCAAGTGCCGT
TCACCTGAGCGAGAGACTTTTTCATGCCACTGGACAGATGAGGTTCATCATGGTACAAA
GAA**CCTAGG**ACCCATACAGCTGTTCTATACCAGAAGGAACACTCAAGAATGGACTCAAG
AATGGAAAGAATGCCCTGATTATGTTTCTGCTGGGGAAAACAGCTGTTACTTTAATTCAT
CGTTTACCTCCATC**gca**ATACCTTATTGTATCAAGCTAACTAGCAATGGTGGTACAGTGG
ATGAAAAGTGTTTCTCTGTTGATGAAATAGTGCAACCAGATCCACCCATTGCCCTCAACT
GGACTTTACTGAACGTCAGTTTAACTGGGATTCATGCA**GATATC**CAAGTGAGATGGGAA
GCACCACGCAATGCAGATATTCAGAAAGGATGGATGGTTCTGGAGTATGAACTTCAATA
CAAAGAAGTAAATGAAACTAAATGGAAAATGATGGACCCTATATTGACAACATCAGTTCC
AGTGTACTCATTGAAAGTGGATAAGGAATATGAAGTGCGTGTGAGATCCAAACAACGAA
ACTCTGGAAATTATGGCGAGTTCAGTGAGGTGCTCTATGTAACACTTCCTCAGATGAGC
CAA*

pSecTag GH-GHBP-W104A-His amino acid sequence of amino acids:

M H W G T L C G F L W L W P Y L F Y V Q A V P I Q K V Q D D T K T L I K T I V T R I N D
I S H T Q S V S S K Q K V T G L D F I P G L H P I L T L S K M D Q T L A V Y Q Q I L T S M P
S R N V I Q I S N D L E N L R D L L H V L A F S K S C H L P W A S G L E T L D S L G G V
L E A S G Y S T E V V A L S R L Q G S L Q D M L W Q L D L S P G C G G R G G G G S G
G G G S G G G G S G G G G S E F F S G S E A T A A I L S R A P W S L Q S V N P G L K
T N S S K E P K F T K C R S P E R E T F S C H W T D E V H H G T K N L G P I Q L F Y T
R R N T Q E W T Q E W K E C P D Y V S A G E N S C Y F N S S F T S I A I P Y C I K L T S
N G G T V D E K C F S V D E I V Q P D P P I A L N W T L L N V S L T G I H A D I Q V R W
E A P R N A D I Q K G W M V L E Y E L Q Y K E V N E T K W K M M D P I L T T S V P V Y
S L K V D K E Y E V R V R S K Q R N S G N Y G E F S E V L Y V T L P Q M S Q K L F D T
G*

(F)

pSecTag GCSF-W104-GHBP-Hist nucleic acid sequence of nucleotides:

GCSF in red, upper case, GHBP in pink, upper case, linker in black, lower case, and signal sequence in purple, lower case; * refers to the stop codon. The AvrII and EcoRV sites (CCTAGG & GATATC, respectively) are shown in bold and underlined. W104A mutation in brown, lower case.

atggctggacctgccacccagagccccatgaagctgatggccctgcagctgctgctgtggcacagtgcactctggacagtgcag
gaagcc

ACCCCCCTGGGCCCTGCCAGCTCCCTGCCCCAGAGCTTCCTGCTCAAGTGCTTAGAGC
AAGTGAGGAAGATCCAGGGCGATGGCGCAGCGCTCCAGGAGAAGCTGTGTGCCACCT
ACAAGCTGTGCCACCCCGAGGAGCTGGTGCTGCTCGGACACTCTCTGGGCATCCCCT
GGGCTCCCCTGAGCAGCTGCCCCAGCCAGGCCCTGCAGCTGGCAGGCTGCTTGAGCC
AACTCCATAGCGGCCTTTTCCTCTACCAGGGGCTCCTGCAGGCCCTGGAAGGGATCTC
CCCCGAGTTGGGTCCCACCTTGGACACACTGCAGCTGGACGTCGCCGACTTTGCCACC
ACCATCTGGCAGCAGATGGAAGAACTGGGAATGGCCCCTGCCCTGCAGCCCACCCAG
GGTGCCATGCCGGCCTTCGCCTCTGCTTTCCAGCGCCGGGCAGGAGGGGTCCTGGTT
GCCTCCCATCTGCAGAGCTTCCTGGAGGTGTCGTACCGCGTTCTACGCCACCTTGCCC
AGCCC
ggcggccgcggtggcggaggtagtggtggcggaggtagcggtggcggaggttctggtggcggaggttccgaattc

TTTTCTGGAAGTGAGGCCACAGCAGCTATCCTTAGCAGAGCACCCTGGAGTCTGCAAA
GTGTTAATCCAGGCCTAAAGACAAATTCTTCTAAGGAGCCTAAATTCACCAAGTGCCGT
TCACCTGAGCGAGAGACTTTTTCATGCCACTGGACAGATGAGGTTCATCATGGTACAAA
GAA**CCTAGG**ACCCATACAGCTGTTCTATACCAGAAGGAACACTCAAGAATGGACTCAAG
AATGGAAAGAATGCCCTGATTATGTTTCTGCTGGGGAAAACAGCTGTTACTTTAATTCAT
CGTTTACCTCCATC**gca**ATACCTTATTGTATCAAGCTAACTAGCAATGGTGGTACAGTGG
ATGAAAAGTGTTTCTCTGTTGATGAAATAGTGCAACCAGATCCACCCATTGCCCTCAACT
GGACTTTACTGAACGTCAGTTTAACTGGGATTCATGCA**GATATC**CAAGTGAGATGGGAA
GCACCACGCAATGCAGATATTCAGAAAGGATGGATGGTTCTGGAGTATGAACTTCAATA
CAAAGAAGTAAATGAAACTAAATGGAAAATGATGGACCCTATATTGACAACATCAGTTCC
AGTGTACTCATTGAAAGTGGATAAGGAATATGAAGTGCGTGTGAGATCCAAACAACGAA
ACTCTGGAAATTATGGCGAGTTCAGTGAGGTGCTCTATGTAACACTTCCTCAGATGAGC
CAA*

pSecTag GCSF-W104-GHBP-Hist amino acid sequence of amino acids:

M A G P A T Q S P M K L M A L Q L L L W H S A L W T V Q E A T P L G P A S S L P Q S F
L L K C L E Q V R K I Q G D G A A L Q E K L C A T Y K L C H P E E L V L L G H S L G I P
W A P L S S C P S Q A L Q L A G C L S Q L H S G L F L Y Q G L L Q A L E G I S P E L G P
T L D T L Q L D V A D F A T T I W Q Q M E E L G M A P A L Q P T Q G A M P A F A S A F
Q R R A G G V L V A S H L Q S F L E V S Y R V L R H L A Q P G G R G G G G S G G G G
S G G G G S G G G G S E F F S G S E A T A A I L S R A P W S L Q S V N P G L K T N S S
K E P K F T K C R S P E R E T F S C H W T D E V H H G T K N L G P I Q L F Y T R R N T
Q E W T Q E W K E C P D Y V S A G E N S C Y F N S S F T S I A I P Y C I K L T S N G G T
V D E K C F S V D E I V Q P D P P I A L N W T L L N V S L T G I H A D I Q V R W E A P R
N A D I Q K G W M V L E Y E L Q Y K E V N E T K W K M M D P I L T T S V P V Y S L K V D
K E Y E V R V R S K Q R N S G N Y G E F S E V L Y V T L P Q M S Q K L F D T G*

References

ABBOUD, M., LAVER, J. & BLAU, C. A. 1998. Granulocytosis causing sickle-cell crisis. *Lancet,* 351**,** 959-959.

ABDOLZADE-BAVIL, A., COOKSEY, B. A., SCHECKERMANN, C., LAMMERICH, A., PUKAC, L., KRASNEY, P., ALLGAIER, H., SHEN, W. D., MULLER, U., LIU, P. & VON KERCZEK, A. 2013. Pegylated versus glycopegylated G-CSFs and their biochemical and physiological properties. *Blood,* 122**,** 4851--4851-.

ABDOLZADE-BAVIL, A., VON KERCZEK, A., COOKSEY, B. A., KAUFMAN, T., KRASNEY, P. A., PUKAC, L., GÖRLACH, M., LAMMERICH, A., SCHECKERMANN, C., ALLGAIER, H., SHEN, W. D. & LIU, P. M. 2016. Differential sensitivity of lipegfilgrastim and pegfilgrastim to neutrophil elastase correlates with differences in clinical pharmacokinetic profile. *The Journal of Clinical Pharmacology,* 56**,** 186-194.

ABEGG, A. L., VICKERY, L. E., BREMER, M. E., DONNELLY, A. M., DOSHI, P. D., EVANS, M. L., THURMAN, T. L., BRAFORD, S. R., CAPARON, M. H., BAUER, S. C., GIRI, J. G., WELPLY, J. K., MCKEARN, J. P. & SMITH, W. G. 2002. The enhanced in vitro hematopoietic activity of leridistim, a chimeric dual G-CSF and IL-3 receptor agonist. *Leukemia,* 16**,** 316-26.

ADUSUMILLI, A., KATTEPOGU, R. R. & KROTHAPALLI, S. R. 2012. The clinical applications of Hematopoietic growth factor - GCSF.

AKBARZADEH, S. 2002. Tyrosine residues of the granulocyte colony-stimulating factor receptor transmit proliferation and differentiation signals in murine bone marrow cells. *Blood,* 99**,** 879-887.

ALVAREZ-RUIZ, S., PEÑAS, P. F., FERNÁNDEZ-HERRERA, J., SÁNCHEZ-PÉREZ, J., FRAGA, J. & GARCÍA-DÍEZ, A. 2004. Maculopapular eruption with enlarged macrophages in eight patients receiving G-CSF or GM-CSF. *Journal of the European Academy of Dermatology and Venereology : JEADV,* 18**,** 310-3.

ANCLIFF, P. J., GALE, R. E., LIESNER, R., HANN, I. & LINCH, D. C. 2003. Long-term follow-up of granulocyte colony-stimulating factor receptor mutations in patients with severe congenital neutropenia: implications for leukaemogenesis and therapy. *British Journal of Haematology,* 120**,** 685-690.

ANDERSEN, D. C. & KRUMMEN, L. 2002. Recombinant protein expression for therapeutic applications. *Current Opinion Biotechnology,* 13**,** 117-23.

ARAKAWA, T., PRESTRELSKI, S. J., NARHI, L. O., BOONE, T. C. & KENNEY, W. C. 1993. Cysteine 17 of recombinant human granulocyte-colony stimulating factor is partially solvent-exposed. *Journal of Protein Chemistry,* 12**,** 525-31.

ARDEN, N. & BETENBAUGH, M. J. 2004. Life and death in mammalian cell culture: strategies for apoptosis inhibition. *Trends Biotechnology,* 22**,** 174-80.

ARMSTRONG, J. K. 2009. The occurrence, induction, specificity and potential effect of antibodies against poly(ethylene glycol). *In:* VERONESE, F. M. (ed.) *PEGylated Protein Drugs: Basic Science and Clinical Applications.* Basel: Birkhäuser Basel.

ARMSTRONG, J. K., HEMPEL, G., KOLING, S., CHAN, L. S., FISHER, T., MEISELMAN, H. J. & GARRATTY, G. 2007. Antibody against poly(ethylene glycol) adversely affects PEG-asparaginase therapy in acute lymphoblastic leukemia patients. *Cancer,* 110**,** 103-111.

ARVEDSON, T., O'KELLY, J. & YANG, B. B. 2015. Design rationale and development approach for pegfilgrastim as a long-acting granulocyte colony-stimulating factor. *BioDrugs,* 29**,** 185-98.

ARVEDSON, T. L. & GIFFIN, M. J. 2012. Structural Biology of G-CSF and Its Receptor. *In:* MOLINEUX, G., FOOTE, M. & ARVEDSON, T. (eds.) *Twenty years of G-CSF: clinical and nonclinical discoveries.* Basel: Springer Basel.

ATAERGIN, S., ARPACI, F., TURAN, M., SOLCHAGA, L., CETIN, T., OZTURK, M., OZET, A., KOMURCU, S. & OZTURK, B. 2008. Reduced dose of lenograstim is as efficacious as standard dose of filgrastim for peripheral blood stem cell mobilization and transplantation: a randomized study in patients undergoing autologous peripheral stem cell transplantation. *American journal of hematology,* 83**,** 644-8.

AVALOS, B. R. 1996. Molecular analysis of the granulocyte colony-stimulating factor receptor. *Blood,* 88**,** 761-77.

AWAYA, N., UCHIDA, H., MIYAKAWA, Y., KINJO, K., MATSUSHITA, H., NAKAJIMA, H., IKEDA, Y. & KIZAKI, M. 2002. Novel variant isoform of G-CSF receptor involved in induction of proliferation of FDCP-2 cells: relevance to the pathogenesis of myelodysplastic syndrome. *Journal of cellular physiology,* 191**,** 327-35.

AZOULAY, E. 2001. Granulocyte colony-stimulating factor or neutrophil-induced pulmonary toxicity: myth or reality? *. *CHEST Journal,* 120**,** 1695-1695.

BACKLIWAL, G., HILDINGER, M., CHENUET, S., WULHFARD, S., DE JESUS, M. & WURM, F. M. 2008a. Rational vector design and multi-pathway modulation of HEK 293E cells yield recombinant antibody titers exceeding 1 g/l by transient transfection under serum-free conditions. *Nucleic Acids Research,* 36**,** e96-e96.

BACKLIWAL, G., HILDINGER, M., KUETTEL, I., DELEGRANGE, F., HACKER, D. L. & WURM, F. M. 2008b. Valproic acid: A viable alternative to sodium butyrate for enhancing protein expression in mammalian cell cultures. *Biotechnology and Bioengineering,* 101**,** 182-189.

BAJRAMI, B., ZHU, H., KWAK, H. J., MONDAL, S., HOU, Q., GENG, G., KARATEPE, K., ZHANG, Y. C., NOMBELA-ARRIETA, C., PARK, S. Y., LOISON, F., SAKAI, J., XU, Y., SILBERSTEIN, L. E. & LUO, H. R. 2016. G-CSF maintains controlled neutrophil mobilization during acute inflammation by negatively regulating CXCR2 signaling. *The Journal of Experimental Medicine,* 213**,** 1999-2018.

BARTH, E., FISCHER, G., SCHNEIDER, E. M., MOLDAWER, L. L., GEORGIEFF, M. & WEISS, M. 2002. Peaks of endogenous G-CSF serum concentrations are followed by an increase in respiratory burst activity of granulocytes in patients with septic shock. *Cytokine,* 17**,** 275-84.

BAUMANN, I., TESTA, N. G., LANGE, C., DE WYNTER, E., LUFT, T., DEXTER, T. M., VAN HOEF, M. E. H. M. & HOWELL, A. 1993. Haemopoietic cells mobilised into the circulation by lenograstim as alternative to bone marrow for allogeneic transplants. *The Lancet,* 341**,** 369.

BEEKMAN, R. & TOUW, I. P. 2010. G-CSF and its receptor in myeloid malignancy. *Blood,* 115**,** 5131-5136.

BENDALL, L. J. & BRADSTOCK, K. F. 2014. G-CSF: From granulopoietic stimulant to bone marrow stem cell mobilizing agent. *Cytokine Growth Factor Rev,* 25**,** 355-67.

BÖHME M1, S. D., RADKE J, MORENZ J, WEISE W 1994. Effect of granulocyte colony stimulating factor (G-CSF) on peripheral blood leukocytes and lymphocytes in patients with chemotherapy-induced leukopenia. *Geburtshilfe Frauenheilkd,* 54**,** 670-4.

BONDOS, S. E. & BICKNELL, A. 2003. Detection and prevention of protein aggregation before, during, and after purification. *Analytical Biochemistry,* 316**,** 223-31.

BONEBERG, E.-M., HARENG, L., GANTNER, F., WENDEL, A. & HARTUNG, T. 2000. Human monocytes express functional receptors for granulocyte colony- stimulating factor that mediate suppression of monokines and interferon-gamma. *Blood,* 95**,** 270-276.

BONIG, H., SILBERMANN, S., WELLER, S., KIRSCHKE, R., KORHOLZ, D., JANSSEN, G., GOBEL, U. & NURNBERGER, W. 2001. Glycosylated vs non-glycosylated granulocyte colony-stimulating factor (G-CSF)--results of a prospective randomised monocentre study. *Bone Marrow Transplant,* 28**,** 259-64.

BORNHORST, J. A. & FALKE, J. J. 2000. [16] Purification of proteins using polyhistidine affinity tags. *Methods in enzymology,* 326**,** 245-254.

BOULAY, J.-L., O'SHEA, J. J. & PAUL, W. E. 2003. Molecular phylogeny within type i cytokines and their cognate receptors. *Immunity,* 19**,** 159-163.

BOWEN, S., TARE, N., INOUE, T., YAMASAKI, M., OKABE, M., HORII, I. & ELIASON, J. F. 1999. Relationship between molecular mass and duration of activity of polyethylene glycol conjugated granulocyte colony-stimulating factor mutein. *Experimental Hematology,* 27**,** 425-432.

BRADFORD, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry,* 72**,** 248-54.

BRADLEY, T. R. & METCALF, D. 1966. The growth of mouse bone marrow cells in vitro. *Australian Journal of Experimental Biology and Medical Science,* 44**,** 287-300.

BRONCHUD, M. H., POTTER, M. R., MORGENSTERN, G., BLASCO, M. J., SCARFFE, J. H., THATCHER, N., CROWTHER, D., SOUZA, L. M., ALTON, N. K. & TESTA, N. G. 1988. In vitro and in vivo analysis of the effects of recombinant human granulocyte colony-stimulating factor in patients. *British Journal of Cancer,* 58**,** 64-69.

BUCHNER, A., LAMMERICH, A., ABDOLZADE-BAVIL, A., MÜLLER, U. & BIAS, P. 2014. Lipegfilgrastim: pharmacodynamics and pharmacokinetics for body-weight-adjusted and 6 mg fixed doses in two randomized studies in healthy volunteers. *Current Medical Research and Opinion,* 30**,** 2523-2533.

BURGESS, A. W., CAMAKARIS, J. & METCALF, D. 1977. Purification and properties of colony-stimulating factor from mouse lung-conditioned medium. *The Journal of Biological Chemistry,* 252**,** 1998-2003.

BUSSOLINO, F., WANG, J. M., DEFILIPPI, P., TURRINI, F., SANAVIO, F., EDGELL, C. J., AGLIETTA, M., ARESE, P. & MANTOVANI, A. 1989. Granulocyte- and granulocyte-macrophage-colony stimulating factors induce human endothelial cells to migrate and proliferate. *Nature,* 337**,** 471-3.

CALHOUN, D. A., DONNELLY, W. H., DU, Y., DAME, J. B., LI, Y. & CHRISTENSEN, R. D. 1999. Distribution of granulocyte colony-stimulating factor (G-CSF) and G-CSF-receptor mRNA and protein in the human fetus. *Pediatric research,* 46**,** 333-8.

CARPENTER, J. F., MANNING, M. C. & RANDOLPH, T. W. 2002. Long-term storage of proteins. *Current Protocols in Protein Science,* Chapter 4**,** Unit 4.6.

CARTER, P. J. 2011. Introduction to current and future protein therapeutics: a protein engineering perspective. *Experimental cell research,* 317**,** 1261-9.

CAVAZZANA-CALVO, M., ANDRE-SCHMUTZ, I. & FISCHER, A. 2013. Haematopoietic stem cell transplantation for SCID patients: where do we stand? *British Journal of Haematology,* 160**,** 146-52.

CAWLEY, P., WILKINSON, I. & ROSS, R. J. 2013. Developing long-acting growth hormone formulations. *Clinical endocrinology,* 79**,** 305-9.

CEBON, J., NICOLA, N., WARD, M., GARDNER, I., DEMPSEY, P., LAYTON, J., DUHRSEN, U., BURGESS, A. W., NICE, E. & MORSTYN, G. 1990. Granulocyte-macrophage colony stimulating factor from human lymphocytes. The effect of glycosylation on receptor binding and biological activity. *Journal of Biological Chemistry,* 265**,** 4483-91.

CHAGA, G. S. 2001. Twenty-five years of immobilized metal ion affinity chromatography: past, present and future. *Journal of Biochemical and Biophysical Methods,* 49**,** 313-34.

CHI, E. Y., KRISHNAN, S., KENDRICK, B. S., CHANG, B. S., CARPENTER, J. F. & RANDOLPH, T. W. 2003. Roles of conformational stability and colloidal stability in the aggregation of recombinant human granulocyte colony-stimulating factor. *Protein Science,* 12**,** 903-13.

CLARK, O. A. C., LYMAN, G. H., CASTRO, A. A., CLARK, L. G. O. & DJULBEGOVIC, B. 2005. Colony-stimulating factors for chemotherapy-induced febrile neutropenia: a meta-analysis of randomized controlled trials. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology,* 23**,** 4198-214.

COLOTTA, F., RE, F., POLENTARUTTI, N., SOZZANI, S. & MANTOVANI, A. 1992. Modulation of granulocyte survival and programmed cell death by cytokines and bacterial products. *Blood,* 80**,** 2012-20.

COOPER, K. L., MADAN, J., WHYTE, S., STEVENSON, M. D. & AKEHURST, R. L. 2011. Granulocyte colony-stimulating factors for febrile neutropenia prophylaxis following chemotherapy: systematic review and meta-analysis. *BMC cancer,* 11**,** 404-404.

CORY, A. H., OWEN, T. C., BARLTROP, J. A. & CORY, J. G. 1991. Use of an aqueous soluble tetrazolium/formazan assay for cell growth assays in culture. *Cancer communications,* 3**,** 207-12.

COSTA, S., ALMEIDA, A., CASTRO, A. & DOMINGUES, L. 2014. Fusion tags for protein solubility, purification and immunogenicity in Escherichia coli: the novel Fh8 system. *Frontiers in microbiology,* 5**,** 63.

COUDERC, L. J., STELIANIDES, S., FRACHON, I., STERN, M., EPARDEAU, B., BAUMELOU, E., CAUBARRERE, I. & HERMINE, O. 1999. Pulmonary toxicity of chemotherapy and G/GM-CSF: a report of five cases. *Respiratory Medicine,* 93**,** 65-68.

COX, G. N., SMITH, D. J., CARLSON, S. J., BENDELE, A. M., CHLIPALA, E. A. & DOHERTY, D. H. 2004. Enhanced circulating half-life and hematopoietic properties of a human granulocyte colony-stimulating factor/immunoglobulin fusion protein. *Experimental Hematology,* 32**,** 441-9.

CROMWELL, M. E., HILARIO, E. & JACOBSON, F. 2006. Protein aggregation and bioprocessing. *Journal of the American Association of Pharmaceutical Scientists,* 8**,** E572-9.

D'SOUZA, A., JAIYESIMI, I., TRAINOR, L. & VENUTURUMILI, P. 2008. Granulocyte colony-stimulating factor administration: adverse events. *Transfusion medicine reviews,* 22**,** 280-90.

DALE, D. C. 2012. Twenty years of the colony-stimulating factors. *Current Opinion in Hematology,* 19**,** 1-2.

DALE, D. C., BONILLA, M. A., DAVIS, M. W., NAKANISHI, A. M., HAMMOND, W. P., KURTZBERG, J., WANG, W., JAKUBOWSKI, A., WINTON, E. & LALEZARI, P. 1993. A randomized controlled phase III trial of recombinant human granulocyte colony-stimulating factor (filgrastim) for treatment of severe chronic neutropenia. *Blood,* 81**,** 2496-2502.

DALE, D. C., COTTLE, T. E., FIER, C. J., BOLYARD, A. A., BONILLA, M. A., BOXER, L. A., CHAM, B., FREEDMAN, M. H., KANNOURAKIS, G., KINSEY, S. E., DAVIS, R., SCARLATA, D., SCHWINZER, B., ZEIDLER, C. & WELTE, K. 2003. Severe chronic neutropenia: treatment and follow-up of patients in the Severe Chronic Neutropenia International Registry. *American journal of hematology,* 72**,** 82-93.

DAMIANI, R., OLIVEIRA, J. E., VORAUER-UHL, K., PERONI, C. N., VIANNA, E. G., BARTOLINI, P. & RIBELA, M. T. 2009. Stable expression of a human-like sialylated recombinant thyrotropin in a Chinese hamster ovary cell line expressing alpha2,6-sialyltransferase. *Protein Expression and Purification,* 67**,** 7-14.

DARIE, C., BOUTALBA, S., FICHTER, P., HURET, J. F., JAILLOT, P., DEPLUS, F., GERENTON, S., ZENONE, T., MOREAU, J. L. & GRAND, A. 2004. [Aortitis after G-CSF injections]. *La Revue de médecine interne / fondée ... par la Société nationale francaise de médecine interne,* 25**,** 225-9.

DE HAAN, G., AUSEMA, A., WILKENS, M., MOLINEUX, G. & DONTJE, B. 2000. Efficient mobilization of haematopoietic progenitors after a single injection of pegylated recombinant human granulocyte colony-stimulating factor in mouse strains with distinct marrow-cell pool sizes. *British Journal of Haematology,* 110**,** 638-46.

DE LA LUZ SIERRA, M., GASPERINI, P., MCCORMICK, P. J., ZHU, J. & TOSATO, G. 2007. Transcription factor Gfi-1 induced by G-CSF is a negative regulator of CXCR4 in myeloid cells. *Blood,* 110**,** 2276-85.

DEJESUS, C. E., EGEN, J., METZGER, M., ALVAREZ, X., COMBS, C. A., MALIDE, D., YU, Z. X., TIAN, X. & DONAHUE, R. E. 2011. Transient neutropenia after granulocyte-colony stimulating factor administration is associated with neutrophil accumulation in pulmonary vasculature. *Experimental Hematology,* 39**,** 142-150.

DELUCA, E., SHERIDAN, W. P., WATSON, D., SZER, J. & BEGLEY, C. G. 1992. Prior chemotherapy does not prevent effective mobilisation by G-CSF of peripheral blood progenitor cells. *British Journal of Cancer,* 66**,** 893-9.

DEMETRI, G. D. & GRIFFIN, J. D. 1991. Granulocyte colony-stimulating factor and its receptor. *Blood,* 78**,** 2791-2808.

DENNING, T. L., WANG, Y.-C., PATEL, S. R., WILLIAMS, I. R. & PULENDRAN, B. 2007. Lamina propria macrophages and dendritic cells differentially induce regulatory and interleukin 17-producing T cell responses. *Nature immunology,* 8**,** 1086-94.

DEREURE, O., BESSIS, D., LAVABRE-BERTRAND, T., EXBRAYAT, C., FEGUEUX, N., BIRON, C. & GUILHOU, J. J. 2000. Thrombotic and necrotizing panniculitis associated with recombinant human granulocyte colony-stimulating factor treatment. *British Journal of Dermatology,* 142**,** 834-836.

DEZENGOTITA, V. M., ABSTON, L. R., SCHMELZER, A. E., SHAW, S. & MILLER, W. M. 2002. Selected amino acids protect hybridoma and CHO cells from elevated carbon dioxide and osmolality. *Biotechnology and Bioengineering,* 78**,** 741-52.

DIEHL, K.-H., HULL, R., MORTON, D., PFISTER, R., RABEMAMPIANINA, Y., SMITH, D., VIDAL, J.-M. & VORSTENBOSCH, C. V. D. 2001. A good practice guide to the administration of substances and removal of blood, including routes and volumes. *Journal of Applied Toxicology,* 21**,** 15-23.

DOEING, D. C., BOROWICZ, J. L. & CROCKETT, E. T. 2003. Gender dimorphism in differential peripheral blood leukocyte counts in mice using cardiac, tail, foot, and saphenous vein puncture methods. *BMC Clinical Pathology,* 3**,** 3.

DONADIEU, J., LEBLANC, T., BADER MEUNIER, B., BARKAOUI, M., FENNETEAU, O., BERTRAND, Y., MAIER-REDELSPERGER, M., MICHEAU, M., STEPHAN, J. L., PHILLIPE, N., BORDIGONI, P., BABIN-BOILLETOT, A., BENSAID, P., MANEL, A. M., VILMER, E., THURET, I., BLANCHE, S., GLUCKMAN, E., FISCHER, A., MECHINAUD, F., JOLY, B., LAMY, T., HERMINE, O., CASSINAT, B., BELLANNÉ-CHANTELOT, C. & CHOMIENNE, C. 2005. Analysis of risk factors for myelodysplasias, leukemias and death from infection among patients with congenital neutropenia. Experience of the French Severe Chronic Neutropenia Study Group. *Haematologica,* 90**,** 45-53.

DONAHUE, R. E., TUSCHONG, L., BAUER, T. R., JR., YAU, Y. Y., LEITMAN, S. F. & HICKSTEIN, D. D. 2011. Leukocyte integrin activation mediates transient neutropenia after G-CSF administration. *Blood,* 118**,** 4209-14.

DONG, F. & LARNER, A. C. 2000. Activation of Akt kinase by granulocyte colony-stimulating factor (G-CSF): evidence for the role of a tyrosine kinase activity distinct from the janus kinases. *Blood,* 95**,** 1656-1662.

DONG, F., LIU, X., DE KONING, J. P., TOUW, I. P., HENNIGHAUSEN, L., LARNER, A. & GRIMLEY, P. M. 1998. Stimulation of Stat5 by granulocyte colony-stimulating factor (G-CSF) is modulated by two distinct cytoplasmic regions of the G-CSF receptor. *Journal of immunology (Baltimore, Md. : 1950),* 161**,** 6503-9.

DREGER, P., HAFERLACH, T., ECKSTEIN, V., JACOBS, S., SUTTORP, M., LÖUFFLER, H., AND, W. M.-R. & SCHMITZ, N. 1994. G-CSF-mobilized peripheral blood progenitor cells for allogeneic transplantation: safety, kinetics of mobilization, and composition of the graft. *British Journal of Haematology,* 87**,** 609-613.

DRUHAN, L. J., AI, J., MASSULLO, P., KINDWALL-KELLER, T., RANALLI, M. A. & AVALOS, B. R. 2005. Novel mechanism of G-CSF refractoriness in patients with severe congenital neutropenia. *Blood,* 105**,** 584-91.

DUNN, C. J. & GOA, K. L. 2000. Lenograstim: an update of its pharmacological properties and use in chemotherapy-induced neutropenia and related clinical settings. *Drugs,* 59**,** 681-717.

EASH, K. J., GREENBAUM, A. M., GOPALAN, P. K. & LINK, D. C. 2010. CXCR2 and CXCR4 antagonistically regulate neutrophil trafficking from murine bone marrow. *Journal of Clinical Investigation,* 120**,** 2423-31.

EL OURIAGHLI, F., FUJIWARA, H., MELENHORST, J. J., SCONOCCHIA, G., HENSEL, N. & BARRETT, A. J. 2003. Neutrophil elastase enzymatically antagonizes the in vitro action of G-CSF: implications for the regulation of granulopoiesis. *Blood,* 101**,** 1752-1758.

ERNST, T. J., RITCHIE, A. R., DEMETRI, G. D. & GRIFFIN, J. D. 1989. Regulation of granulocyte- and monocyte-colony stimulating factor mRNA levels in human blood monocytes is mediated primarily at a post- transcriptional level. *Journal of Biological Chemistry,* 264**,** 5700-5703.

ESTEY, E. & DÖHNER, H. 2006. Acute myeloid leukaemia. *The Lancet,* 368**,** 1894-1907.

FALKENBURG, J. H., HARRINGTON, M. A., DE PAUS, R. A., WALSH, W. K., DAUB, R., LANDEGENT, J. E. & BROXMEYER, H. E. 1991. Differential transcriptional and posttranscriptional regulation of gene

expression of the colony-stimulating factors by interleukin-1 and fetal bovine serum in murine fibroblasts. *Blood,* 78**,** 658-665.

FAZEKAS DE ST GROTH, S., WEBSTER, R. G. & DATYNER, A. 1963. Two new staining procedures for quantitative estimation of proteins on electrophoretic strips. *Biochimica et Biophysica Acta,* 71**,** 377-91.

FIBBE, W. E., PRUIJT, J. F., VELDERS, G. A., OPDENAKKER, G., VAN KOOYK, Y., FIGDOR, C. G. & WILLEMZE, R. 1999. Biology of IL-8-induced stem cell mobilization. *Annals of the New York Academy of Sciences,* 872**,** 71-82.

FIEVEZ, L., DESMET, C., HENRY, E., PAJAK, B., HEGENBARTH, S., GARZE, V., BEX, F., JASPAR, F., BOUTET, P., GILLET, L., VANDERPLASSCHEN, A., KNOLLE, P. A., LEO, O., MOSER, M., LEKEUX, P. & BUREAU, F. 2007. STAT5 is an ambivalent regulator of neutrophil homeostasis. *Public Library of Science One,* 2**,** e727.

FISHBURN, C. S. 2008. The pharmacology of PEGylation: balancing PD with PK to generate novel therapeutics. *Journal of pharmaceutical sciences,* 97**,** 4167-83.

FORBES, L. V., GALE, R. E., PIZZEY, A., POUWELS, K., NATHWANI, A. & LINCH, D. C. 2002. An activating mutation in the transmembrane domain of the granulocyte colony-stimulating factor receptor in patients with acute myeloid leukemia. *Oncogene,* 21**,** 5981-9.

FORNO, G., BOLLATI FOGOLIN, M., OGGERO, M., KRATJE, R., ETCHEVERRIGARAY, M., CONRADT, H. S. & NIMTZ, M. 2004. N- and O-linked carbohydrates and glycosylation site occupancy in recombinant human granulocyte-macrophage colony-stimulating factor secreted by a Chinese hamster ovary cell line. *European Journal of Biochemistry,* 271**,** 907-19.

FREEDMAN, M. H. & ALTER, B. P. 2002. Risk of myelodysplastic syndrome and acute myeloid leukemia in congenital neutropenias. *Seminars in Hematology,* 39**,** 128-133.

FROKJAER, S. & OTZEN, D. E. 2005. Protein drug stability: a formulation challenge. *Nature Reviews Drug Discovery,* 4**,** 298-306.

FUKUNAGA, R., ISHIZAKA-IKEDA, E., SETO, Y. & NAGATA, S. 1990. Expression cloning of a receptor for murine granulocyte colony-stimulating factor. *Cell,* 61**,** 341-350.

FUSSENEGGER, M., SCHLATTER, S., DATWYLER, D., MAZUR, X. & BAILEY, J. E. 1998. Controlled proliferation by multigene metabolic engineering enhances the productivity of Chinese hamster ovary cells. *Nature Biotechnology,* 16**,** 468-72.

GABRIELSON, J. P., BRADER, M. L., PEKAR, A. H., MATHIS, K. B., WINTER, G., CARPENTER, J. F. & RANDOLPH, T. W. 2007. Quantitation of aggregate levels in a recombinant humanized monoclonal antibody formulation by size-exclusion chromatography, asymmetrical flow field flow fractionation, and sedimentation velocity. *Journal of Pharmaceutical Sciences,* 96**,** 268-79.

GALLUPPI, G. 2001. Integration of pharmacokinetic and pharmacodynamic studies in the discovery, development, and review of protein therapeutic agents: A conference report. *Clinical Pharmacology & Therapeutics,* 69**,** 387-399.

GARAY, R. P., EL-GEWELY, R., ARMSTRONG, J. K., GARRATTY, G. & RICHETTE, P. 2012. Antibodies against polyethylene glycol in healthy subjects and in patients treated with PEG-conjugated agents. *Expert Opinion on Drug Delivery,* 9**,** 1319-1323.

GARAY, R. P. & LABAUNE, J. 2011. Immunogenicity of polyethylene glycol (PEG). *The Open Conference Proceedings Journal,* 2**,** 104-107.

GASCOYNE, R. D., MOSKOWITZ, C. & SHEA, T. C. 2013. Controversies in BMT for Lymphoma. *Biology of Blood and Marrow Transplantation,* 19**,** S26-S32.

GEORGE, S., GEORGE, B. & GORAK, E. 2005. Erythema nodosum secondary to granulocyte colony-stimulating factor in a patient with Hodgkin lymphoma during CD34+ cell mobilization for autologous peripheral blood stem cell transplantation: a dose-mediated effect. *Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation,* 11**,** 816-7.

GERTZ, M. A., LACY, M. Q., BJORNSSON, J. & LITZOW, M. R. 2000. Fatal pulmonary toxicity related to the administration of granulocyte colony-stimulating factor in amyloidosis: a report and review of growth factor-induced pulmonary toxicity. *Journal of hematotherapy & stem cell research,* 9**,** 635-43.

GLADKOV, O., MOISEYENKO, V., BONDARENKO, I. N., SHPARYK, Y., BARASH, S., ADAR, L. & AVISAR, N. 2016. A phase iii study of balugrastim versus pegfilgrastim in breast cancer patients receiving chemotherapy with doxorubicin and docetaxel. *The Oncologist,* 21**,** 7-15.

GLASPY, J., TANG, T., RUTTY, D., YAN, X., BONDARENKO, I. & KRASNOZHON, D. 2014. A phase ii, randomized, multi-centre, open-label, active-controlled, dose-finding trial of F-627 (benefilgrastim) in women with breast cancer receiving myelotoxic chemotherapy. *Blood,* 124**,** 1584-1584.

GLASS, L. F., FOTOPOULOS, T. & MESSINA, J. L. 1996. A generalized cutaneous reaction induced by granulocyte colony-stimulating factor. *Journal of the American Academy of Dermatology,* 34**,** 455-459.

GREGORY, A. D., HOGUE, L. A., FERKOL, T. W. & LINK, D. C. 2007. Regulation of systemic and local neutrophil responses by G-CSF during pulmonary Pseudomonas aeruginosa infection. *Blood,* 109**,** 3235-3243.

GRIGG, A. P., ROBERTS, A. W., RAUNOW, H., HOUGHTON, S., LAYTON, J. E., BOYD, A. W., MCGRATH, K. M. & MAHER, D. 1995. Optimizing dose and scheduling of filgrastim (granulocyte colony-stimulating factor) for mobilization and collection of peripheral blood progenitor cells in normal volunteers. *Blood,* 86**,** 4437-45.

GÜRMAN, G., DILEK, İ., ARSLAN, Ö., ARAT, M., BEKSAÇ, M., İLHAN, O., ÖZCAN, M., AKAN, H., DALVA, K., KONUK, N., UYSAL, A. & KOÇ, H. 2001. The effect of G-CSF on lymphocyte subsets and CD34+ cells in allogeneic stem cell transplantation. *Transfusion and Apheresis Science,* 24**,** 23-28.

HAGHIGHAT, A., WEISS, D., WHALIN, M. K., COWAN, D. P. & TAYLOR, W. R. 2007. Granulocyte colony-stimulating factor and granulocyte macrophage colony-stimulating factor exacerbate atherosclerosis in apolipoprotein E-deficient mice. *Circulation,* 115**,** 2049-54.

HALPERN, W., RICCOBENE, T., AGOSTINI, H., BAKER, K. & STOLOW D, G. M., HIRSCH J, MAHONEY A, CARRELL J, BOYD E, GRZEGORZEWSKI KJ. 2002. Albugranin, a recombinant human granulocyte colony stimulating factor (G-CSF) genetically fused to recombinant human albumin induces prolonged myelopoietic effects in mice and monkeys. *Pharmaceutical Research,* 19**,** 1720-9.

HARADA, M., QIN, Y., TAKANO, H., MINAMINO, T., ZOU, Y., TOKO, H., OHTSUKA, M., MATSUURA, K., SANO, M., NISHI, J.-I., IWANAGA, K., AKAZAWA, H., KUNIEDA, T., ZHU, W., HASEGAWA, H., KUNISADA, K., NAGAI, T., NAKAYA, H., YAMAUCHI-TAKIHARA, K. & KOMURO, I. 2005. G-CSF prevents cardiac remodeling after myocardial infarction by activating the Jak-Stat pathway in cardiomyocytes. *Nature medicine,* 11**,** 305-11.

HARI, P. N. & MCCARTHY, P. L. 2013. Multiple myeloma: future directions in autologous transplantation and novel agents. *Biology of Blood and Marrow Transplantation,* 19**,** S20-S25.

HARRINGTON, L. E., HATTON, R. D., MANGAN, P. R., TURNER, H., MURPHY, T. L., MURPHY, K. M. & WEAVER, C. T. 2005. Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. *Nature immunology,* 6**,** 1123-32.

HASENBERG, A., HASENBERG, M., MANN, L., NEUMANN, F., BORKENSTEIN, L., STECHER, M., KRAUS, A., ENGEL, D. R., KLINGBERG, A., SEDDIGH, P., ABDULLAH, Z., KLEBOW, S., ENGELMANN, S., REINHOLD, A., BRANDAU, S., SEELING, M., WAISMAN, A., SCHRAVEN, B., GOTHERT, J. R., NIMMERJAHN, F. & GUNZER, M. 2015. Catchup: a mouse model for imaging-based tracking and modulation of neutrophil granulocytes. *Nature Methods,* 12**,** 445-452.

HAYAT, S. Q., HEARTH-HOLMES, M. & WOLF, R. E. 1995. Flare of arthritis with successful treatment of Felty's syndrome with granulocyte colony stimulating factor (GCSF). *Clinical Rheumatology,* 14**,** 211-212.

HERMAN, A. C., BOONE, T. C. & LU, H. S. 1996. Characterization, formulation, and stability of Neupogen (Filgrastim), a recombinant human granulocyte-colony stimulating factor. *Pharmaceutical Biotechnology,* 9**,** 303-28.

HERMANS, M. H. A., VAN DE GEIJN, G.-J., ANTONISSEN, C., GITS, J., VAN LEEUWEN, D., WARD, A. C. & TOUW, I. P. 2003. Signaling mechanisms coupled to tyrosines in the granulocyte colony-stimulating factor receptor orchestrate G-CSF-induced expansion of myeloid progenitor cells. *Blood,* 101**,** 2584-90.

HIERHOLZER, C., KELLY, E., LYONS, V., ROEDLING, E., DAVIES, P., BILLIAR, T. R. & TWEARDY, D. J. 1998. G-CSF instillation into rat lungs mediates neutrophil recruitment, pulmonary edema, and hypoxia. *Journal of Leukocyte Biology,* 63**,** 169-174.

HILL, C. P., OSSLUND, T. D. & EISENBERG, D. 1993. The structure of granulocyte-colony-stimulating factor and its relationship to other growth factors. *Proceedings of the National Academy of Sciences,* 90**,** 5167-5171.

HILL, J. M., SYED, M. A., ARAI, A. E., POWELL, T. M., PAUL, J. D., ZALOS, G., READ, E. J., KHUU, H. M., LEITMAN, S. F., HORNE, M., CSAKO, G., DUNBAR, C. E., WACLAWIW, M. A. & CANNON, R. O. 2005. Outcomes and risks of granulocyte colony-stimulating factor in patients with coronary artery disease. *Journal of the American College of Cardiology,* 46**,** 1643-8.

HILTON, D. J., WATOWICH, S. S., KATZ, L. & LODISH, H. F. 1996. Saturation Mutagenesis of the WSXWS Motif of the Erythropoietin Receptor. *Journal of Biological Chemistry,* 271**,** 4699-4708.

HIROKAWA, M., LEE, M., MOTEGI, M. & MIURA, A. B. 1996. Reversible renal impairment during leukocytosis induced by G-CSF in non-Hodgkin's lymphoma. *American Journal of Hematology,* 51**,** 328-329.

HOGGATT, J. & PELUS, L. M. 2014. New G-CSF agonists for neutropenia therapy. *Expert opinion on investigational drugs,* 23**,** 21-35.

HÖGLUND M1, S. B., SIMONSSON B, TÖTTERMAN T, BENGTSSON M. 1996. Dose-dependent mobilisation of haematopoietic progenitor cells in healthy volunteers receiving glycosylated rHuG-CSF. *Bone Marrow Transplant.,* 18**,** 19-27.

HORTNER, M., NIELSCH, U., MAYR, L. M., JOHNSTON, J. A., HEINRICH, P. C. & HAAN, S. 2002. Suppressor of cytokine signaling-3 is recruited to the activated granulocyte-colony stimulating factor receptor and modulates its signal transduction. *The Journal of Immunology,* 169**,** 1219-1227.

HUNTER, M. G. & AVALOS, B. R. 2000. Granulocyte colony-stimulating factor receptor mutations in severe congenital neutropenia transforming to acute myelogenous leukemia confer resistance to apoptosis and enhance cell survival. *Blood,* 95**,** 2132-2137.

HUNTER, M. G., DRUHAN, L. J., MASSULLO, P. R. & AVALOS, B. R. 2003. Proteolytic cleavage of granulocyte colony-stimulating factor and its receptor by neutrophil elastase induces growth inhibition and decreased cell surface expression of the granulocyte colony-stimulating factor receptor. *American Journal of Hematology,* 74**,** 149-155.

HÜTTMANN, A., DÜHRSEN, U., STYPMANN, J., NOPPENEY, R., NÜCKEL, H., NEUMANN, T., GUTERSOHN, A., NIKOL, S. & ERBEL, R. 2006. Granulocyte colony-stimulating factor-induced blood stem cell mobilisation in patients with chronic heart failure--Feasibility, safety and effects on exercise tolerance and cardiac function. *Basic research in cardiology,* 101**,** 78-86.

HÜTTMANN, A., SCHIRSAFI, K., SEEBER, S. & BOJKO, P. 2005. Comparison of lenograstim and filgrastim: effects on blood cell recovery after high-dose chemotherapy and autologous peripheral blood stem cell transplantation. *Journal of Cancer Research and Clinical Oncology,* 131**,** 152-156.

ICHIKAWA, Y., PLUZNIK, D. H. & SACHS, L. 1966. In vitro control of the development of macrophage and granulocyte colonies. *Proceedings of the National Academy of Sciences of the United States of America,* 56**,** 488-95.

IHLE, J. N., KELLER, J., HENDERSON, L., KLEIN, F. & PALASZYNSKI, E. 1982. Procedures for the purification of interleukin 3 to homogeneity. *Journal of immunology (Baltimore, Md. : 1950),* 129**,** 2431-6.

IMAI, N., HIGUCHI, M., KAWAMURA, A., TOMONOH, K., OH-EDA, M., FUJIWARA, M., SHIMONAKA, Y. & OCHI, N. 1990. Physicochemical and biological characterization of asialoerythropoietin. Suppressive effects of sialic acid in the expression of biological activity of human erythropoietin in vitro. *European Journal of Biochemistry,* 194**,** 457-62.

INANO, H., KAMEYAMA, S., YASUI, S. & NAGAI, A. 1998. Granulocyte colony-stimulating factor induces neutrophil sequestration in rabbit lungs. *American journal of respiratory cell and molecular biology,* 19**,** 167-74.

INOKUCHI, R., MANABE, H., OHTA, F., NAKAMURA, K., NAKAJIMA, S. & YAHAGI, N. 2014. Granulocyte colony-stimulating factor-producing lung cancer and acute respiratory distress syndrome. *The clinical respiratory journal***,** 3-5.

ISHIKAWA, M., IIJIMA, H., SATAKE-ISHIKAWA, R., TSUMURA, H., IWAMATSU, A., KADOYA, T., SHIMADA, Y., FUKAMACHI, H., KOBAYASHI, K., MATSUKI, S. & ET AL. 1992. The substitution of cysteine 17 of recombinant human G-CSF with alanine greatly enhanced its stability. *Cell Structure and Function,* 17**,** 61-5.

IVANOV, I. I., MCKENZIE, B. S., ZHOU, L., TADOKORO, C. E., LEPELLEY, A., LAFAILLE, J. J., CUA, D. J. & LITTMAN, D. R. 2006. The orphan nuclear receptor RORgammat directs the differentiation program of proinflammatory IL-17+ T helper cells. *Cell,* 126**,** 1121-33.

JEVSEVAR, S., KUNSTELJ, M. & POREKAR, V. G. 2010. PEGylation of therapeutic proteins. *Biotechnology journal,* 5**,** 113-28.

JIAO, H., BERRADA, K., YANG, W., TABRIZI, M., PLATANIAS, L. C. & YI, T. 1996. Direct association with and dephosphorylation of Jak2 kinase by the SH2-domain-containing protein tyrosine phosphatase SHP-1. *Molecular and Cellular Biology,* 16**,** 6985-6992.

JOHNSTON, E., CRAWFORD, J., BLACKWELL, S., BJURSTROM, T., LOCKBAUM, P., ROSKOS, L., YANG, B.-B., GARDNER, S., MILLER-MESSANA, M. A., SHOEMAKER, D., GARST, J. & SCHWAB, G. 2000. Randomized, Dose-Escalation Study of SD/01 Compared With Daily Filgrastim in Patients Receiving Chemotherapy. *Journal of Clinical Oncology,* 18**,** 2522-2528.

JONSSON, F., MANCARDI, D. A., KITA, Y., KARASUYAMA, H., IANNASCOLI, B., VAN ROOIJEN, N., SHIMIZU, T., DAERON, M. & BRUHNS, P. 2011. Mouse and human neutrophils induce anaphylaxis. *Journal of Clinical Investigation,* 121**,** 1484-96.

JOYCE, A. P., WANG, M., LAWRENCE-HENDERSON, R., FILLIETTAZ, C., LEUNG, S. S., XU, X. & O'HARA, D. M. 2014. One mouse, one pharmacokinetic profile: quantitative whole blood serial sampling for biotherapeutics. *Pharmaceutical Research,* 31**,** 1823-1833.

JUDGE, A., MCCLINTOCK, K., PHELPS, J. R. & MACLACHLAN, I. 2006. Hypersensitivity and loss of disease site targeting caused by antibody responses to PEGylated liposomes. *Molecular Therapy,* 13**,** 328-337.

JUN-ICHI, T., KATSUMI, H., ETSUKO, K., MAKI, N. & ITARY, Y. 1985. Studies on antigenicity of the polyethylene glycol (PEG)-modified uricase. *International Journal of Immunopharmacology,* 7**,** 725-730.

KANG, H.-J., KIM, H.-S., ZHANG, S.-Y., PARK, K.-W., CHO, H.-J., KOO, B.-K., KIM, Y.-J., SOO LEE, D., SOHN, D.-W., HAN, K.-S., OH, B.-H., LEE, M.-M. & PARK, Y.-B. 2004. Effects of intracoronary infusion of peripheral blood stem-cells mobilised with granulocyte-colony stimulating factor on left ventricular systolic function and restenosis after coronary stenting in myocardial infarction: the MAGIC cell randomised clinical. *Lancet,* 363**,** 751-6.

KAUFMANN, H., MAZUR, X., MARONE, R., BAILEY, J. E. & FUSSENEGGER, M. 2001. Comparative analysis of two controlled proliferation strategies regarding product quality, influence on tetracycline-regulated gene expression, and productivity. *Biotechnology and Bioengineering,* 72**,** 592-602.

KAWACHI, Y., WATANABE, A., UCHIDA, T., YOSHIZAWA, K., KUROOKA, N. & SETSU, K. 1996. Acute arterial thrombosis due to platelet aggregation in a patient receiving granulocyte colony-stimulating factor. *British Journal of Haematology,* 94**,** 413-416.

KELLY, S. & WHEATLEY, D. 2009. Prevention of febrile neutropenia: use of granulocyte colony-stimulating factors. *British journal of cancer,* 101 Suppl**,** S6-10.

KENDRICK, T. S., LIPSCOMBE, R. J., RAUSCH, O., NICHOLSON, S. E., LAYTON, J. E., GOLDIE-CREGAN, L. C. & BOGOYEVITCH, M. A. 2004. Contribution of the membrane-distal tyrosine in intracellular signaling by the granulocyte colony-stimulating factor receptor. *The Journal of biological chemistry,* 279**,** 326-40.

KILE, B. T., MASON-GARRISON, C. L. & JUSTICE, M. J. 2003. Sex and strain-related differences in the peripheral blood cell values of inbred mouse strains. *Mammalian Genome,* 14**,** 81-5.

KIM, H. K., DE LA LUZ SIERRA, M., WILLIAMS, C. K., GULINO, A. V. & TOSATO, G. 2006. G-CSF down-regulation of CXCR4 expression identified as a mechanism for mobilization of myeloid cells. *Blood,* 108**,** 812-20.

KIM, Y. S., JONES, L. S., DONG, A., KENDRICK, B. S., CHANG, B. S., MANNING, M. C., RANDOLPH, T. W. & CARPENTER, J. F. 2003. Effects of sucrose on conformational equilibria and fluctuations within the native-state ensemble of proteins. *Protein Science,* 12**,** 1252-61.

KIMPLE, M. E., BRILL, A. L. & PASKER, R. L. 2013. Overview of Affinity Tags for Protein Purification. *Current protocols in protein science / editorial board, John E. Coligan ... [et al.],* 73**,** Unit-9.9.

KIMPLE, M. E. & SONDEK, J. 2004. Overview of affinity tags for protein purification. *Current protocols in protein science,* Chapter 9**,** Unit 9.9.

KNUDSEN, E., IVERSEN, P. O., BØYUM, A., SEIERSTAD, T., NICOLAYSEN, G. & BENESTAD, H. B. 2011. G-CSF enhances the proliferation and mobilization, but not the maturation rate, of murine myeloid cells. *European journal of haematology,* 87**,** 302-11.

KOAY, D. C., NGUYEN, T.-H. & SARTORELLI, A. C. 2002. Distinct region of the granulocyte colony-stimulating factor receptor mediates proliferative signaling through activation of Janus kinase 2 and p44/42 mitogen-activated protein kinase. *Cellular Signalling,* 14**,** 239-247.

KOTA, J., MACHAVARAM, K. K., MCLENNAN, D. N., EDWARDS, G. A., PORTER, C. J. H. & CHARMAN, S. A. 2007. Lymphatic absorption of subcutaneously administered proteins: influence of different injection sites on the absorption of darbepoetin alfa using a sheep model. *Drug Metabolism and Disposition,* 35**,** 2211-2217.

KRISHNAN, S., CHI, E. Y., WEBB, J. N., CHANG, B. S., SHAN, D., GOLDENBERG, M., MANNING, M. C., RANDOLPH, T. W. & CARPENTER, J. F. 2002. Aggregation of granulocyte colony stimulating factor under physiological conditions: characterization and thermodynamic inhibition. *Biochemistry,* 41**,** 6422-31.

KUWABARA, T., KOBAYASHI, S. & SUGIYAMA, Y. 1996. Pharmacokinetics and pharmacodynamics of a recombinant human granulocyte colony-stimulating factor. *Drug metabolism reviews,* 28**,** 625-58.

LAKEN, H. A. & LEONARD, M. W. 2001. Understanding and modulating apoptosis in industrial cell culture. *Current opinion in biotechnology,* 12**,** 175-9.

LAMBERTINI, M., DEL MASTRO, L., BELLODI, A. & PRONZATO, P. 2014. The five "Ws" for bone pain due to the administration of granulocyte-colony stimulating factors (G-CSFs). *Critical reviews in oncology/hematology,* 89**,** 112-28.

LANGRISH, C. L., CHEN, Y., BLUMENSCHEIN, W. M., MATTSON, J., BASHAM, B., SEDGWICK, J. D., MCCLANAHAN, T., KASTELEIN, R. A. & CUA, D. J. 2005. IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. *The Journal of experimental medicine,* 201**,** 233-40.

LAYTON, J. E. & HALL, N. E. 2006. The interaction of G-CSF with its receptor. *Front Biosci,* 11**,** 3181-9.

LEIBUNDGUT-LANDMANN, S., GROSS, O., ROBINSON, M. J., OSORIO, F., SLACK, E. C., TSONI, S. V., SCHWEIGHOFFER, E., TYBULEWICZ, V., BROWN, G. D., RULAND, J. & REIS E SOUSA, C. 2007. Syk- and CARD9-dependent coupling of innate immunity to the induction of T helper cells that produce interleukin 17. *Nature immunology,* 8**,** 630-8.

LI, H. & NICHOLAS, J. 2002. Identification of amino acid residues of gp130 signal transducer and gp80 receptor subunit that are involved in ligand binding and signaling by human herpesvirus 8-encoded interleukin-6. *Journal of Virology,* 76**,** 5627-5636.

LI, Y. 2010. Commonly used tag combinations for tandem affinity purification. *Biotechnology and Applied Biochemistry,* 55**,** 73-83.

LIESCHKE, G. J., GRAIL, D., HODGSON, G., METCALF, D., STANLEY, E., CHEERS, C., FOWLER, K. J., BASU, S., ZHAN, Y. F. & DUNN, A. R. 1994. Mice lacking granulocyte colony-stimulating factor have chronic neutropenia, granulocyte and macrophage progenitor cell deficiency, and impaired neutrophil mobilization. *Blood,* 84**,** 1737-1746.

LITAM, P. P. 1993. Splenic extramedullary hematopoiesis in a patient receiving intermittently administered granulocyte colonystimulating factor. *Annals of Internal Medicine,* 118**,** 954-954.

LLAMAS-VELASCO, M., GARCÍA-MARTÍN, P., SÁNCHEZ-PÉREZ, J., FRAGA, J. & GARCÍA-DIEZ, A. 2013. Sweet's syndrome with subcutaneous involvement associated with pegfilgrastim treatment: first reported case. *Journal of cutaneous pathology,* 40**,** 46-9.

LORD, B. I., WOOLFORD, L. B. & MOLINEUX, G. 2001. Kinetics of neutrophil production in normal and neutropenic animals during the response to filgrastim (r-metHu G-CSF) or filgrastim SD/01 (PEG-r-metHu G-CSF). *Clinical Cancer Research,* 7**,** 2085-2090.

LOVING, C. L., KEHRLI, M. E., JR., BROCKMEIER, S. L., BAYLES, D. O., MICHAEL, D. D., SCHLINK, S. N. & LAGER, K. M. 2013. Porcine granulocyte-colony stimulating factor (G-CSF) delivered via replication-defective adenovirus induces a sustained increase in circulating peripheral blood neutrophils. *Biologicals,* 41**,** 368-76.

LÖWENBERG , B., VAN PUTTEN , W., THEOBALD , M., GMÜR , J., VERDONCK , L., SONNEVELD , P., FEY , M., SCHOUTEN , H., DE GREEF , G., FERRANT , A., KOVACSOVICS , T., GRATWOHL , A., DAENEN , S., HUIJGENS , P. & BOOGAERTS , M. 2003. Effect of priming with granulocyte colony-stimulating factor on the outcome of chemotherapy for acute myeloid leukemia. *New England Journal of Medicine,* 349**,** 743-752.

MAHMOOD, I. & GREEN, M. D. 2005. Pharmacokinetic and pharmacodynamic considerations in the development of therapeutic proteins. *Clinical Pharmacokinetics,* 44**,** 331-47.

MANJASETTY, B. A., TURNBULL, A. P., PANJIKAR, S., BUSSOW, K. & CHANCE, M. R. 2008. Automated technologies and novel techniques to accelerate protein crystallography for structural genomics. *Proteomics,* 8**,** 612-25.

MANZ, M. G., MIYAMOTO, T., AKASHI, K. & WEISSMAN, I. L. 2002. Prospective isolation of human clonogenic common myeloid progenitors. *Proceedings of the National Academy of Sciences of the United States of America,* 99**,** 11872-7.

MARTIN, C., BURDON, P. C., BRIDGER, G., GUTIERREZ-RAMOS, J. C., WILLIAMS, T. J. & RANKIN, S. M. 2003. Chemokines acting via CXCR2 and CXCR4 control the release of neutrophils from the bone marrow and their return following senescence. *Immunity,* 19**,** 583-93.

MASTRANGELO, A. J., HARDWICK, J. M., ZOU, S. & BETENBAUGH, M. J. 2000. Part II. Overexpression of bcl-2 family members enhances survival of mammalian cells in response to various culture insults. *Biotechnol Bioeng,* 67**,** 555-64.

MCCRACKEN, S., LAYTON, J. E., SHORTER, S. C., STARKEY, P. M., BARLOW, D. H. & MARDON, H. J. 1996. Expression of granulocyte-colony stimulating factor and its receptor is regulated during the development of the human placenta. *Journal of Endocrinology,* 149**,** 249-258.

MCCRACKEN, S. A. 1999. Gestational regulation of granulocyte-colony stimulating factor receptor expression in the human placenta. *Biology of Reproduction,* 60**,** 790-796.

MCLENNAN, D. N., PORTER, C. J. H. & CHARMAN, S. A. 2005. Subcutaneous drug delivery and the role of the lymphatics. *Drug Discovery Today: Technologies,* 2**,** 89-96.

METCALF, D. 2008. Hematopoietic cytokines. *Blood,* 111**,** 485-91.

METCALF, D. 2010. The colony-stimulating factors and cancer. *Nature reviews. Cancer,* 10**,** 425-34.

MILLA, P., DOSIO, F. & CATTEL, L. 2012. PEGylation of proteins and liposomes: a powerful and flexible strategy to improve the drug delivery. *Current Drug Metabolism,* 13**,** 105-119.

MOLINEUX, G. 2004. The design and development of pegfilgrastim (PEG-rmetHuG-CSF, Neulasta®). *Current Pharmaceutical Design,* 10**,** 1235-1244.

MOLINEUX, G. 2011. Granulocyte colony-stimulating factors. *Cancer treatment and research,* 157**,** 33-53.

MOLINEUX, G., KINSTLER, O., BRIDDELL, B., HARTLEY, C., MCELROY, P., KERZIC, P., SUTHERLAND, W., STONEY, G., KERN, B., FLETCHER, F. A., COHEN, A., KORACH, E., ULICH, T., MCNIECE, I., LOCKBAUM, P., MILLER-MESSANA, M. A., GARDNER, S., HUNT, T. & SCHWAB, G. 1999. A new form of Filgrastim with sustained duration in vivo and enhanced ability to mobilize PBPC in both mice and humans. *Experimental Hematology,* 27**,** 1724-34.

MOONEN, P., MERMOD, J. J., ERNST, J. F., HIRSCHI, M. & DELAMARTER, J. F. 1987. Increased biological activity of deglycosylated recombinant human granulocyte/macrophage colony-stimulating factor produced by yeast or animal cells. *Proceedings of the National Academy of Sciences of the United States of America,* 84**,** 4428-31.

MORIKAWA, K., MORIKAWA, S., NAKAMURA, M. & MIYAWAKI, T. 2002. Characterization of granulocyte colony-stimulating factor receptor expressed on human lymphocytes. *British Journal of Haematology,* 118**,** 296-304.

MORSTYN G, C. L., DÜHRSEN U, SOUZA LM, ALTON NK, VILLEVAL JL, NICOLA NA, BOYD AW, KANNOURAKIS G, CEBON J, ET AL. 1988. Clinical studies with granulocyte colony stimulating factor (G-CSF) in patients receiving cytotoxic chemotherapy. *Behring Institute Mitteilungen.,* 83**,** 234-9.

NAGATA, S. 1989. Gene structure and function of granulocyte colony-stimulating factor. *BioEssays : news and reviews in molecular, cellular and developmental biology,* 10**,** 113-7.

NAGATA, S., TSUCHIYA, M., ASANO, S., KAZIRO, Y., YAMAZAKI, T., YAMAMOTO, O., HIRATA, Y., KUBOTA, N., OHEDA, M. & NOMURA, H. 1986. Molecular cloning and expression of cDNA for human granulocyte colony-stimulating factor. *Nature,* 319**,** 415-8.

NAGHOOSI, H., BEHZADIAN, F., SAEEDINIA, A. & GHORASHI, S. A. 2008. Site-directed mutagenesis in human granulocyte- colony stimulating factor , cloning and expression in Escherichia coli. 6**,** 229-234.

NEMZEK, J. A., BOLGOS, G. L., WILLIAMS, B. A. & REMICK, D. G. 2001. Differences in normal values for murine white blood cell counts and other hematological parameters based on sampling site. *Inflammation Research,* 50**,** 523-527.

NGUYEN-JACKSON, H., PANOPOULOS, A. D., ZHANG, H., LI, H. S. & WATOWICH, S. S. 2010. STAT3 controls the neutrophil migratory response to CXCR2 ligands by direct activation of G-CSF-induced CXCR2 expression and via modulation of CXCR2 signal transduction. *Blood,* 115**,** 3354-63.

NICOLA, N. A., METCALF, D., MATSUMOTO, M. & JOHNSON, G. R. 1983. Purification of a factor inducing differentiation in murine myelomonocytic leukemia cells. Identification as granulocyte colony-stimulating factor. *Journal of Biological Chemistry.,* 258**,** 9017-9023.

NIITSU, N., IKI, S., MUROI, K., MOTOMURA, S., MURAKAMI, M., TAKEYAMA, H., OHSAKA, A. & URABE, A. 1997. Interstitial pneumonia in patients receiving granulocyte colony-stimulating factor during chemotherapy: survey in Japan 1991-96. *British journal of cancer,* 76**,** 1661-6.

NIMUBONA, S., GRULOIS, I., BERNARD, M., DRÉNOU, B., GODARD, M., FAUCHET, R. & LAMY, T. 2002. Complete remission in hypoplastic acute myeloid leukemia induced by G-CSF without chemotherapy: report on three cases. *Leukemia,* 16**,** 1871-3.

NISHIZAWA, M., TSUCHIYA, M., WATANABE-FUKUNAGA, R. & NAGATA, S. 1990. Multiple elements in the promoter of granulocyte colony-stimulating factor gene regulate its constitutive expression in human carcinoma cells. *Journal of Biological Chemistry.,* 265**,** 5897-5902.

NOBLE, J. E. & BAILEY, M. J. A. 2009. Chapter 8 Quantitation of Protein. *In:* RICHARD, R. B. & MURRAY, P. D. (eds.) *Methods in Enzymology.* Academic Press.

NOHYNEK, G. J., PLARD, J. P., WELLS, M. Y., ZERIAL, A. & ROQUET, F. 1996. Comparison of the potency of glycosylated and nonglycosylated recombinant human granulocyte colony-stimulating

factors in neutropenic and nonneutropenic CD rats. *Cancer Chemotherapy and Pharmacology,* 39**,** 259-266.

NOMIYAMA, J., SHINOHARA, K. & INQUE, H. 1994. Erythema nodosum caused by the administration of granulocyte colony-stimulating factor in a patient with refractory anemia. *American Journal of Hematology,* 47**,** 333-333.

O'MALLEY, D. P., WHALEN, M. & BANKS, P. M. 2003. Spontaneous splenic rupture with fatal outcome following G-CSF administration for myelodysplastic syndrome. *American journal of hematology,* 73**,** 294-5.

OH-EDA, M., HASEGAWA, M., HATTORI, K., KUBONIWA, H., KOJIMA, T., ORITA, T., TOMONOU, K., YAMAZAKI, T. & OCHI, N. 1990. O-linked sugar chain of human granulocyte colony-stimulating factor protects it against polymerization and denaturation allowing it to retain its biological activity. *Journal of Biological Chemistry,* 265**,** 11432-11435.

OHDO, S., FURUKUBO, T., ARATA, N., YUKAWA, E., HIGUCHI, S., NAKANO, S. & OGAWA, N. 1998. Influence of dosing time on pharmacological action of G-CSF in mice. *Life Sciences,* 62**,** PI163-8.

OHLSON, M., SÖRENSSON, J., LINDSTRÖM, K., BLOM, A. M., FRIES, E. & HARALDSSON, B. 2001. Effects of filtration rate on the glomerular barrier and clearance of four differently shaped molecules. *American Journal of Physiology - Renal Physiology,* 281**,** F103-F113.

OKABE, M., ASANO, M., KUGA, T., KOMATSU, Y., YAMASAKI, M., YOKOO, Y., ITOH, S., MORIMOTO, M. & OKA, T. 1990. In vitro and in vivo hematopoietic effect of mutant human granulocyte colony-stimulating factor [see comments]. *Blood,* 75**,** 1788-1793.

OKAMOTO, M., NAKAI, M., NAKAYAMA, C., YANAGI, H., MATSUI, H., NOGUCHI, H., NAMIKI, M., SAKAI, J., KADOTA, K., FUKUI, M. & ET AL. 1991. Purification and characterization of three forms of differently glycosylated recombinant human granulocyte-macrophage colony-stimulating factor. *Archives of Biochemistry and Biophysics,* 286**,** 562-8.

PANOPOULOS, A. D. & WATOWICH, S. S. 2008. Granulocyte colony-stimulating factor: molecular mechanisms of action during steady state and 'emergency' hematopoiesis. *Cytokine,* 42**,** 277-88.

PARMAR, S. & RITCHIE, D. S. 2014. Allogeneic transplantation as anticancer immunotherapy. *Current Opinion in Immunology,* 27**,** 38-45.

PASSAFIUME, M., VULLIEZ-LE NORMAND, B., RIOTTOT, M. M. & BENTLEY, G. A. 1998. Sequence analysis of a monoclonal antibody specific for the preS2 region of hepatitis B surface antigen, and the cloning, expression and characterisation of its single-chain Fv construction. *The Federation of European Biochemical Societies,* 441**,** 407-12.

PEDRAZZOLI, P., GIBELLI, N., PAVESI, L., PRETI, P., PIOLINI, M., BERTOLINI, F. & ROBUSTELLI DELLA CUNA, G. 1996. Effects of glycosylated and non-glycosylated G-CSFs, alone and in combination with other cytokines, on the growth of human progenitor cells. *Anticancer research,* 16**,** 1781-5.

PIKE, B. L. & ROBINSON, W. A. 1970. Human bone marrow colony growth in agar-gel. *Journal of cellular physiology,* 76**,** 77-84.

PIPER, M. G., MASSULLO, P. R., LOVELAND, M., DRUHAN, L. J., KINDWALL-KELLER, T. L., AI, J., COPELAN, A. & AVALOS, B. R. 2010. Neutrophil elastase downmodulates native G-CSFR expression and granulocyte-macrophage colony formation. *Journal of Inflammation (London, England),* 7**,** 5-5.

PISAL, D. S., KOSLOSKI, M. P. & BALU-IYER, S. V. 2010. Delivery of therapeutic proteins. 99**,** 2557-2575.

PRENDIVILLE, J., THIESSEN, P. & MALLORY, S. B. 2001. Neutrophilic dermatoses in two children with idiopathic neutropenia: association with granulocyte colony-stimulating factor (G-CSF) therapy. *Pediatric Dermatology,* 18**,** 417-421.

RASO, S. W., ABEL, J., BARNES, J. M., MALONEY, K. M., PIPES, G., TREUHEIT, M. J., KING, J. & BREMS, D. N. 2005. Aggregation of granulocyte-colony stimulating factor in vitro involves a conformationally altered monomeric state. *Protein Science,* 14**,** 2246-57.

REISNER, A. H., NEMES, P. & BUCHOLTZ, C. 1975. The use of Coomassie Brilliant Blue G250 perchloric acid solution for staining in electrophoresis and isoelectric focusing on polyacrylamide gels. *Analytical Biochemistry,* 64**,** 509-516.

RIA, R., REALE, A., MELACCIO, A., RACANELLI, V., DAMMACCO, F. & VACCA, A. 2015. Filgrastim, lenograstim and pegfilgrastim in the mobilization of peripheral blood progenitor cells in patients with lymphoproliferative malignancies. *Clinical and Experimental Medicine,* 15**,** 145-150.

RIBARSKA, J. T., JOLEVSKA, S. T., PANOVSKA, A. P. & DIMITROVSKA, A. 2008. Studying the formation of aggregates in recombinant human granulocyte-colony stimulating factor (rHuG-CSF), lenograstim, using size-exclusion chromatography and SDS-PAGE. *Acta Pharmaceutica,* 58**,** 199-206.

RICHTER AW, A. E. 1984. Polyethylene glycol reactive antibodies in man: titer distribution in allergic patients treated with monomethoxy polyethylene glycol modified allergens or placebo, and in healthy blood donors. *International Archives of Allergy and Immunology.,* 74**,** 36-39.

RISS, T. L. M., R. A 1992. Comparison of MTT, XTT, and a novel tetrazolium compound MTS for in vitro proliferation and chemosensitivity assays. *Molecular Biology of the Cell 3 (SUPPL),* 184A.

ROBERTS, A. W. 2005. G-CSF: A key regulator of neutrophil production, but that's not all! *Growth Factors,* 23**,** 33-41.

ROBERTS, C. J. 2014. Protein aggregation and its impact on product quality. *Current Opinion in Biotechnology,* 30**,** 211-7.

ROBINSON, W., METCALF, D. & BRADLEY, T. R. 1967. Stimulation by normal and leukemic mouse sera of colony formationin vitro by mouse bone marrow cells. *Journal of Cellular Physiology,* 69**,** 83-91.

ROSKOS, L. K. 2012. The clinical pharmacology of filgrastim and pegfilgrastim. *In:* MOLINEUX, G., FOOTE, M. & ARVEDSON, T. (eds.) *Twenty Years of G-CSF: Clinical and Nonclinical Discoveries.* Basel: Springer Basel.

SANDOR, V., HASSAN, R. & KOHN, E. 1996. Exacerbation of pseudogout by granulocyte colony-stimulating factor. *Annals of internal medicine,* 125**,** 781-781.

SANFELIU, A. & STEPHANOPOULOS, G. 1999. Effect of glutamine limitation on the death of attached Chinese hamster ovary cells. *Biotechnology and Bioengineering,* 64**,** 46-53.

SANTINI, V., SCAPPINI, B., INDIK, Z. K., GOZZINI, A., FERRINI, P. R. & SCHREIBER, A. D. 2003. The carboxy-terminal region of the granulocyte colony-stimulating factor receptor transduces a phagocytic signal. *Blood,* 101**,** 4615-22.

SARKAR, C. A., LOWENHAUPT, K., HORAN, T., BOONE, T. C., TIDOR, B. & LAUFFENBURGER, D. A. 2002. Rational cytokine design for increased lifetime and enhanced potency using pH-activated "histidine switching". *Nature Biotechnology,* 20**,** 908-13.

SAUERWALD, T. M., OYLER, G. A. & BETENBAUGH, M. J. 2003. Study of caspase inhibitors for limiting death in mammalian cell culture. *Biotechnology and Bioengineering,* 81**,** 329-40.

SCARAMUZZA, S., TONON, G., OLIANAS, A., MESSANA, I., SCHREPFER, R., ORSINI, G. & CALICETI, P. 2012. A new site-specific monoPEGylated filgrastim derivative prepared by enzymatic conjugation: Production and physicochemical characterization. *Journal of Controlled Release,* 164**,** 355-363.

SCHNEIDER, A., KRÜGER, C., STEIGLEDER, T., WEBER, D., PITZER, C., LAAGE, R., ARONOWSKI, J., MAURER, M. H., GASSLER, N., MIER, W., HASSELBLATT, M., KOLLMAR, R., SCHWAB, S., SOMMER, C., BACH, A., KUHN, H.-G. & SCHÄBITZ, W.-R. 2005. The hematopoietic factor G-CSF is a neuronal ligand that counteracts programmed cell death and drives neurogenesis. *The Journal of clinical investigation,* 115**,** 2083-98.

SCHOLZ, M., ACKERMANN, M., ENGEL, C., EMMRICH, F., LOEFFLER, M. & KAMPRAD, M. 2009. A pharmacokinetic model of filgrastim and pegfilgrastim application in normal mice and those with cyclophosphamide-induced granulocytopaenia. *Cell Proliferation,* 42**,** 813-22.

SEDMAK, J. J. & GROSSBERG, S. E. 1977. A rapid, sensitive, and versatile assay for protein using Coomassie brilliant blue G250. *Analytical Biochemistry,* 79**,** 544-552.

SEMPLE, S. C., HARASYM, T. O., CLOW, K. A., ANSELL, S. M., KLIMUK, S. K. & HOPE, M. J. 2005. Immunogenicity and rapid blood clearance of liposomes containing polyethylene glycol-lipid conjugates and nucleic acid. *Journal of Pharmacology and Experimental Therapeutics,* 312**,** 1020-1026.

SHAH, J. & WELSH, S. J. 2014. The clinical use of granulocyte-colony stimulating factor. *British journal of hospital medicine (London, England : 2005),* 75**,** C29-32.

SHANNON, M. F., COLES, L. S., FIELKE, R. K., GOODALL, G. J., LAGNADO, C. A. & VADAS, M. A. 2009. Three Essential promoter elements mediate tumour necrosis factor and interleukin-1 activation of the granulocyte-colony stimulating factor gene. *Growth Factors*. 1992;7(3):181-93.

SHARMA, S. K., EVANS, D. B., VOSTERS, A. F., MCQUADE, T. J. & TARPLEY, W. G. 1991. Metal affinity chromatography of recombinant HIV-1 reverse transcriptase containing a human renin cleavable metal binding domain. *Biotechnology and Applied Biochemistry,* 14**,** 69-81.

SHERIDAN, W. P., BEGLEY, C. G., JUTTNER, C. A., SZER, J., TO, L. B., MAHER, D., MCGRATH, K. M., MORSTYN, G. & FOX, R. M. 1992. Effect of peripheral-blood progenitor cells mobilised by filgrastim (G-CSF) on platelet recovery after high-dose chemotherapy. *Lancet,* 339**,** 640-4.

SIMPSON, R. J. 2010. Stabilization of proteins for storage. *Cold Spring Harbor Protocols,* 2010**,** pdb.top79.

SMITH, W. B., GAMBLE, J. R. & VADAS, M. A. 1994. The role of granulocyte-macrophage and granulocyte colony-stimulating factors in neutrophil transendothelial migration: comparison with interleukin-8. *Experimental Hematology,* 22**,** 329-34.

STANLEY, E. R. & HEARD, P. M. 1977. Factors regulating macrophage production and growth. Purification and some properties of the colony stimulating factor from medium conditioned by mouse L cells. *Journal of Biological Chemistry,* 252**,** 4305-4312.

STARK, M. A., HUO, Y., BURCIN, T. L., MORRIS, M. A., OLSON, T. S. & LEY, K. 2005. Phagocytosis of apoptotic neutrophils regulates granulopoiesis via IL-23 and IL-17. *Immunity,* 22**,** 285-94.

STRONCEK, D. F., CLAY, M. E., PETZOLDT, M. L., SMITH, J., JASZCZ, W., OLDHAM, F. B. & MCCULLOUGH, J. 1996a. Treatment of normal individuals with granulocyte-colony-stimulating factor: donor experiences and the effects on peripheral blood CD34+ cell counts and on the collection of peripheral blood stem cells. *Transfusion,* 36**,** 601-610.

STRONCEK, D. F., CLAY, M. E., SMITH, J., ILSTRUP, S., OLDHAM, F. & MCCULLOUGH, J. 1996b. Changes in blood counts after the administration of granulocyte-colony- stimulating factor and the collection of peripheral blood stem cells from healthy donors. *Transfusion,* 36**,** 596-600.

SUNDY, J. S., BARAF, H. B., YOOD, R. A. & ET AL. 2011. Efficacy and tolerability of pegloticase for the treatment of chronic gout in patients refractory to conventional treatment: Two randomized controlled trials. *Journal of the American Medical Association,* 306**,** 711-720.

SUNSTROM, N. A., GAY, R. D., WONG, D. C., KITCHEN, N. A., DEBOER, L. & GRAY, P. P. 2000. Insulin-like growth factor-I and transferrin mediate growth and survival of Chinese hamster ovary cells. *Biotechnology Progress,* 16**,** 698-702.

SUPERSAXO, A., HEIN, W. R. & STEFFEN, H. 1990. Effect of molecular weight on the lymphatic absorption of water-soluble compounds following subcutaneous administration. *Pharmaceutical Research,* 7**,** 167-9.

TABATA, H., KUBO, M., IZUMISAWA, S., NARITA, K., MATSUZAWA, T. & SAKAI, T. 1998. A parallel comparison of three automated multichannel blood cell counting systems for analysis of blood of laboratory animals. *Comparative Haematology International,* 8**,** 66-71.

TAMADA, T., HONJO, E., MAEDA, Y., OKAMOTO, T., ISHIBASHI, M., TOKUNAGA, M. & KUROKI, R. 2006. Homodimeric cross-over structure of the human granulocyte colony-stimulating factor (GCSF) receptor signaling complex. *Proceedings of the National Academy of Sciences of the United States of America,* 103**,** 3135-40.

TANAKA, H., TANAKA, Y., SHINAGAWA, K., YAMAGISHI, Y., OHTAKI, K. & ASANO, K. 1997. Three types of recombinant human granulocyte colony-stimulating factor have equivalent biological activities in monkeys. *Cytokine,* 9**,** 360-369.

THOMAS, J. W., BAUM, C. M., HOOD, W. F., KLEIN, B., MONAHAN, J. B., PAIK, K., STATEN, N., ABRAMS, M. & MCKEARN, J. P. 1995. Potent interleukin 3 receptor agonist with selectively enhanced hematopoietic activity relative to recombinant human interleukin 3. *Proceedings of the National Academy of Sciences of the United States of America,* 92**,** 3779-83.

TIAN, S. S., LAMB, P., SEIDEL, H. M., STEIN, R. B. & ROSEN, J. 1994. Rapid activation of the STAT3 transcription factor by granulocyte colony-stimulating factor. *Blood,* 84**,** 1760-4.

TILLMANN, H., GANSON, N. J., PATEL, K., THOMPSON, A. J., ABDELMALEK, M., MOODY, T., MCHUTCHISON, J. G. & HERSHFIELD, M. S. 2010. 307 High prevalence of pre-existing antibodies against polyethylene glycol (peg) in hepatitis c (hcv) patients which is not associated with impaired response to peg-interferon. *Journal of Hepatology,* 52**,** S129.

TOUW, I. P. 2007. Granulocyte colony-stimulating factor and its receptor in normal myeloid cell development, leukemia and related blood cell disorders. *Frontiers in Bioscience,* 12**,** 800-800.

TOUW, I. P. & BONTENBAL, M. 2007. Granulocyte colony-stimulating factor: key (f)actor or innocent bystander in the development of secondary myeloid malignancy? *Journal of the National Cancer Institute,* 99**,** 183-6.

TOUW, I. P. & VAN DE GEIJN, G. J. 2007. Granulocyte colony-stimulating factor and its receptor in normal myeloid cell development, leukemia and related blood cell disorders. *Frontiers in Bioscience,* 12**,** 800-15.

VALTIERI, M., BOCCOLI, G., TESTA, U., BARLETTA, C. & PESCHLE, C. 1991. Two-step differentiation of AML-193 leukemic line: terminal maturation is induced by positive interaction of retinoic acid with granulocyte colony-stimulating factor (CSF) and vitamin D3 with monocyte CSF. *Blood,* 77**,** 1804-12.

VAN DE GEIJN, G.-J. M., GITS, J., AARTS, L. H. J., HEIJMANS-ANTONISSEN, C. & TOUW, I. P. 2004. G-CSF receptor truncations found in SCN/AML relieve SOCS3-controlled inhibition of STAT5 but leave suppression of STAT3 intact. *Blood,* 104**,** 667-74.

VAN DE GEIJN, G. J. M., AARTS, L. H. J., ERKELAND, S. J., PRASHER, J. M. & TOUW, I. P. 2003. Granulocyte colony-stimulating factor and its receptor in normal hematopoietic cell development and myeloid disease. *Reviews of physiology, biochemistry and pharmacology,* 149**,** 53-71.

VAN DER AUWERA, P., PLATZER, E., XU, Z. X., SCHULZ, R., FEUGEAS, O., CAPDEVILLE, R. & EDWARDS, D. J. 2001. Pharmacodynamics and pharmacokinetics of single doses of subcutaneous pegylated human G-CSF mutant (Ro 25-8315) in healthy volunteers: Comparison with single and multiple daily doses of filgrastim. *American Journal of Hematology,* 66**,** 245-251.

VAN WOENSEL, J. B., KNOESTER, H., LEEUW, J. A. & VAN AALDEREN, W. M. 1994. Acute respiratory insufficiency during doxorubicin, cyclophosphamide, and G-CSF therapy. *Lancet,* 344**,** 759-60.

VERONESE, F. M. & MERO, A. 2008. The Impact of PEGylation on Biological Therapies. *BioDrugs,* 22**,** 315-329.

VIDARSSON, B., GEIRSSON, A. J. & ÖNUNDARSON, P. T. 1995. Reactivation of rheumatoid arthritis and development of leukocytoclastic vasculitis in a patient receiving granulocyte colony-stimulating factor for felty's syndrome. *The American Journal of Medicine,* 98**,** 589-591.

WADHWA, M., BIRD, C., DOUGALL, T., RIGSBY, P., BRISTOW, A. & THORPE, R. 2015. Establishment of the first international standard for PEGylated granulocyte colony stimulating factor (PEG-G-CSF): report of an international collaborative study. *Journal of Immunological Methods,* 416**,** 17-28.

WALLS, D. & LOUGHRAN, S. T. 2011. Tagging recombinant proteins to enhance solubility and aid purification. *Methods in Molecular Biology,* 681**,** 151-75.

WALLS, D., MCGRATH, R. & LOUGHRAN, S. T. 2011. A digest of protein purification. *Methods in Molecular Biology,* 681**,** 3-23.

WANG, W. 2005. Protein aggregation and its inhibition in biopharmaceutics. *International Journal of Pharmaceutics,* 289**,** 1-30.

WANG, X., ISHIDA, T. & KIWADA, H. 2007. Anti-PEG IgM elicited by injection of liposomes is involved in the enhanced blood clearance of a subsequent dose of PEGylated liposomes. *Journal of Controlled Release,* 119**,** 236-244.

WANG, Y., TÄGIL, K., RIPA, R. S., NILSSON, J. C., CARSTENSEN, S., JØRGENSEN, E., SØNDERGAARD, L., HESSE, B., JOHNSEN, H. E. & KASTRUP, J. 2005. Effect of mobilization of bone marrow stem cells by granulocyte colony stimulating factor on clinical symptoms, left ventricular perfusion and function in patients with severe chronic ischemic heart disease. *International journal of cardiology,* 100**,** 477-83.

WARD, A. C. 2007. The role of the granulocyte colony-stimulating factor receptor (G-CSF-R) in disease. *Frontiers in Bioscience*, 12,608-618.

WARD, A. C., OOMEN, S. P. M. A., SMITH, L., GITS, J., VAN LEEUWEN, D., SOEDE-BOBOK, A. A., ERPELINCK-VERSCHUEREN, C. A. J., YI, T. & TOUW, I. P. 2000. The SH2 domain-containing protein tyrosine phosphatase SHP-1 is induced by granulocyte colony-stimulating factor (G-CSF) and modulates signaling from the G-CSF receptor. *Leukemia,* 14**,** 1284-1291.

WARD, A. C., VAN AESCH, Y. M., GITS, J., SCHELEN, A. M., DE KONING, J. P., VAN LEEUWEN, D., FREEDMAN, M. H. & TOUW, I. P. 1999a. Novel point mutation in the extracellular domain of the granulocyte colony-stimulating factor (G-Csf) receptor in a case of severe congenital neutropenia hyporesponsive to G-Csf treatment. *Journal of Experimental Medicine,* 190**,** 497-508.

WARD, A. C., VAN AESCH, Y. M., SCHELEN, A. M. & TOUW, I. P. 1999b. Defective internalization and sustained activation of truncated granulocyte colony-stimulating factor receptor found in severe congenital neutropenia/acute myeloid leukemia. *Blood,* 93**,** 447-458.

WEI, A. 2001. Granulocyte colony-stimulating factor-induced sickle cell crisis and multiorgan dysfunction in a patient with compound heterozygous sickle cell/beta + thalassemia. *Blood,* 97**,** 3998-3999.

WELTE, K. 2012. Twenty Years of G-CSF. *Milestones in Drug Therapy*, 15-25.

WELTE, K., GABRILOVE, J., BRONCHUD, M., PLATZER, E. & MORSTYN, G. 1996. Filgrastim (r-metHuG-CSF): the first 10 years. *Blood,* 88**,** 1907-1929.

WELTE, K., PLATZER, E., LU, L., GABRILOVE, J. L., LEVI, E., MERTELSMANN, R. & MOORE, M. A. 1985. Purification and biochemical characterization of human pluripotent hematopoietic colony-stimulating factor. *Proceedings of the National Academy of Sciences,* 82**,** 1526-1530.

WERNER, J. M., BREEZE, A. L., KARA, B., ROSENBROCK, G., BOYD, J., SOFFE, N. & CAMPBELL, I. D. 1994. Secondary structure and backbone dynamics of human granulocyte colony-stimulating factor in solution. *Biochemistry,* 33**,** 7184-7192.

WHITE, J. M. L., MUFTI, G. J., SALISBURY, J. R. & DU VIVIER, A. W. P. 2006. Cutaneous manifestations of granulocyte colony-stimulating factor. *Clinical and experimental dermatology,* 31**,** 206-7.

WICZLING, P., LOWE, P., PIGEOLET, E., LÜDICKE, F., BALSER, S. & KRZYZANSKI, W. 2009. Population pharmacokinetic modelling of filgrastim in healthy adults following intravenous and subcutaneous administrations. *Clinical Pharmacokinetics,* 48**,** 817-826.

WILKINSON, I. R., FERRANDIS, E., ARTYMIUK, P. J., TEILLOT, M., SOULARD, C., TOUVAY, C., PRADHANANGA, S. L., JUSTICE, S., WU, Z., LEUNG, K. C., STRASBURGER, C. J., SAYERS, J. R. & ROSS, R. J. 2007. A ligand-receptor fusion of growth hormone forms a dimer and is a potent long-acting agonist. *Nature medicine,* 13**,** 1108-13.

WÖLFLER, A., ERKELAND, S. J., BODNER, C., VALKHOF, M., RENNER, W., LEITNER, C., OLIPITZ, W., PFEILSTÖCKER, M., TINCHON, C., EMBERGER, W., LINKESCH, W., TOUW, I. P. & SILL, H. 2005. A functional single-nucleotide polymorphism of the G-CSF receptor gene predisposes individuals to high-risk myelodysplastic syndrome. *Blood,* 105**,** 3731-6.

WURM, F. M. 2004. Production of recombinant protein therapeutics in cultivated mammalian cells. *Nature Biotechnology,* 22**,** 1393-8.

WURM, F. M. & HACKER, D. 2011. First CHO genome. *Nature Biotechnology,* 29**,** 718-20.
YAMAGUCHI, T., MIYATA, Y., HAYAMIZU, K., HASHIZUME, J., MATSUMOTO, T., TASHIRO, H. & OHDAN, H. 2012. Preventive effect of G-CSF on acute lung injury via alveolar macrophage regulation. *The Journal of surgical research,* 178**,** 378-84.
YANG, B.-B. 2006. Integration of pharmacokinetics and pharmacodynamics into the drug development of pegfilgrastim, a pegylated protein. *Pharmacokinetics and Pharmacodynamics of Biotech Drugs*.
YANG, B.-B., LUM, P. K., HAYASHI, M. M. & ROSKOS, L. K. 2004. Polyethylene glycol modification of filgrastim results in decreased renal clearance of the protein in rats. *Journal of Pharmaceutical Sciences,* 93**,** 1367-1373.
YANG, T. 2013. Modern chemistry & applications pegylation – successful approach for therapeutic protein conjugation. *Modern Chemistry & Applications,* 1**,** 1-2.
YANG, W. C., LU, J., NGUYEN, N. B., ZHANG, A., HEALY, N. V., KSHIRSAGAR, R., RYLL, T. & HUANG, Y. M. 2014. Addition of valproic acid to CHO cell fed-batch cultures improves monoclonal antibody titers. *Molecular Biotechnology,* 56**,** 421-8.
YOKOSE, N., OGATA, K., TAMURA, H., AN, E., NAKAMURA, K., KAMIKUBO, K., KUDOH, S., DAN, K. & NOMURA, T. 1998. Pulmonary toxicity after granulocyte colony-stimulating factor-combined chemotherapy for non-Hodgkin's lymphoma. *British journal of cancer,* 77**,** 2286-90.
YOON, S. K., SONG, J. Y. & LEE, G. M. 2003. Effect of low culture temperature on specific productivity, transcription level, and heterogeneity of erythropoietin in Chinese hamster ovary cells. *Biotechnology and Bioengineering,* 82**,** 289-298.
YU, Z., KASTENMULLER, G., HE, Y., BELCREDI, P., MOLLER, G., PREHN, C., MENDES, J., WAHL, S., ROEMISCH-MARGL, W., CEGLAREK, U., POLONIKOV, A., DAHMEN, N., PROKISCH, H., XIE, L., LI, Y., WICHMANN, H. E., PETERS, A., KRONENBERG, F., SUHRE, K., ADAMSKI, J., ILLIG, T. & WANG-SATTLER, R. 2011. Differences between human plasma and serum metabolite profiles. *Public Library of Science One,* 6**,** e21230.
ZANGHI, J. A., RENNER, W. A., BAILEY, J. E. & FUSSENEGGER, M. 2000. The growth factor inhibitor suramin reduces apoptosis and cell aggregation in protein-free CHO cell batch cultures. *Biotechnology Progress,* 16**,** 319-25.
ZEIDLER, C., GERMESHAUSEN, M., KLEIN, C. & WELTE, K. 2009. Clinical implications of ELA2-, HAX1-, and G-CSF-receptor (CSF3R) mutations in severe congenital neutropenia. *British Journal of Haematology,* 144**,** 459-67.
ZHANG, C. C. & LODISH, H. F. 2008. Cytokines regulating hematopoietic stem cell function. *Current opinion in hematology,* 15**,** 307-311.
ZHANG, T., SHE, Z., HUANG, Z., LI, J., LUO, X. & DENG, Y. 2014. Application of sialic acid/polysialic acid in the drug delivery systems. *Asian Journal of Pharmaceutical Sciences,* 9**,** 75-81.
ZHAO, S., ZHANG, Y., TIAN, H., CHEN, X., CAI, D., YAO, W. & GAO, X. 2013. Extending the serum half-life of G-CSF via fusion with the domain III of human serum albumin. *Biomedical Research International***,** 8.
ZHUANG, D., QIU, Y., HAQUE, S. J. & DONG, F. 2005. Tyrosine 729 of the G-CSF receptor controls the duration of receptor signaling: involvement of SOCS3 and SOCS1. *Journal of leukocyte biology,* 78**,** 1008-15.
ZSEBO, K. M., COHEN, A. M., MURDOCK, D. C., BOONE, T. C., INOUE, H., CHAZIN, V. R., HINES, D. & SOUZA, L. M. 1986. Recombinant human granulocyte colony stimulating factor: molecular and biological characterization. *Immunobiology,* 172**,** 175-84.