Generation of a long acting GCSF for treatment of neutropenia and stem cell harvest

Abdulrahman Alshehri, Richard Ross and Ian R Wilkinson
Department of Human Metabolism, School of Medicine, Royal Hallamshire Hospital, University of Sheffield, UK.

Background

Over the last 20 years, granulocyte colony-stimulating factor (GCSF) has become a recognized therapy in the treatment of patients suffering from neutropenia. Current therapies require daily injections of GCSF to stimulate stem cell production and response to treatment is often unpredictable as GCSF is rapidly cleared. A number of approaches to reducing GCSF clearance have been tried mainly through conjugation with another moiety. The technologies already being employed, include PEGylation, linking to immunoglobulins and glycosylation to increase the half-life of rhGCSF. However although these approaches have reduced clearance the pharmacokinetic profile has remained unpredictable.

Aim and hypothesis

The aim of this project is to create a long acting GCSF with predictable pharmacokinetic profile to provide a more effective treatment for generating HSCs for bone marrow transplantation. We will achieve this via incorporation of variable glycosylated linkers between two GCSF molecules. This will create a hyperglycosylated construct with a high molecular weight and protected from proteolysis resulting in reduced clearance while retaining bioactivity. This approach also alleviates potential problems with direct glycosylation of the ligand which may inhibit bioactivity and potentially introduce immunogenic sites.

Methodology

GCSF tandem molecules with linkers containing between 2-8 NAT glycosylation motifs and their respective controls (Q replaces N in the sequence motif NAT) were cloned, and sequenced (Figure 1 & Table 1). Following expression in Chinese hamster ovary (CHO) cells, expressed protein was quantified by ELISA and analysed by western blot and SDS-PAGE to confirm molecular weights. Bioactivity of purified proteins was tested using AML-193 proliferation assay.

Figure 1:

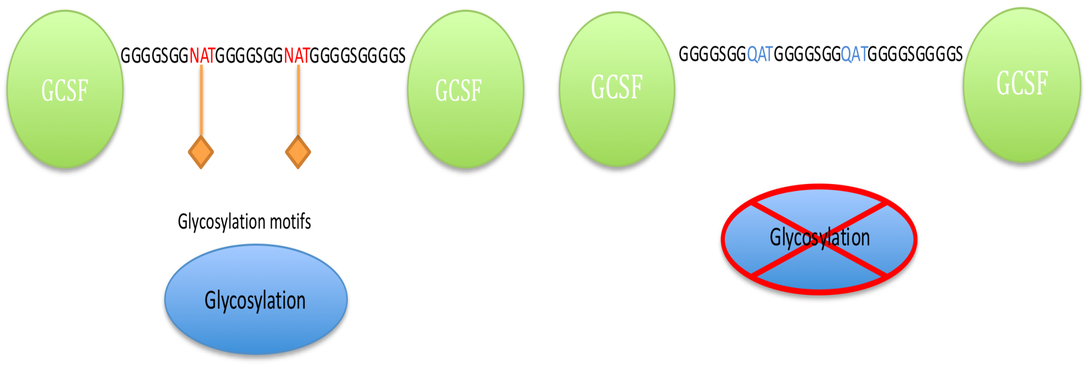


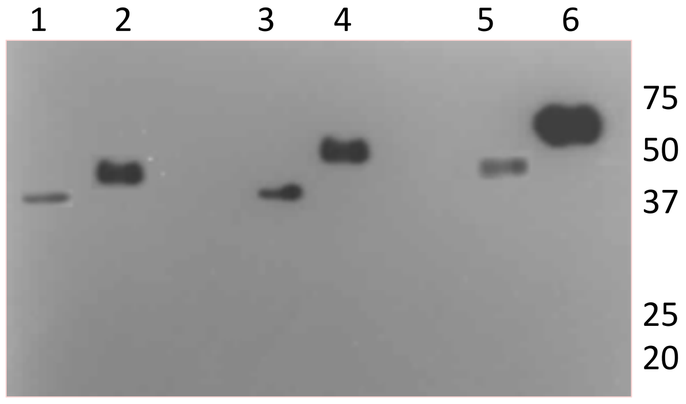
Table 1: List of constructs

Construct name	Number of NAT/ QAT motifs in linker region	Molecular weight (kDa)	Description
GCSF (2NAT)	2 × NAT motifs	**45.2**	GCSF tandem agonist
GCSF (2QAT)	2 × QAT motifs	**45.4**	Control for GCSF (2NAT)
GCSF (4NAT)	4 × NAT motifs	**45.2**	GCSF tandem agonist
GCSF (4QAT)	4 × QAT motifs	**45.4**	Control for GCSF (4NAT)
GCSF (8NAT)	8 × NAT motifs	**48.5**	GCSF tandem agonist
GCSF (8QAT)	8 × QAT motifs	**48.5**	Control for GCSF (8NAT)

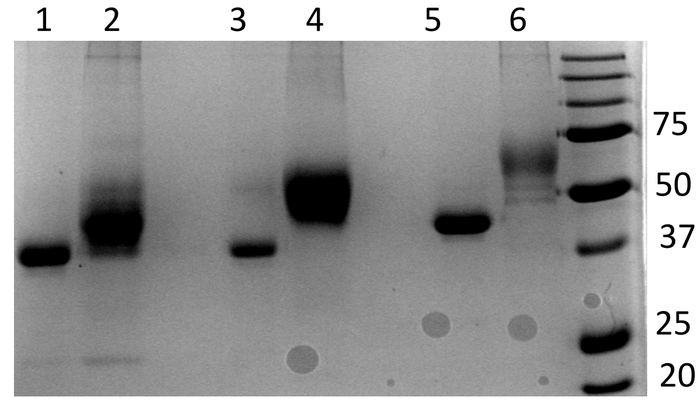
Results

Figure 2: Western blot & SDS-PAGE analysis

It is possible to express GCSF tandem molecules linked by a flexible linker $(Gly_4Ser)_n$ in mammalian cell lines. The glycosylated tandem molecules show an increased in molecular weight above that of their controls (non-glycosylated tandem molecules).



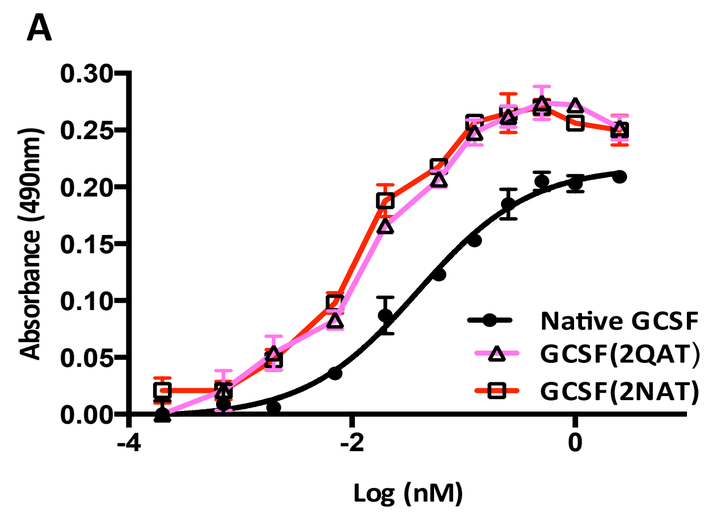
Western blot of crude media from transiently transfected CHO Flp-In cells expressing GCSF tandem molecules. Lane 1; GCSF (2QAT). Lane 2; GCSF (2NAT). Lane 3; GCSF (4QAT). Lane 4; GCSF (4NAT). Lane 5; GCSF (8QAT). Lane 6; GCSF (8NAT).

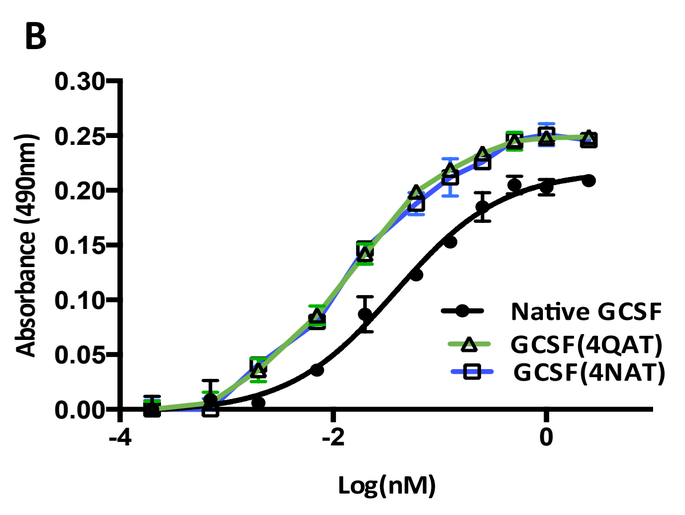


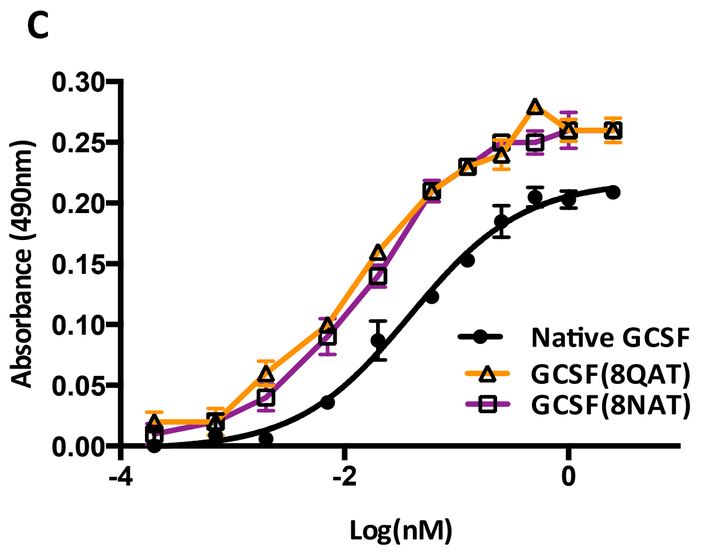
Purified GCSF tandem molecules analyzed by SDS-PAGE. Lane 1; GCSF (2QAT). Lane 2; GCSF (2NAT). Lane 3; GCSF (4QAT). Lane 4; GCSF (4NAT). Lane 5; GCSF (8QAT). Lane 6; GCSF (8NAT).

Figure 3: AML-193 proliferation assay

GCSF stimulates the proliferation of the AML-193 cell line (Human acute myeloid leukemic cell line). All purified GCSF tandems show increased bioactivity with all standard curves shifted to the left in comparison to native GCSF.







Construct name	EC50 (nM)	SD
Native GCSF	0.041 (n=12)	0.0031
GCSF (2QAT)	0.016 (n=2)	0.0035
GCSF (2NAT)	0.011 (n=2)	0.0007
GCSF (4QAT)	0.011 (n=3)	0.0022
GCSF (4NAT)	0.013 (n=3)	0.0006
GCSF (8QAT)	0.012 (n=3)	0.0029
GCSF (8NAT)	0.016 (n=3)	0.0006

Figure 3 (A,B,C): Proliferation of AML-193 cells in the presence of GCSF tandems and native GCSF.

Table 2: shows EC50 values calculated for each GCSF tandem & native GCSF using Graph Pad Prism software.

Conclusion

Results show that the use of glycosylated linkers to generate GCSF tandems results in molecules with increased molecular weight and increased bioactivity compared to native GCSF. Future studies will test protein clearance using a rat model system.

References

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