Title: Generation of long acting therapies using glycosylated linkers

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Rationale: The current therapeutic drugs such as, growth hormone (GH), granulocyte colony-stimulating factor (GCSF) and leptin require once-daily injections, which are inconvenient and expensive. Therefore, a number of approaches to reducing therapeutic regimens clearance have been tried mainly through conjugation with another moiety. One such technology already being employed is PEGylation; however this has been shown to be non-biodegradable and toxic. A previous study by Asterion has shown that the use of glycosylated-linkers between two GH ligands to create protein-tandems resulted in their glycosylation and an increased molecular weight (MW) whilst maintaining biological activity. The use of this technology using GCSF as an example will be presented, but can be easily applied to other molecules such as leptin.

Hypothesis: The incorporation of variable glycosylated linkers between two GCSF ligands will create a construct with high molecular weight and protected from proteolysis resulting in reduced clearance with out blocking bioactivity.

Methodology: GCSF tandems with linkers containing between 2-8 NAT glycosylation motifs and their respective controls (Q replaces N in the sequence motif NAT so there is no glycosylation) were cloned, and sequenced. Following expression in Chinese hamster ovary (CHO) cells, expressed protein was analysed by SDS-PAGE to confirm molecular weights. *In vitro* bioactivity was tested using an AML-193 proliferation assay. Immobilised Metal Affinity Chromatography (IMAC) was used to purify the protein. Pharmacokinetic and pharmacodynamics properties of the purified GCSF tandem proteins were measured in normal Sprague Dawley rats with full ethical approval.

Results: Purified glycosylated tandems show increased molecular weight above that of controls when analysed by SDS-PAGE. All GCSF tandems show increased bioactivity in comparison to native GCSF. Following intravenous administration to rats, GCSF2NAT, GCSF4NAT, GCSF8NAT containing 2, 4 & 8 glycosylation sites respectively and GCSF8QAT (non-glycosylated GCSF tandem control) showed approximately 3-fold longer circulating half-life compared to that reported for the native GCSF (1.79 hours). Both GCSF2NAT and GCSF4NAT show a significant increase in the percentage of neutrophils over controls at 12 hours post injection. This effect however is short lived as the counts at 24+ hours are not significantly different to controls. GCSF8NAT shows an increase in the percentage of neutrophils that is only significant at 48 hours.

Conclusion: Results show that the use of glycosylated linkers to generate GCSF tandems results in molecules with increased molecular weight, improved *in vitro* bioactivity, longer circulating half-lives and enhanced neutrophilic population when compared to both native GCSF and the non-glycosylated tandem protein.

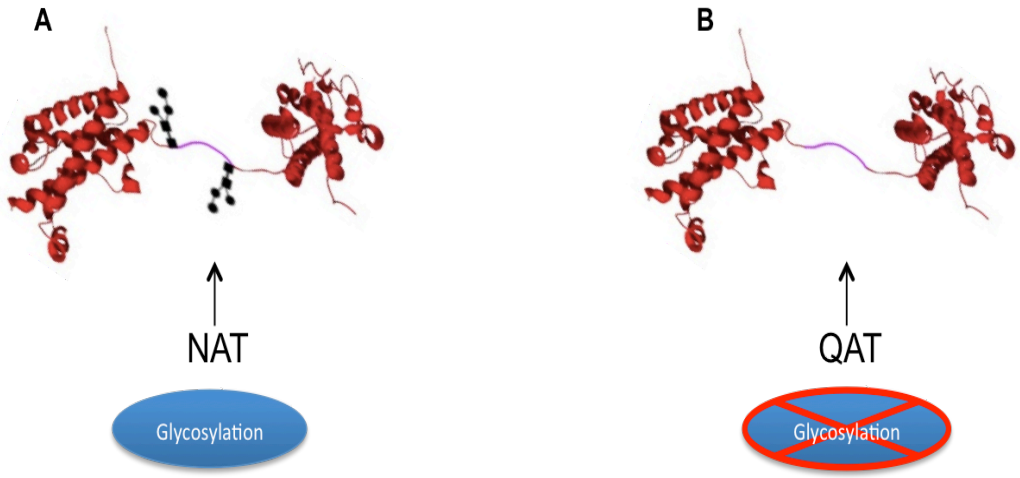


Figure 1: An example of 2NAT glycosylation motifs and its control 2QAT within a flexible linker $(Gly4Ser)_n$ between two GCSF ligands. (A) The glycosylation motif 2NAT inserted to the linker (glycosylated linker). (B) Non-glycosylation motif 2QAT control.

1. 1. LI & D'ANJOU 2009. Pharmacological significance of glycosylation in therapeutic proteins. *Curr Opin Biotechnol,* 20**,** 678-84.

2. 2. SINCLAIR & ELLIOTT 2005. Glycoengineering: the effect of glycosylation on the properties of therapeutic proteins. *J Pharm Sci,* 94**,** 1626-35.

3. 3. TANAKA *et al.* 1991. Pharmacokinetics of recombinant human granulocyte colony-stimulating factor conjugated to polyethylene glycol in rats. *Cancer Res,* 51**,** 3710-4.

4. CHUNG, H. K., KIM, S. W., BYUN, S. J., KO, E. M., CHUNG, H. J., WOO, J. S., YOO, J. G., LEE, H. C., YANG, B. C., KWON, M., PARK, S. B., PARK, J. K. & KIM, K. W. 2011. Enhanced biological effects of Phe140Asn, a novel human granulocyte colony-stimulating factor mutant, on HL60 cells. *BMB Rep,* 44**,** 686-91.
5. 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.
6. 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 Commun,* 3**,** 207-12.

7. COX, G. N., CHLIPALA, E. A., SMITH, D. J., CARLSON, S. J., BELL, S. J. & DOHERTY, D. H. 2014. Hematopoietic properties of
granulocyte colony-stimulating factor/immunoglobulin (G-CSF/IgG-Fc) fusion.

Abdulrahman Alshehri (Boseit@hotmail.com) working at security Forces Hospital, Makkah, Saudi Arabia for 12 years. I did my Phd at sheffield University, UK. I have been working in creating long acting therapies using different stratigies such as, glycosylated linkers. Using multiple techniquies like PCR, gene cloning, cell culture, pharmacodynamic and pharmacokinetic to generate these therapies. This research has awarded the prize for the best research amongst 500 researches around the world from the Society for Endocrinology conference held in Edinburgh in November 2015.