P75 NEUROTROPHIN RECEPTOR-FC FUSION PROTEIN FOR USE IN PAIN THERAPY

The present disclosure describes a glycosylated protein which possesses advantageous glycosylation when expressed from a particular cell line under specific conditions, an advantageous process for manufacture of the glycosylated protein and the glycosylated proteins use in the treatment of a range of conditions.

The glycosylated protein described in the present disclosure is in development for a range of indications. Glycosylation of a protein is a post-translation modification in which carbohydrates (including glycans and monosaccharides) are linked to the component protein typically through either O-glycosidic bonds (in particular to the oxygen atom of a hydroxyl group within the amino acid side chain of, for example, serine or threonine) or N-glycosidic bonds (in particular to the nitrogen atom of an amide group within the amino acid side chain of, for example, asparagine, or of a guanidino group within the amino acid side chain of arginine) Glycosylated proteins are typically produced by an enzyme catalysed reaction, in particular fermentation and expression from a cell line. Glycosylated proteins are expressed from a cell as the protein goes through post translational modification. Sugars attach themselves to side chains on the glycosylated protein. There can be variation in the glycosylated protein depending on the process by which the peptide has been expressed. Factors can include the cell from which the glycosylated protein is expressed and the environmental conditions such as nutrients, oxygen levels and lysis.

The present disclosure relates to a particularly advantageous glycosylated form of the protein described in WO2015/040398. The application describes the protein and a method of manufacture. The protein is disclosed as Sequence ID No. 3.

WO2013/136078 describes the use of p75NTR neurotrophin binding protein in the treatment of pain.

WO2016/009222 discusses the use of p75NTR neurotrophin binding protein in the treatment of osteoarthritis. Treatment includes curative or reversal of the disease as well as relief from symptoms and reflects disease modifying effect.

The current invention relates to a glycosylated p75NTR neurotrophin binding protein (NBP)-Fc fusion protein. The p75NTR(NBP)-Fc fusion protein finds use in the treatment of pain and other neurotrophic factor related pathologies such as psoriasis, eczema, rheumatoid arthritis, cystitis, endometriosis and osteoarthritis.

Figures

Fig 1 references Seq ID No. 1, the amino acid sequence for the glycosylated protein of the present disclosure.
Fig 2
Table 1 Seq ID No. 1 amino acid sequence indicating N-linked and O-linked glycosylation sites
Fig 3 Seq ID No. 2, the amino acid sequence for the comparator protein of the present disclosure.
Fig 4 describes the forms of glycosylation and the accepted notation for describing them
Fig 5 describes a P75NTR(NBP) sequence
Fig 6 describes Seq ID No. 3

Description

After a protein is expressed in the cell it goes through a process called post translational modification. Molecules of sugars and carbohydrates attach themselves to the protein (a process called glycosylation). Glycosylated proteins tend to fold themselves into a particular conformation. Glycosylation can influence the way the protein folds, or act as a steric block, changing the ability of

the protein to bind to other molecules in the body. Accordingly, glycosylation can have a great effect on the ability of a protein to be used for a particular indication. The choice of a particular cell to culture and the specific fermentation conditions can result in a glycosylated protein that is more appropriate for use.

The present disclosure provides:

A glycosylated p75NTR neurotrophin binding protein (NBP)-Fc fusion protein, comprising:
a p75NTR(NBP) portion, having at least 85% sequence identity with Seq ID No. 3; and
an immunoglobulin Fc portion, wherein, the p75NTR(NBP) and Fc portions are connected via a linker, the linker comprises a peptide of formula G_x, where x is 1, 2, 3, 4, 5 or 6 and wherein the linker does not comprise or consist of the sequence GGGGS.

Suitably in one aspect of the disclosure the linker portion is GGG

Suitably in one aspect of the disclosure the Fc is a human Fc

Suitably, the p75NTR(NBP) portion, has at least 90% sequence identity with Seq ID No. 3

Suitably, the p75NTR(NBP) portion, has at least 95% sequence identity with Seq ID No. 3

Seq ID No. 3 is:

KEACPTGLYTHSGECCKACNLGEGVAQPCGANQTVCEPCLDSVTFSDVVSATEPCKPCTECV
GLQSMSAPCVEADDAVCRCAYGYYQDETTGRCEACRVCEAGSGLVFSCQDKQNTVCEECP
DGTYSDEANHVDPCLPCTVCEDTERQLRECTRWADAECEEIPGRWITRSTPPEGSDSTAPSTQ
EPEAPPEQDLIASTVAGVVTTVMG

Another aspect of the present disclosure provides a glycosylated protein having at least 85% sequence identity with the protein of Seq ID No. 1. Another aspect of the present disclosure provides a glycosylated protein having at least 90% sequence identity with the protein of Seq ID No. 1. Another aspect of the present disclosure provides a glycosylated protein having at least 95% sequence identity with the protein of Seq ID No. 1.

In another aspect the present disclosure provides a glycosylated protein as described herein wherein the glycosylated protein binds to NT3 with a binding affinity (K_d) of between about 0.001 nM to about 50 nM

The present disclosure also provides a glycosylated protein, of SEQ ID No. 1, with the sequence:

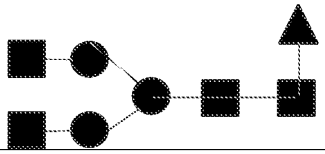
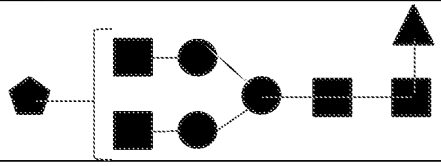
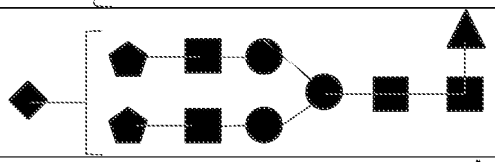
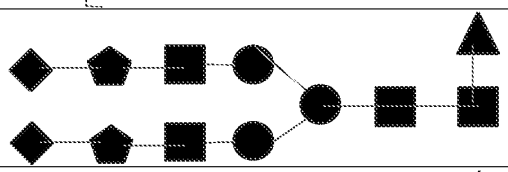
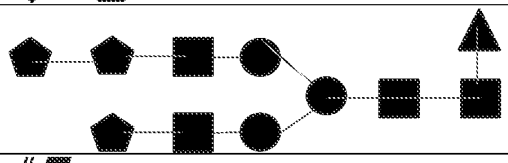
KEACPTGLYTHSGECCKACNLGEGVAQPCGANQTVCEPCLDSVTFSDVVSATEPCKPCTECV
GLQSMSAPCVEADDAVCRCAYGYYQDETTGRCEACRVCEAGSGLVFSCQDKQNTVCEECP
DGTYSDEANHVDPCLPCTVCEDTERQLRECTRWADAECEEIPGRWITRSTPPEGSDSTAPSTQ
EPEAPPEQDLIASTVAGVVTTVMGGGGGEPKSSDKTHTCPPCPAPELLGGPSVFLFPPKPKDTL
MISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQ
DWLNGKEYKCKVSNKALPAPI
EKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTP
PVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPG

Due to the branching of the chains and post- translational modifications, their structures are very complex and difficult to characterise. The glycosylated protein will likely be a mixture due to differences in the amount of glycosylation; both in terms of the length of the various glycosylation chain and variation in the sites at which glycosylation can take place.

It is the convention to number the constituent amino acids of a protein from the N terminus to the COOH terminus. Particularly preferred embodiments include glycosylated proteins of Seq ID No. 1, further characterised by glycosylation occurring at specific amino acid sites. Typically, glycosylation occurs on the side chain of an amino acid constituent of the protein. Sugars bind to either the N or O containing side chains (or both), such as the $CH_2C(O)NH_2$ side chain which defines asparagine, CH_2OH side chain which defines serine, and/or $CH(OH)CH_3$ side chain which defines threonine . Within this specification, the N or O atom on the side chain will be referred to by the position of the constituent amino acid within the protein. So, the nitrogen containing moiety on the side chain of the 32nd amino acid from the NH_2 terminus of the constituent protein, will be referenced as 'N32'.

In this context, N refers to an nitrogen containing moiety on the amino side chain and does not refer to a single letter amino acid code. In this context, specific amino acids will be referred to by three letter amino acid codes other than in SEQ ID Nos.

Some common glycans in mAb Fc are abbreviated as G0F, G1F, G2FS2, G3F & G2FS1 to indicate differences in monosaccharide composition.

G0F	
G1F	
G2FS1	
G2FS2	
G3F	
[illegible]	

The present disclosure provides a glycosylated protein with the sequence: (Seq ID No. 1)

KEACPTGLYTHSGECCKACNLGEGVAQPCGANQTVCEPCLDSVTFSDVVSATEPCKPCTECV

GLQSMSAPCVEADDAVCRCAYGYYQDETTGRCEACRVCEAGSGLVFSCQDKQNTVCEECP
DGTYSDEANHVDPCLPCTVCEDTERQLRECTRWADAECEEIPGRWITRSTPPEGSDSTAPSTQ
EPEAPPEQDLIASTVAGVVTTVMGGGGEPKSSDKTHTCPPCPAPELLGGPSVFLFPPKPKDTL
MISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQ
DWLNGKEYKCKVSNKALPAPI
EKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTP
PVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPG

It is understood that the glycosylated protein may be a mixture of glycosylated proteins.

The neutral N glycosylation profile in which sialic acid residues have been removed from the molecule ahead of analysis) includes G0F (representing 19-32%, suitably 20-25%,particularly suitably 22-24 % of the total neutral N-glycan population), G1F (representing 21-31%, suitably 26-28 % of the total neutral N-glycan population), G3F (representing 5-10%, suitably 7-8 % of the total neutral N-glycan population), G2FS1 representing 2-8% , suitably 4-6% of the total neutral glycan population; and G2FS2 representing 10-20% , suitably 14-17% of the total neutral glycan population

In one particularly suitable embodiment, the glycosylated protein is distinguished by glycosylation at both the N moieties, ‘N32’ and ‘N294’.

Suitably N32 and/or N294 are located in the side chain of an asparagine (Asn) amino acid residue.

Suitably the glycosylated protein is glycosylated at Asn32
Suitably the glycosylated protein is glycosylated at Asn294.
Suitably the glycosylated protein is glycosylated at Asn32 and Asn294.

Suitably, where the predominant glycan structure at Asn32 is G2FS2
Suitably the glycan structure at Asn294 is G1F
Suitably the glycan structure at Asn294 is G0F
Suitably the glycosylated protein is a mixture of glycosylation where the glycan structure at Asn294 are G0F and G1F

The glycans at the N32 site, in particular Asn32, have a MW of 1565or 3857 Da. In further embodiments, the glycans at the N294 site, in particular Asn294 have a molecular weight of 1419 or 2471 Da.

There are multiple constituent amino acids of the glycosylated protein of Seq ID 1 which possess a hydroxyl group on the side chain, in particular serine and threonine. In further embodiments, the peptide is characterized by O-linked glycosylation at between 1 and 12 of the OH containing amino acid side chains, in particular sides chains of serine and/or threonine residues.

In another embodiment wherein the protein is N-glycosylated, in particular as described in suitable embodiments described hereinabove, the N glycosylated protein also has O-linked glycosylation at 1 to 6 sites on the glycosylated protein, in particular at between 1 and 6 of the OH containing amino acid side chains, in particular sides chains of serine and/or threonine residues.
Suitably, there is O-linked glycosylation at 3 sites on the glycosylated protein.

In another embodiment, O linked glycosylation occurs at specific amino acids. Numbering the amino acids from N-terminus of the protein, with K being amino acid residue 1, O-linked glycosylation occurs at O44, O169, O171, O172, O179, O180, O183, O184, O198, O205, O206, ie the oxygen containing moiety on the side chain of amino acid residues 44, 169, 171, 172, 179, 180, 183, 184,

198, 205, 206, in particular the OH containing amino acid side chains, particularly sides chains of serine and/or threonine residues .

In a further embodiment, O-linked glycosylation occurs at at least 1 site, suitably 3 sites, suitably 6 sites, suitably all of these sites.

In a yet further embodiment, O-linked glycosylation occurs at position 169. This is independent of glycosylation at any other sites.

One of the glycans that can be found at an O linked position is HexNAc1Neu5NAc1Hex1

Another of the glycans that can be found at an O-linked position is HexNAc1Neu5NAc2Hex1

The O-linked glycans can be sialylated with 1-2 sialic acid molecules.

In suitable embodiments, the peptide of the present disclosure is characterized by glycosylation at the N containing sidechain on the amino acids at N32, in particular the side chain of an asparagine (Asn) amino acid residue,and N294 in particular the side chain of an asparagine (Asn) amino acid residue and at between 1 and 12 of the OH containing amino acid sidechains, in particular the side chain of a serine (Ser) amino acid residue and/or threonine (Thr) residue, .

In particular, the present disclosure is characterized by peptide of Seq ID No. 1 with glycosylation at the N32 site, Asn32,, the N294 site, Asn294, and between 1 and 6 of the OH containing amino acid side chains, in particular the side chain of a serine (Ser) amino acid residue and/or threonine (Thr) residue.

In suitable embodiments the peptide of Seq ID No. 1 with glycosylation at the Asn32 site, the Asn294 site and the between 1 and 3 of the OH containing amino acid side chains, in particular the side chain of a serine (Ser) amino acid residue and/or threonine (Thr) residue..

In suitable embodiments the peptide of Seq ID No. 1 with glycosylation at the Asn32 site, the Asn294 site and 3 of the OH containing amino acid side chains, in particular the side chain of a serine (Ser) amino acid residue and/or threonine (Thr) residue.

In a particularly suitable embodiment the disclosure provides a glycosylated protein wherein 90% of the glycosylated protein has a MW of 109kDa, the glycosylated protein is glycosylated at Asn294 where the glycan structures at Asn294 are G0F and G1F; and glycosylated at Asn32 where the predominant glycan structure is G2FS1 and G2FS2

In another particularly suitable embodiment the disclosure provides a glycosylated protein wherein 90% of the glycosylated protein has a MW of 109kDa, the glycosylated protein is glycosylated at Asn294 where the glycan structures at Asn294 are G0F and G1F; and glycosylated at ASN_{32} where the predominant glycan structure is G2FS1 and there is O-linked glycosylation at 3 sites on the glycosylated protein, in particular at 3 of the OH containing amino acid side chains, in particular sides chains of serine and/or threonine residues.

In another particularly suitable embodiment the disclosure provides a glycosylated protein wherein 90% of the glycosylated protein has a MW of 109kDa, the glycosylated protein is glycosylated at Asn294 where the glycan structures at Asn294 are G0F and G1F; and glycosylated at Asn32 where the predominant glycan structure is G2FS1 and there is O-linked glycosylation at 3 sites on the glycosylated protein, in particular at 3 of the OH containing amino acid side chains, in particular sides chains of serine and/or threonine residues; wherein the O-linked glycans comprise: HexNAc1Neu5NAc1Hex1 and HexNAc1Neu5NAc2Hex1.

The present invention provides for a molecule of Seq ID No. 1, with both N and O linked glycosylation. Fig 2 and below, show Seq ID No. 1, with N linked glycosylation sites shown in bold and O linked glycosylation sites shown underlined and in italics:

KEACPTGLYT_HSGECCKACN_LGEGVAQPCG_ANQTVCEPCL_DSVTFSDVVS_ATEPCKPC
TE 60
CVGLQSMSAP_CVEADDAVCR_CAYGYYQDET_TGRCEACRVC_EAGSGLVFSC_QDKQNT
VCEE 120
CPDGTYSDEA_NHVDPCLPCT_VCEDTERQLR_ECTRWADAEC_EEIPGRWITR_*ST*PPEGSD*S*
T 180
AP*ST*QEPEAP_PEQDLIA*ST*V_AGVV*TT*VMGG_GGEPK*SS*DK*T*_H*T*CPPCPAPE_LLGGPSVFLF
240
PPKPKDTLMI_SRTPEVTCVV_VDVSHEDPEV_KFNWYVDGVE_VHNAKTKPRE_EQYNSTY
RVV 300
SVLTVLHQDW_LNGKEYKCKV_SNKALPAPIE_KTISKAKGQP_REPQVYTLPP_SRDELTKN
QV_360
SLTCLVKGFY_PSDIAVEWES_NGQPENNYKT_TPPVLDSDGS_FFLYSKLTV_DKSRWQQGN
V_420
FSCSVMHEAL_HNHYTQKSL_SLSPG

The present invention provides for a molecule, having 90% or greater homology with sequence ID No. 1 with glycosylation at one or more of positions 32, 169, 171, 172, 179, 180, 183, 184, 198, 199, 205, 206, 216, 217, 222 & 294.

The present invention further provides for a molecule, having 95% or greater homology with of sequence ID No. 3 with glycosylation at one or more of positions 32, 169, 171, 172, 179, 180, 183, 184, 198, 199, 205, 206, 216, 217, 222 & 294.

The present invention provides for a molecule of sequence ID No. 3 with glycosylation at one or more of positions 32, 169, 171, 172, 179, 180, 183, 184, 198, 199, 205, 206, 216, 217, 222 & 294.

The present invention provides for a molecule as described above, with glycosylation at one of more of positions 169, 171, 172, 179, 180, 183, 184, 198, 199, 205, 206.

Suitably the present invention provides for a molecule as described above wherein there is glycosylation at 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 & 16 of the positions.
Suitably the present invention provides for a molecule as described above wherein there is glycosylation at 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 & 11 of the positions.

The present invention provides for a molecule as described above wherein there is glycosylation at 1-3 of the positions
The present invention provides for a molecule as described above wherein there is glycosylation at 1-6 of the positions
The present invention provides for a molecule as described above wherein there is glycosylation at 3-6 of the positions
The present invention provides for a molecule as described above wherein there is glycosylation at 1-9 of the positions
The present invention provides for a molecule as described above wherein there is glycosylation at 6-9 of the positions
The present invention provides for a molecule as described above wherein there is glycosylation at 6-12 of the positions
The present invention provides for a molecule as described above wherein there is glycosylation at 1-12 of the positions

The present invention provides for a molecule as described above wherein there is glycosylation at 1-16 of the positions
The present invention provides for a molecule as described above wherein there is glycosylation at 6-16 of the positions.

A molecule as described above wherein:

The N glycan at position 32 is selected from
F(6)A2G(4)2S(3)2, F(6)A2G(4)2S(3)1
Suitably glycan is F(6)A2G(4)2S(3)2

The Glycan at position 169 is selected from HexNAc(1), HexNAc(1)Hex(1), HexNAc(1)NeuAc(1), HexNAc(1)Hex(1)NeuAc(1), HexNAc(1)Hex(1)NeuAc(2)

Suitably the glycan is HexNAc(1), HexNAc(1)Hex(1), HexNAc(1)Hex(1)NeuAc(1) (HexNAc(1)Hex(1)NeuAc(1), HexNAc(1)Hex(1)NeuAc(2)

Suitably the glycan is HexNAc(1)Hex(1)NeuAc(2)

The Glycan at position 171 is selected from HexNAc(1), HexNAc(1)Hex(1), HexNAc(1)NeuAc(1), HexNAc(1)Hex(1)NeuAc(1), HexNAc(1)Hex(1)NeuAc(2)

Suitably the glycan is selected from HexNAc(1), HexNAc(1)Hex(1), HexNAc(1)Hex(1)NeuAc(1) , HexNAc(1)Hex(1)NeuAc(2)
Suitably the glycan is HexNAc(1)Hex(1)NeuAc(2)

The Glycan at position 172 is selected from HexNAc(1), HexNAc(1)Hex(1), HexNAc(1)NeuAc(1), HexNAc(1)Hex(1)NeuAc(1), HexNAc(1)Hex(1)NeuAc(2)

Suitably the glycan is selected from HexNAc(1) , HexNAc(1)Hex(1), HexNAc(1)Hex(1)NeuAc(2)
Suitably the glycan is HexNAc(1)Hex(1)NeuAc(1)

The Glycan at position 179 is selected from HexNAc(1), HexNAc(1)Hex(1), HexNAc(1)NeuAc(1), HexNAc(1)Hex(1)NeuAc(1), HexNAc(1)Hex(1)NeuAc(2)

Suitably the glycan is selected from HexNAc(1)Hex(1) or HexNAc(1)Hex(1)NeuAc(2)
Suitably the glycan is HexNAc(1)Hex(1)NeuAc(2)

The Glycan at position 180 is selected from HexNAc(1), HexNAc(1)Hex(1), HexNAc(1)NeuAc(1), HexNAc(1)Hex(1)NeuAc(1), HexNAc(1)Hex(1)NeuAc(2)

Suitably the glycan is selected from HexNAc(1) , HexNAc(1)Hex(1) , HexNAc(1)NeuAc(1) (N1S1), HexNAc(1)Hex(1)NeuAc(1) , HexNAc(1)Hex(1)NeuAc(2)
Suitably the glycan is HexNAc(1)Hex(1)NeuAc(2)

The Glycan at position 183 is selected from HexNAc(1), HexNAc(1)Hex(1), HexNAc(1)NeuAc(1), HexNAc(1)Hex(1)NeuAc(1), HexNAc(1)Hex(1)NeuAc(1), HexNAc(1)Hex(1)NeuAc(2)

Suitably the glycan is selected from HexNAc(1) , HexNAc(1)Hex(1) , HexNAc(1)Hex(1)NeuAc(1) , HexNAc(1)Hex(1)NeuAc(2)
Suitably the glycan is selected from HexNAc(1) HexNAc(1)Hex(1) and HexNAc(1)Hex(1)NeuAc(2)

The Glycan at position 184 is selected from HexNAc(1), HexNAc(1)Hex(1), HexNAc(1)NeuAc(1), HexNAc(1)Hex(1)NeuAc(1), HexNAc(1)Hex(1)NeuAc(1), HexNAc(1)Hex(1)NeuAc(2)

Suitably the glycan is selected from HexNAc(1) , HexNAc(1)Hex(1) , N1S1, HexNAc(1)Hex(1)NeuAc(1) , HexNAc(1)Hex(1)NeuAc(2)
Suitably the glycan is selected from HexNAc(1) , H1N1, HexNAc(1)Hex(1)NeuAc(1) , HexNAc(1)Hex(1)NeuAc(2)

The Glycan at position 185 is selected from HexNAc(1), HexNAc(1)Hex(1), HexNAc(1)NeuAc(1), HexNAc(1)Hex(1)NeuAc(1), HexNAc(1)Hex(1)NeuAc(2)

Suitably the glycan is selected from HexNAc(1) , HexNAc(1)Hex(1) , N1S1, HexNAc(1)Hex(1)NeuAc(1) , HexNAc(1)Hex(1)NeuAc(2)
Suitably the glycan is selected from HexNAc(1) , H1N1, HexNAc(1)Hex(1)NeuAC(1) , HexNAc(1)Hex(1)NeuAc(2)

The Glycan at position 198 is selected from HexNAc(1), HexNAc(1)Hex(1), HexNAc(1)NeuAc(1), HexNAc(1)Hex(1)NeuAc(1), HexNAc(1)Hex(1)NeuAc(2)

Suitably the glycan is selected from HexNAc(1), HexNAc(1)Hex(1)NeuAc(1) , HexNAc(1)Hex(1)NeuAc(2)
Suitably the glycan is selected from HexNAc(1) , HexNAc(1)Hex(1) , HexNAc(1)Hex(1)NeuAc(1) , HexNAc(1)Hex(1)NeuAc(2)

The Glycan at position 199 is selected from HexNAc(1), HexNAc(1)Hex(1), HexNAc(1)NeuAc(1), HexNAc(1)Hex(1)NeuAc(1), HexNAc(1)Hex(1)NeuAc(2)

Suitably the glycan is selected from HexNAc(1), HexNAc(1)Hex(1)NeuAc(1) , HexNAc(1)Hex(1)NeuAc(2)
Suitably the glycan is selected from HexNAc(1) , HexNAc(1)Hex(1) , HexNAc(1)Hex(1)NeuAc(1) , HexNAc(1)Hex(1)NeuAc(2)

The Glycan at position 205 is selected from HexNAc(1), HexNAc(1)Hex(1), HexNAc(1)NeuAc(1), HexNAc(1)Hex(1)NeuAc(1), HexNAc(1)Hex(1)NeuAc(2)

Suitably the glycan is selected from HexNAc(1) , HexNAc(1)Hex(1) , N1S1, HexNAc(1)Hex(1)NeuAc(1) , HexNAc(1)Hex(1)NeuAc(2)

The Glycan at position 206 is selected from HexNAc(1), HexNAc(1)Hex(1), HexNAc(1)NeuAc(1), HexNAc(1)Hex(1)NeuAc(1), HexNAc(1)Hex(1)NeuAc(2)

Suitably the glycan is selected from HexNAc(1) , HexNAc(1)Hex(1) , N1S1, HexNAc(1)Hex(1)NeuAc(1) , HexNAc(1)Hex(1)NeuAc(2)

The Glycan at position 216 is selected from HexNAc(1), HexNAc(1)Hex(1), HexNAc(1)NeuAc(1), HexNAc(1)Hex(1)NeuAc(1), HexNAc(1)Hex(1)NeuAc(2)

Suitably the glycan is selected from HexNAc(1) or HexNAc(1)Hex(1)
Suitably the glycan is HexNAc(1)

The Glycan at position 217 is selected from HexNAc(1), HexNAc(1)Hex(1), HexNAc(1)NeuAc(1), HexNAc(1)Hex(1)NeuAc(1), HexNAc(1)Hex(1)NeuAc(2)

Suitably the glycan is selected from HexNAc(1) , HexNAc(1)Hex(1) , HexNAc(1)Hex(1)NeuAc(1) , HexNAc(1)Hex(1)NeuAc(2)
Suitably the glycan is selected from HexNAc(1) , HexNAc(1)Hex(1) , HexNAc(1)Hex(1)NeuAc(1) ,

The Glycan at position 222 is selected from HexNAc(1), HexNAc(1)Hex(1), HexNAc(1)NeuAc(1), HexNAc(1)Hex(1)NeuAc(1), HexNAc(1)Hex(1)NeuAc(2)

Suitably the glycan is selected from HexNAc(1) , HexNAc(1)Hex(1) , HexNAc(1)Hex(1)NeuAc(1) , HexNAc(1)Hex(1)NeuAc(2)
Suitably the glycan is selected from HexNAc(1) , HexNAc(1)Hex(1) , HexNAc(1)Hex(1)NeuAc(2)
Suitably the glycan is selected from HexNAc(1) , HexNAc(1)Hex(1) , HexNAc(1)Hex(1)NeuAc(1)
Suitably the glycan is selected from HexNAc(1) , HexNA(1)Hex(1) ,

The N glycan at position 294 is selected from
F(6)A2, F(6)A2[6}G(4)1 & F(6)A2[3}G(4)1, F(6)A2G(4)2

Suitably the glycan is F(6)A2

The present invention describes molecules with every combination of the disclosed glycans at 1-16 of the described glycan sites.

In one form of the invention is described a molecule having 90% or greater homology with sequence ID No. 1, with glycosylation at position 32, 294 and at 1-11 of each of positions 169, 171, 172, 179, 180, 183, 184, 198, 199, 205, 206.

In one form of the invention is described a molecule having 95% or greater homology with sequence ID No. 1, with glycosylation at position 32, 294 and at 1-11 of each of positions 169, 171, 172, 179, 180, 183, 184, 198, 199, 205, 206.

In one form of the invention is described a molecule of sequence ID No. 1, with glycosylation at position 32, 294 and at 1-11 of each of positions 169, 171, 172, 179, 180, 183, 184, 198, 199, 205, 206.

Suitably the present invention provides for a molecule as described above wherein there is glycosylation at 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 & 11 of the positions.

Suitably the present invention provides for a molecule as described above wherein there is glycosylation at 1-6 of the positions
Suitably the present invention provides for a molecule as described above wherein there is glycosylation at 3-6 of the positions
Suitably the present invention provides for a molecule as described above wherein there is glycosylation at 1-9 of the positions
Suitably the present invention provides for a molecule as described above wherein there is glycosylation at 6-9 of the positions
Suitably the present invention provides for a molecule as described above wherein there is glycosylation at 7-10 of the positions
Suitably the present invention provides for a molecule as described above wherein there is glycosylation at 9-9 of the positionsSuitably the present invention provides for a molecule as described above wherein there is glycosylation at 6-11 of the positions

In one form of the invention is described a molecule having 90% or greater homology with sequence ID No. 1, with glycosylation at each of positions 169, 171, 172, 177, 179, 180, 183, 184, 198, 199, 205, 206.

In one form of the invention is described a molecule having 95% or greater homology with sequence ID No. 1, with glycosylation at each of positions 169, 171, 172, 177, 179, 180, 183, 184, 198, 199, 205, 206.

In one form of the invention is described a molecule of sequence ID No. 1, with glycosylation at each of positions 169, 171, 172, 179, 180, 183, 184, 198, 199, 205, 206

In a further form of the invention, is described a molecule having 90% or greater homology with sequence ID No. 1, with glycosylation at each of positions 169, 171, 172, 179, 180, 183, 184, 198, 199, 205, 206, wherein

The Glycan at position 169 is selected from HexNAc(1), HexNAc(1)Hex(1), HexNAc(1)NeuAc(1), HexNAc(1)Hex(1)NeuAc(1), HexNAc(1)Hex(1)NeuAc(2)

Suitably the glycan is HexNAc(1), HexNAc(1)Hex(1), HexNAc(1)Hex(1)NeuAc(1) (HexNAc(1)Hex (1)NeuAc(1), HexNAc(1)Hex(1)NeuAc(2)

Suitably the glycan is HexNAc(1)Hex(1)NeuAc(2)

The Glycan at position 171 is selected from HexNAc(1), HexNAc(1)Hex(1), HexNAc(1)NeuAc(1), HexNAc(1)Hex(1)NeuAc(1), HexNAc(1)Hex(1)NeuAc(2)

Suitably the glycan is selected from HexNAc(1), HexNAc(1)Hex(1) , HexNAc(1)Hex(1)NeuAc(2)
Suitably the glycan is HexNAc(1)Hex(1)NeuAc(2)

The Glycan at position 172 is selected from HexNAc(1), HexNAc(1)Hex(1), HexNAc(1)NeuAc(1), HexNAc(1)Hex(1)NeuAc(1), HexNAc(1)Hex(1)NeuAc(2)

Suitably the glycan is selected from HexNAc(1) , HexNAc(1)Hex(1) , HexNAc(1)Hex(1)NeuAc(1) , HexNAc(1)Hex(1)NeuAc(2)
Suitably the glycan is HexNAc(1)Hex(1)NeuAc(1)

The Glycan at position 179 is selected from HexNAc(1), HexNAc(1)Hex(1), HexNAc(1)NeuAc(1), HexNAc(1)Hex(1)NeuAc(1), HexNAc(1)Hex(1)NeuAc(2)

Suitably the glycan is selected from HexNAc(1)Hex(1) or HexNAc(1)Hex(1)NeuAc(2)
Suitably the glycan is HexNAc(1)Hex(1)NeuAc(2)

The Glycan at position 180 is selected from HexNAc(1), HexNAc(1)Hex(1), HexNAc(1)NeuAc(1), HexNAc(1)Hex(1)NeuAc(1), HexNAc(1)Hex(1)NeuAc(2)

Suitably the glycan is selected from HexNAc(1) , HexNAc(1)Hex(1) , HexNAc(1)NeuAc(1) (N1S1), HexNAc(1)Hex(1)NeuAc(1) , HexNAc(1)Hex(1)NeuAc(2)
Suitably the glycan is HexNAc(1)Hex(1)NeuAc(2)

The Glycan at position 183 is selected from HexNAc(1), HexNAc(1)Hex(1), HexNAc(1)NeuAc(1), HexNAc(1)Hex(1)NeuAc(1), HexNAc(1)Hex(1)NeuAc(1), HexNAc(1)Hex(1)NeuAc(2)

Suitably the glycan is selected from HexNAc(1) , HexNAc(1)Hex(1) , HexNAc(1)Hex(1)NeuAc(1) , HexNAc(1)Hex(1)NeuAc(2)
Suitably the glycan is selected from HexNAc(1) HexNAc(1)Hex(1) and HexNAc(1)Hex(1)NeuAc(2)

The Glycan at position 184 is selected from HexNAc(1), HexNAc(1)Hex(1), HexNAc(1)NeuAc(1), HexNAc(1)Hex(1)NeuAc(1), HexNAc(1)Hex(1)NeuAc(1), HexNAc(1)Hex(1)NeuAc(2)

Suitably the glycan is selected from HexNAc(1) , HexNAc(1)Hex(1) , N1S1, HexNAc(1)Hex(1)NeuAc(1) , HexNAc(1)Hex(1)NeuAc(2)
Suitably the glycan is selected from HexNAc(1) , H1N1, HexNAc(1)Hex(1)NeuAc(1) , HexNAc(1)Hex(1)NeuAc(2)

The Glycan at position 185 is selected from HexNAc(1), HexNAc(1)Hex(1), HexNAc(1)NeuAc(1), HexNAc(1)Hex(1)NeuAc(1), HexNAc(1)Hex(1)NeuAc(2)

Suitably the glycan is selected from HexNAc(1) , HexNAc(1)Hex(1) , N1S1, HexNAc(1)Hex(1)NeuAc(1) , HexNAc(1)Hex(1)NeuAc(2)
Suitably the glycan is selected from HexNAc(1) , HexNAc(1)Hex(1), HexNAc(1)Hex(1)NeuAC(1) , HexNAc(1)Hex(1)NeuAc(2)

The Glycan at position 198 is selected from HexNAc(1), HexNAc(1)Hex(1), HexNAc(1)NeuAc(1), HexNAc(1)Hex(1)NeuAc(1), HexNAc(1)Hex(1)NeuAc(2)

Suitably the glycan is selected from HexNAc(1), HexNAc(1)Hex(1)NeuAc(1) , HexNAc(1)Hex(1)NeuAc(2)
Suitably the glycan is selected from HexNAc(1) , HexNAc(1)Hex(1) , HexNAc(1)Hex(1)NeuAc(1) , HexNAc(1)Hex(1)NeuAc(2)

The Glycan at position 199 is selected from HexNAc(1), HexNAc(1)Hex(1), HexNAc(1)NeuAc(1), HexNAc(1)Hex(1)NeuAc(1), HexNAc(1)Hex(1)NeuAc(2)

Suitably the glycan is selected from HexNAc(1), HexNAc(1)Hex(1)NeuAc(1) , HexNAc(1)Hex(1)NeuAc(2)
Suitably the glycan is selected from HexNAc(1) , HexNAc(1)Hex(1) , HexNAc(1)Hex(1)NeuAc(1) , HexNAc(1)Hex(1)NeuAc(2)

The Glycan at position 205 is selected from HexNAc(1), HexNAc(1)Hex(1), HexNAc(1)NeuAc(1), HexNAc(1)Hex(1)NeuAc(1), HexNAc(1)Hex(1)NeuAc(2)

Suitably the glycan is selected from HexNAc(1) , HexNAc(1)Hex(1) , N1S1, HexNAc(1)Hex(1)NeuAc(1) , HexNAc(1)Hex(1)NeuAc(2)

The Glycan at position 206 is selected from HexNAc(1), HexNAc(1)Hex(1), HexNAc(1)NeuAc(1), HexNAc(1)Hex(1)NeuAc(1), HexNAc(1)Hex(1)NeuAc(2)

Suitably the glycan is selected from HexNAc(1) , HexNAc(1)Hex(1) , N1S1, HexNAc(1)Hex(1)NeuAc(1) , HexNAc(1)Hex(1)NeuAc(2)

Suitably the molecule has 95% homology with Seq ID No. 1
Suitably the molecule is Seq ID No. 1

In a further form of the invention, is described a molecule having 90% or greater homology with sequence ID No. 1, with glycosylation at each of positions 169, 171, 172, 177, 179, 180, 183, 184, 198, 199, 205, 206, wherein

The Glycan at position 169 is HexNAc(1)Hex(1)NeuAc(2)

The Glycan at position 171 is HexNAc(1)Hex(1)NeuAc(2)

The Glycan at position 172 is HexNAc(1)Hex(1)NeuAc(1)

The Glycan at position 179 is HexNAc(1)Hex(1)NeuAc(2)

The Glycan at position 180 is selected from HexNAc(1)Hex(1)NeuAc(2)

The Glycans at position 183 are selected from
HexNAc(1) HexNAc(1)Hex(1) and HexNAc(1)Hex(1)NeuAc(2)

The Glycans at position 184 are selected from HexNAc(1) , H1N1, HexNAc(1)Hex(1)NeuAc(1) , HexNAc(1)Hex(1)NeuAc(2)

The Glycans at position 185 is selected from
HexNAc(1) , HexNAc(1)Hex(1), HexNAc(1)Hex(1)NeuAC(1) , HexNAc(1)Hex(1)NeuAc(2)

The Glycans at position 198 are selected from HexNAc(1) , HexNAc(1)Hex(1) , HexNAc(1)Hex(1)NeuAc(1) , HexNAc(1)Hex(1)NeuAc(2)

The Glycans at position 199 are selected from HexNAc(1) , HexNAc(1)Hex(1) , HexNAc(1)Hex(1)NeuAc(1) , HexNAc(1)Hex(1)NeuAc(2)

The Glycans at position 205 are selected from HexNAc(1) , HexNAc(1)Hex(1) , N1S1, HexNAc(1)Hex(1)NeuAc(1) , HexNAc(1)Hex(1)NeuAc(2)

The Glycans at position 206 are selected from HexNAc(1) , HexNAc(1)Hex(1) , N1S1, HexNAc(1)Hex(1)NeuAc(1) , HexNAc(1)Hex(1)NeuAc(2)

Suitably the molecule has 95% homology with Seq ID No. 1
Suitably the molecule is Seq ID No. 1

Its understood that the molecule is expressed and that thus will result in molecules with the same amino acid backbone, but that there will be variation in the glycosylation seen, with the end product a mixture of different glycosylated forms of the invention.

The molecule may also be found as a dimer.

Glycosylation affects glycosylated protein binding, solubility, stability, pharmacokinetics, and pharmacodynamics (PK/PD), bioactivity and safety (e.g., immunogenicity). Importantly, Fc glycoforms impact antibody structure and effector functions. Thus, the glycosylation pattern of a therapeutic mAb is a critical quality attribute (CQA) that is frequently discussed and reviewed. Overall, glycan structural analysis and heterogeneity control are critical for the quality of all glycosylated therapeutic proteins and are especially important for biosimilar products, where the chemical similarity to the reference product can eliminate or decrease the scope of clinical studies needed for marketing approval.

The glycans on the molecule are covalently bound to sialic acid molecules. One of the effects of sialic acids is to add a charge to the molecule. The effect of charge on a molecule is two fold. Firstly in influences the folding of the molecule and its arrangement in space. The structure a molecule takes is key to its ability to bind, both in terms of how strongly it binds to a site and its selectivity for a site over other binding positions. Secondly the charge itself has an effect on binding and can be a key determinant of a molecules half life within the body.

In one embodiment, there are 16-17 sialic acid molecules on each glycosylated monomer of Seq ID No. 1
Suitably there are 16.3-16.9 sialic acid molecules on each glycosylated monomer of Seq ID No. 1

In particularly suitable embodiment there are 16.55-16.65 sialic acid molecules on each glycosylated monomer of Seq ID No. 1

There are also variations in the sialic acids bound to the glycan.

The predominant sialic acid is N-Acetylneuraminic acid (Neu5Ac) and the minor version is N-glycolyl neuraminic acid (Neu5Gc). Neu5Gc is not expressed in humans and therefore there is an immunogenicity risk associated with this sialic acid. Having a very low level is a critical quality attribute for a therapeutic glycosylated protein.

Suitably there are 16.85-16.1, more suitably 16.65-16.3, even more suitably 16.1-16.35 molecules of Neu5Ac for each monomer of Seq ID No. 1.

Suitably there are 0.2-28, more suitably 0.24-0.28, even more suitably 0.255-0.265 molecules of Neu5G for each monomer of Seq ID No. 1.

As used herein, the term, "Fc" or "immunoglobulin Fc" or "Ig Fc" is understood to mean the carboxyl-terminal portion of an immunoglobulin chain constant region, in particular an immunoglobulin heavy chain constant region, or a portion thereof. Particularly the immunoglobulin Fc comprises 1) a CH1 domain, a CH2 domain, and a CH3 domain, optionally with an immunoglobulin hinge region, 2) a CH1 domain and a CH2 domain, optionally with an immunoglobulin hinge region, 3) a CH1 domain and a CH3 domain, optionally with an immunoglobulin hinge region, 4) a CH2 domain and a CH3 domain, optionally with an immunoglobulin hinge region or 5) a combination of two or more domains selected from but not limited to CH1, CH2 and CH3 optionally combined with an immunoglobulin hinge region. Particularly the immunoglobulin Fc comprises at least an immunoglobulin hinge region, a CH2 domain and a CH3 domain, and optionally a CH1 domain. Particularly the immunoglobulin Fc comprises or consists of an Fc or a portion of an Fc of an immunoglobulin of isotype including but not limited to IgG, IgM, IgA, IgD, IgE, more particularly, IgG1, IgG2, IgG3, IgG4, IgA1, IgA2, sIgA, more particularly IgG1, IgG2 or IgG4, most particularly IgG1. Optionally the immunoglobulin Fc also comprises amino acid mutations, deletions, substitutions or chemical modifications which serve to minimise complement fixation or antibody-dependent cellular cytotoxicity or which improve affinity of binding to the Fc receptor.

Further particularly the immunoglobulin Fc comprises or consists of any of: (a) a CH2 domain or portion thereof and a CH3 domain or portion thereof, (b) a CH2 domain or portion thereof, or (c) a CH3 domain or portion thereof, wherein the immunoglobulin Fc or portion thereof is of isotype including but not limited to IgG, IgM, IgA, IgD, IgE, further particularly, IgG1, IgG2, IgG3, IgG4, IgA1, IgA2, sIgA, more particularly, IgG, IgG2 or IgG4, most particularly IgG1.

Particularly the immunoglobulin Fc comprises or consists of the carboxy terminal region of an immunoglobulin heavy chain and may comprise the CH2 and/or CH3 domains, or parts thereof, from IgG, IgA or IgD antibody isotypes, or the CH2 and/or CH3 and/or CH4 domains, or parts thereof from IgM or IgE. Particularly the immunoglobulin Fc comprises or consists of a fragment of the Fc, comprising mainly CH3 and a small portion of CH2, as is derivable by pepsin digestion of the immunoglobulin. Particularly the immunoglobulin Fc comprises or consists of the full Fc region, comprising CH2 and CH3, additionally connected to the hinge region which is a short segment of heavy chain connecting the CH1 and CH2 regions in the intact immunoglobulin, as may be produced by papain digestion of the immunoglobulin. Particularly the immunoglobulin hinge region comprises or consists of a hinge region or part of a hinge region derived from an IgG particularly human IgG,

more particularly selected from but not limited to IgG1, IgG2, IgG3, or IgG4, most particularly IgG1 or is alternatively a species or allelic variant of the foregoing hinge region embodiments. The hinge region or a part of an immunoglobulin hinge region can be located at the C or N-terminal end of the Fc region, particularly at the N-terminal end.

In addition, the skilled person would understand that the therapeutic potential of the molecule of the present disclosure may be enhanced by the introduction of defined mutations in the crystallizable fragment (Fc) domains eg YTE (M252Y/S254T/T256E) and LS (M428L/N434S). Such techniques are well known to one skilled in the art. The effect of introducing such a mutation typically results in a molecule with increased half-life and prolonged duration of action. The effects of introducing the mutation are not limited to increased half life and duration of action.

The p75NTR(NBP)-Fc fusion protein of the present invention suitably binds NT3 with a binding affinity (K_d) of between about 0.001 nM to about 50 nM. In some preferred embodiments, the binding affinity (K_d) is between about 0.001 nM and any of about 0.01nM, 0.02 nM, 0.05 nM, 1 nM, 1.5 nM, 2 nM, 2.5 nM, 3 nM, 3.5 nM, 4 nM, 4.5 nM, 5 nM, 5.5 nM, 6 nM, 6.5 nM, 7 nM, 7.5 nM, 8 nM, 8.5 nM, 9 nM, 9.5 nM, 10 nM, 15 nM, 20 nM, 25 nM, 30 nM, 35 nM, 40 nM, 45 nM or 50 nM as measured in an in vitro binding assay for NGF, BDNF, NT3 or NT4/5 such as described herein suitably as measured by surface plasmon resonance at 20 °C. In some further preferred embodiments, binding affinity (K_d) is or is less than any of about 1 pM 10pM 25 pM, 50 pM 100 pM 250 pM, 300 pM, 350 pM, 400 pM, 450 pM, 500 pM, 550 pM, 600 pM, 650 pM, 700 pM, 750 pM, 800 pM, 850 pM, 950 pM or 1 nM as measured in an in vitro binding assay for p75NTR(NBP)-Fc fusion protein with the neurotrophins such as described herein, suitably as measured by surface plasmon resonance at 20 °C. In a further more preferred embodiment the binding affinity (K_d) is about 10 pM or about 1 nM, as measured in an in vitro binding assay for p75NTR(NBP)-Fc fusion protein with the neurotrophin. such as described herein, suitably as measured by surface plasmon resonance at 20 °C.

Suitably the p75NTR(NBP)-Fc fusion protein of the invention is for use in the treatment of pain. Without wishing to be bound by any particular theory, the inventors believe that the p75NTR(NBP)-Fc fusion protein achieves efficacy in the treatment of pain by effecting the functional activity of the aforementioned neurotrophins, (defined as modulating or up or down regulating the functional activity of the neurotrophins, NT3 ,for example the functional activity of the aforementioned neurotrophin resulting from the interaction with the respective receptor. By inhibiting NT3, the molecule can provide pain relief. By not suppressing NGF levels the molecule does not interfere with the bodies natural joint regeneration process. This avoids the side effects seen with anti-NGF drugs such as Tanezumab and can lead to regeneration of the joint, that is disease modifying treatment of osteoarthritis.

Suitably the p75NTR(NBP)-Fc fusion protein is selective for NT3 over NGF.
Suitably the fusion protein is 25 times more selective for NT3 over NGF
Suitably the fusion protein is 50 times more selective for NT3 over NGF
Suitably the fusion protein is 500 times more selective for NT3 over NGF
Suitably the fusion protein is 1000 times more selective for NT3 over NGF
Suitably the fusion protein binding to NT3 (Kd) is 1-20pm and binding to NGF is 500-1000pM.

Binding is assessed by measuring NGF binding to and activation of TrkA, as demonstrated in classical neuron survival assays (such as provided in Cowan *et al. Annu. Rev. Neurosci.* 2001;24:551–600).

BINDING LEVELS
Suitably the p75NTR(NBP)-Fc fusion protein effects the functional activity of NT3 as assessed by measuring NT3 binding to and activation of endogenous Trk receptor activity, as demonstrated in Trk receptor phosphorylation, mitogen-activated protein kinase phosphorylation reporter assays or cell survival and neurite extension assays.
Suitably the NT3 Kd for a molecule of the present invention is generated using the Biacore binding assay as described herein.

The p75NTR(NBP)-Fc fusion protein of the invention may also comprise a proteolytic cleavage site, optionally interposed between the p75NTR(NBP) portion and the immunoglobin Fc portion. The proteolytic cleavage site may be located in the linker or at the junction of the linker with either the p75NTR(NBP) portion or/and the immunoglobin Fc portion. The p75NTR(NBP) may optionally be cleaved from the immunoglobin Fc portion prior to formulation and or administration for therapeutic purposes.

The glycosylated proteins of the present disclosure have been found to have numerous, surprising advantages when compared to close analogues.

Without being bound by theory, its believed that glycosylation significantly influences the stereochemistry of the molecule. This leads to a number of advantages including:

- Vastly enhanced stability;
- Increased and advantageous selectivity for key receptors;
- Greater activity at key receptors;
- Stronger and selective binding to key targets; and significantly
- Disease modification in osteoarthritis patients is seen only with the glycosylated protein.

Suitably, glycosylation increases the ability of the molecule to inhibit NT3, and decreases the ability to inhibit NGF.
Suitably the glycosylated molecule has affinity for NT3with a binding affinity of between about 1 pM to about 500 pM
Suitably the glycosylated molecule selectivity for NT3 over NGF of 50-1000 x over NGF Kd

There are numerous additional advantages not detailed here.

Turning to the process by which the glycosylated protein is expressed, methods for expressing glycosylated proteins are well known to those skilled in the art. Such processes typically go through a number of stages; inoculation, fermentation and harvesting. The, cells, feeds and media used may be obtained from the company 'Lonza'. Addressing each individually:

Vector Construction
The process starts with codon optimisation of the DNA sequence to allow efficient expression in CHO cell lines with a signal sequence at the N-terminus that directs the protein for secreted expression and appropriate restriction enzyme sites to enable cloning into cloning into the glutamine synthetase (GS) vectors and a Kozak sequence between the 5' restriction enzyme site and the 'ATG' start codon. These sequences were submitted to and synthesised by Life Technologies and provided within a cloning vector. Other vectors with different secretion sequences and a different selectable marker (example dihydrofolate reductase (DHFR) instead of GS). The 1405 base pair (bp) DNA fragment encoding SEQ ID No. 1 was removed from the cloning vector into the GS expression vector pXC-17 via HindIII and EcoR1 digestion. The digested products (SEQ ID No. 1 DNA fragment and vector were ligated and the ligated products used to transform chemically competent *Escherichia Coli*

(E. coli) cells (for example TOP10 cells). Single colonies were analysed for the correct insert and one positive clone was selected and plasmid DNA prepared and sequence (in forward reverse orientation) to ensure the correct sequence was present. The plasmid was renamed p SEQ ID No. 1 /SGV. Sufficient linearised DNA for the cell line construction process was generated by linearisation of p SEQ ID No 1 /SGV with the restriction enzyme PvuI.

Lonza Biologics' mammalian Chinese Hamster Ovary (CHO) K1SV Glutamine Synthetase – Knock Out (GS-KO) expression system was used to produce SEQ ID No. 1. The CHOK1SV GS-KO host cell line is a derivative of the CHOK1SV host cell line with the endogenous gene for GS 'knocked out'. The host cell line was derived from Lonza Biologics' CHOK1SV GS-KO host working cell bank designated the code 760-W (prepared from the master host cell bank 760-M).

Other suitable CHO Cell lines include for example CHO-DGB with a DHFR selectable marker on the expression vector. There are also other GS-CHO knock out cell lines available that could be used (for example CHOSOURCE GS KO (Horizon discovery) and CHOZN (MilliporeSigma). There are also other mammalian systems available (for example PER.C6® human cells (Crucell).

Transfection by electroporation was performed via a single pulse using linearised plasmid DNA to generate stable CHOK1SV GS-KO transfectant minipools expressing SEQ ID No. 1. CaPO4 or lipid-based reagents (Lipofectamine, Fugene, Transfectin) can also be used to transfect cells.

A number of transfections was performed and split into a larger number of minipools. The day after transfection, selective medium containing methionine sulphoximine (MSX) was added to each transfectant minipool. The addition of MSX, which inhibits GS, to the growth medium increases the selective pressure in the transfectant minipools; following cloning this selective pressure is no longer required. If one used the same vector with a CHOK1SV cell line, the selective pressure would need to be applied throughout the cell line development and included in the early growth steps (before the inoculation of the fermenter).

After ~14 days of incubation, >500 transfectant minipools are expanded to suspension culture in wells of shaken 96 deep well plates. 3 days after transfer to suspension culture, samples from >500 wells were assayed for protein production using the Octet® method and the product concentration data generated were used to identify the overall highest producer transfectant minipools. Multiple high producing transfectant minipools were combined to generate >4 enriched transfectant pools.

The enriched transfectant pools were single cell sorted using a fluorescence activated cell sorter (FACS). Following incubation and imaging, supernatant samples from wells identified as containing a single colony were screened for SEQ ID No 1 production. >500 clonal cell lines were subsequently selected for further evaluation in an abridged fed-batch suspension culture productivity screen and transferred to suspension culture in shaken 96 deep well plates. Other methods can be used to derive monoclonal cell lines (for example a two step dilution cloning in 96-well plates or using semi-solid media plates.

>500 clonal cell lines were successfully adapted to suspension culture and were screened for productivity in a fed batch media. Following this screen, 20 clonal cell lines were selected for further evaluation, based upon their productivity ranking and the parental transfectant pool from which they were derived; these were expanded to culture in shake-flasks and their growth during routine subculture assessed. 9 lead candidate cell lines were selected for progression and cryopreservation of associated research cell banks (RCB). Selection was based on the expression levels of SEQ ID No. 1, acceptable growth characteristics and image evidence that each cell line arose from a single colony. A 70-generation cell line stability study was initiated with all 9 lead candidate cell lines in which 2 independently subcultured lineages of each cell line were then established. The growth and productivity characteristics for each lineage were then evaluated at two points (~5 and ~35 generations beyond that of the associated RCBs) in fed-batch miniature bioreactor culture screens designed to mimic the cGMP manufacturing bioreactor culture process. Product from cultures of

each of the 9 cell lines evaluated at ~5 generations was also assessed for product quality using a range of assays.

After a review of all available data, six lead candidate cell lines were selected for continuation in the 70-generation cell line stability study. Once the 6 cell lines had all accrued greater than seventy generations beyond the associated RCB, a final fed-batch miniature bioreactor culture screen was undertaken in which early and late generation cultures were evaluated concurrently.

Following evaluation of the consistency of the growth, productivity and product characteristics data across the 70-generation study, the lead cell line was chosen. A 200 vial GMP master cell bank was manufactured from a single vial of the relevant RCB and tested in accordance with current regulatory requirements. When sufficient cells were obtained, cells were aliquoted in cryopreservation medium (92.5 % CM66 / 7.5% DMSO) into polypropylene vials (each containing approximately 1.5 x 10^7 viable cells) and cryopreserved in a controlled manner to -100.0°C. Vials are stored in a vapour phase liquid nitrogen autofill dewar in a good manufacturing practice (GMP) controlled area.

Inoculum

The molecule of the present disclosure is expressed from Chinese Hamster Ovary (CHO) cell lines, in particular the cell line CHOK1SV GS-KO. The cells are grown in medium CM16 (UKSL-7212).

Suitable alternative media include:
CD CHO (Thermofisher)
CD FortiCHO™ Medium
CD OptiCHO™ MediumActiCHO (Cytiva)
EX-CELL® Advanced Medium (Merck Millipore)
Cellvento CHO (Merck Millipore)

The inoculum is grown then sequentially transferred via a series of containers of increasing volume:

a) 5-30ml
b) 30-50ml
c) 50-100ml
d) 100-200ml
e) 200-400ml

The containers are on a shaker platform and should be maintained at a temperature range of 34-38C, suitably 36-37C, most suitably at 36.5C.

Fermentation

The grown inoculum material is transferred to 100L cell bag, in CN68 (UKSL-8689) The material should be maintained at a temperature range of 34-38C, suitably 36-37C, most suitably 36.5C. From there it is transferred to a standard air lift type bioreactor.

The pH should be maintained between 6.7-7.3.

Antifoam agents as known to those skilled in the art may be used. Suitable agents include:

Foam away (Thermofisher) & HyClone™ Antifoam (Cytiva)

A constant O_2 flow should be maintained within the reactor, such that the dissolved O_2 level in the medium is 25-65%.

For days 0-7 after transfer to the bioreactor, the pH is suitably 6.84-6.96
Most suitably pH is 6.9.

For days 7-harvest, pH is suitably 7.04-7.16
Most suitably pH is 7.10

Whilst in the bioreactor, the N2 flow rate is 2.8-4.0L/min, suitably 3.2L/min
In another embodiment the N2 flow rate is 0.6L/min

Whilst in the bioreactor, the cells are fed. The feeds are provided at different times and with a variety of constituents:

a) Lonza's SF96 (UKSL-17272) provided continuously
b) D-glucose provided variably but continuously. Feed is stimulated if levels drop to <3.0g/L. Feed is provided as 400g/L
c) Lonza's SF71(UKSL-8683). This is added on days 3, 6, 8 and 10 in 1.04Kg doses in 5L
d) Lonza's SF54 (UKSL-5118). This is added on days 3, 6, 8 and 10 in 0.352Kg doses in 5L
e) Lonza's SF72 (UKSL-8710). This is added on days 3, 6, 8 and 10 in 1kg doses in 10L.

Suitable alternative feeds include:

Efficient feeds (A+ B+ or C+) Gibco; EfficientFeed™ (A+ B+ or C)+ AGT™ Supplement; Cellvento®4 Feed COMP (Merck Millipore); EX-CELL® Advanced CHO Feed 1 (Merck Millipore); ActiCHO feeds A and B (Cytiva)

These can be added singly or in combination and at different times in the process.

Harvesting

Cells are harvested within

a) 30 hours of cell culture viability reaching 75% max cell concentration; or
b) Maximum of 12 days' post inoculation.

The cell medium is cooled to 12-18C, prior to harvest. There is no feed; pH is maintained at 7.25 and N_2 flow is set to sparge. The pH can be adjusted to and maintained at 7.25 if necessary by use of dissolved CO_2

The supernatant is filtered in a three-part process.

Stage 1, 1.1m2 Millipore Millistak D0HC (6 filters/rack)
Stage 2, 1.1m2 Millipore Millistak B1HC (2 filters/rack)
Stage 3 10" Millipore Duropore KVGL (0.22Um filter into 500L Bioprocess container).

The material is sparged at max air flow rate.

Filtrate is stored at 5-3C

After filtration from the cells after fermentation, the mixture is purified. Techniques for purification are well known to those skilled in the art. Suitable techniques would include the following 5 step process:

1 Running through a column, which binds the Fc region of the glycosylated protein.
2 Low pH hold
3 Concentrating the material in a buffer then:
4 Running through a first column, from which the product is eluted

5 Run through a second ion exchange column using a gradient elution.

Confirmation that this is the correct glycosylated protein can be obtained by a Sandwich Elisa, which will confirm the presence of the P75 region and the Fc.

Additional confirmation of the glycosylated protein weight may be obtained by Mass Spectroscopy. Suitable techniques are known to those skilled in the art.

Specific identification of the glycan groups can be done by techniques known to those skilled in the art. One such technique would be to reduce the glycosylated protein and alkylate in the presence of urea. The N-glycan's are released with buffer made up in deuterated water. The release of an N-glycan from an asparagine residue results in deamidation of that residue. This results in a +0.98 Da mass shift when released in water and +2.99 Da mass shift in deuterated water. The samples can then be digested with trypsin or glu-C to generate peptides for the Fc and fusion glycosylated protein glycosylation sites. The proteinase digested samples were subjected to LC-MS analysis.

Preferred CHO cells for use in the disclosure are CHO1SV GSKO cells. Particularly suitable for use are Lonza's CHO cells from their GS Xceed™ cell line.

Suitable bioreactors or fermentation vessels are known to those skilled in the art and include stirred tank or airlift fermenters. Preferred vessels are airlift fermenters.

The glycosylated protein of the present invention is known to be efficacious in the treatment of disease.

Accordingly a further embodiment provides the protein for use in the treatment of eczema, psoriasis, dermatitis, prurigo lesions of atopic dermatitis, obsessive-compulsive disorder, depression, schizophrenia, anorexia nervosa, bulimia nervosa, cardiovascular diseases, coronary atherosclerosis, acute coronary syndromes, obesity, type 2 diabetes, metabolic syndrome, multiple sclerosis, accelerating wound healing, treatment of skin ulcers and corneal ulcers, neurodegeneration, neurodevelopmental or neurological conditions, Huntington's disease, Rett syndrome, dementia, Alzheimer's disease, autism, development neurodegenerative disorders, primary open angle glaucoma, to reduce neural degeneration and to promote peripheral nerve regeneration, cancer, breast cancer, rheumatoid arthritis, osteoarthritis, cystitis and endometriosis.

The present disclosure provides a method of treating eczema, psoriasis, dermatitis, prurigo lesions of atopic dermatitis, obsessive-compulsive disorder, depression, schizophrenia, anorexia nervosa, bulimia nervosa, cardiovascular diseases, coronary atherosclerosis, acute coronary syndromes, obesity, type 2 diabetes, metabolic syndrome, multiple sclerosis, accelerating wound healing, treatment of skin ulcers and corneal ulcers, neurodegeneration, neurodevelopmental or neurological conditions, Huntington's disease, Rett syndrome, dementia, Alzheimer's disease, autism, development neurodegenerative disorders, primary open angle glaucoma, to reduce neural degeneration and to promote peripheral nerve regeneration, cancer, breast cancer, rheumatoid arthritis, osteoarthritis, cystitis and endometriosis in a subject, the method comprising administering to a subject in need thereof a therapeutically effective amount of a glycosylated protein of the present disclosure.

The present disclosure is applicable in both human and veterinary medical fields. Suitably the individual is a mammal, for example a companion animal such as a horse, cat or dog or a farm animal such as a sheep, cow or pig. Most suitably the individual is a human.

According to another preferred aspect of the disclosure there is provided the glycosylated protein for use in the treatment of pain.

The present disclosure provides a method of treating pain in a subject, the method comprising administering to a subject in need thereof a therapeutically effective amount of a glycosylated protein of the present disclosure.

Pain may include but is not limited to:
(a) acute pain and/or spontaneous pain,
(b) chronic pain and or on-going pain,
(c) inflammatory pain including any one of arthritic pain, pain resulting from osteoarthritis or rheumatoid arthritis, resulting from inflammatory bowel diseases, psoriasis and eczema
(d) nociceptive pain,
(e) neuropathic pain, including painful diabetic neuropathy or pain associated with post-herpetic neuralgia,
(f) hyperalgesia,
(g) allodynia,
(h) central pain, central post-stroke pain, pain resulting from multiple sclerosis, pain resulting from spinal cord injury, or pain resulting from Parkinson's disease or epilepsy,
(i) cancer pain,
(j) post-operative pain,
(k) visceral pain, including digestive visceral pain and non-digestive visceral pain, pain due to gastrointestinal (GI) disorders, pain resulting from functional bowel disorders (FBD), pain resulting from inflammatory bowel diseases (IBD), pain resulting from dysmenorrhea, pelvic pain, cystitis, interstitial cystitis or pancreatitis,
(l) musculo-skeletal pain, myalgia, fibromyalgia, spondylitis, sero-negative (non-rheumatoid) arthroplasties, non-articular rheumatism, dystrophinopathy, Glycogenolysis, polymyositis, pyomyositis,
(m) heart or vascular pain, pain due to angina, myocardial infarction, mitral stenosis, pericarditis, Raynaud's phenomenon, scleroderma, scleroderma or skeletal muscle ischemia,
(n) head pain including migraine, migraine with aura, migraine without aura cluster headache, tension-type headache.
(o) orofacial pain, including dental pain, temporomandibular myofascial pain or tinnitus, or
(p) back pain, bursitis, menstrual pain, migraine, referred pain, trigeminal neuralgia, hypersensitisation, pain resulting from spinal trauma and/or degeneration or stroke.
Treatment of pain includes, but is not limited to, preventing, ameliorating, controlling, reducing incidence of, or delaying the development or progression of pain and/or a symptom of pain.

In a further embodiment there is provided a glycosylated protein of the present disclosure for use in the treatment of osteoarthritis.

In suitable embodiments, the treatment of osteoarthritis includes relief from the symptoms of osteoarthritis. Suitably relief from the symptoms of osteoarthritis include, but are not limited to reduction in pain, inflammation, swelling, tenderness, joint stiffness or increase in joint mobility or any combination of these.

In a particularly suitable embodiment, treatment of osteoarthritis includes slowing or arresting of disease progression and/or reduction in cartilage loss. Suitably treatment of osteoarthritis includes reversal of disease progression, regrowth of cartilage and/or curative treatment. Suitably disease progression is determined by the rate of cartilage loss or regrowth. In other preferred embodiments, disease progression may be monitored by determining the number of chondrocytes present in a joint.

In other preferred embodiments, the treatment of osteoarthritis includes prophylactic treatment.

The present disclosure provides a method of treating osteoarthritis in a subject, the method comprising administering to a subject in need thereof a therapeutically effective amount of a glycosylated glycosylated protein of the present disclosure.

In a further embodiment there is provided a glycosylated protein of the present disclosure for use in the treatment of Alzheimer's.

The present disclosure provides a method of treating disease Alzheimers in a subject, the method comprising administering to a subject in need thereof a therapeutically effective amount of a glycosylated protein of the present disclosure.

According to another aspect of the disclosure there is provided the glycosylated protein for separate, sequential or simultaneous use in a combination combined with a second pharmacologically active compound. Suitably the second pharmacologically active compound of the combination may include but is not limited to;

- an opioid analgesic, e.g. morphine, heroin, hydromorphone, oxymorphone, levorphanol, levallorphan, methadone, meperidine, fentanyl, cocaine, codeine, dihydrocodeine, oxycodone, hydrocodone, propoxyphene, nalmefene, nalorphine, naloxone, naltrexone, buprenorphine, butorphanol, nalbuphine or pentazocine;
- a nonsteroidal anti-inflammatory drug (NSAID), e.g. aspirin, diclofenac, diflusinal, etodolac, fenbufen, fenoprofen, flufenisal, flurbiprofen, ibuprofen, indomethacin, ketoprofen, ketorolac, meclofenamic acid, mefenamic acid, meloxicam, nabumetone, naproxen, nimesulide, nitroflurbiprofen, olsalazine, oxaprozin, phenylbutazone, piroxicam, sulfasalazine, sulindac, tolmetin or zomepirac;
- a barbiturate sedative, e.g. amobarbital, aprobarbital, butabarbital, butabital, mephobarbital, metharbital, methohexital, pentobarbital, phenobartital, secobarbital, talbutal, theamylal or thiopental;
- a benzodiazepine having a sedative action, e.g. chlordiazepoxide, clorazepate, diazepam, flurazepam, lorazepam, oxazepam, temazepam or triazolam;
- an H_1 antagonist having a sedative action, e.g. diphenhydramine, pyrilamine, promethazine, chlorpheniramine or chlorcyclizine;
- a sedative such as glutethimide, meprobamate, methaqualone or dichloralphenazone;
- a skeletal muscle relaxant, e.g. baclofen, carisoprodol, chlorzoxazone, cyclobenzaprine, methocarbamol or orphrenadine;

- an NMDA receptor antagonist, e.g. dextromethorphan ((+)-3-hydroxy-N-methylmorphinan) or its metabolite dextrorphan ((+)-3-hydroxy-N-methylmorphinan), ketamine, memantine, pyrroloquinoline quinine, cis-4-(phosphonomethyl)-2-piperidinecarboxylic acid, budipine, EN-3231 (MorphiDex®, a combination formulation of morphine and dextromethorphan), topiramate, neramexane or perzinfotel including an NR2B antagonist, e.g. ifenprodil, traxoprodil or (–)-(R)-6-{2-[4-(3-fluorophenyl)-4-hydroxy-1-piperidinyl]-1-hydroxyethyl-3,4-dihydro-2(1H)-quinolinone;
- an alpha-adrenergic, e.g. doxazosin, tamsulosin, clonidine, guanfacine, dexmetatomidine, modafinil, or 4-amino-6,7-dimethoxy-2-(5-methane-sulfonamido-1,2,3,4-tetrahydroisoquinol-2-yl)-5-(2-pyridyl) quinazoline;
- a tricyclic antidepressant, e.g. desipramine, imipramine, amitriptyline or nortriptyline;
- an anticonvulsant, e.g. carbamazepine, lamotrigine, topiratmate or valproate;
- a tachykinin (NK) antagonist, particularly an NK-3, NK-2 or NK-1 antagonist, e.g. (αR,9R)-7-[3,5-bis(trifluoromethyl)benzyl]-8,9,10,11-tetrahydro-9-methyl-5-(4-methylphenyl)-7H-[1,4]diazocino[2,1-g][1,7]-naphthyridine-6-13-dione (TAK-637), 5-[[(2R,3S)-2-[(1R)-1-[3,5-bis(trifluoromethyl)phenyl]ethoxy-3-(4-fluorophenyl)-4-morpholinyl]-methyl]-1,2-dihydro-3H-1,2,4-triazol-3-one (MK-869), aprepitant, lanepitant, dapitant or 3-[[2-methoxy-5-(trifluoromethoxy)phenyl]-methylamino]-2-phenylpiperidine (2S,3S);
- a muscarinic antagonist, e.g. oxybutynin, tolterodine, propiverine, tropsium chloride, darifenacin, solifenacin, temiverine and ipratropium;
- a COX-2 selective inhibitor, e.g. celecoxib, rofecoxib, parecoxib, valdecoxib, deracoxib, etoricoxib, or lumiracoxib;
- a coal-tar analgesic, in particular paracetamol;
- a neuroleptic such as droperidol, chlorpromazine, haloperidol, perphenazine, thioridazine, mesoridazine, trifluoperazine, fluphenazine, clozapine, olanzapine, risperidone, ziprasidone, quetiapine, sertindole, aripiprazole, sonepiprazole, blonanserin, iloperidone, perospirone, raclopride, zotepine, bifeprunox, asenapine, lurasidone, amisulpride, balaperidone, palindore, eplivanserin, osanetant, rimonabant, meclinertant, Miraxion® or sarizotan;
- a vanilloid receptor agonist (e.g. resinferatoxin) or antagonist (e.g. capsazepine);
- a beta-adrenergic such as propranolol;
- a local anaesthetic such as mexiletine;
- a corticosteroid such as dexamethasone;
- a 5-HT receptor agonist or antagonist, particularly a 5-$HT_{1B/1D}$ agonist such as eletriptan, sumatriptan, naratriptan, zolmitriptan or rizatriptan;
- a 5-HT_{2A} receptor antagonist such as R(+)-alpha-(2,3-dimethoxy-phenyl)-1-[2-(4-fluorophenylethyl)]-4-piperidinemethanol (MDL-100907);

- a cholinergic (nicotinic) analgesic, such as ispronicline (TC-1734), (E)-N-methyl-4-(3-pyridinyl)-3-buten-1-amine (RJR-2403), (R)-5-(2-azetidinylmethoxy)-2-chloropyridine (ABT-594) or nicotine;
- Tramadol®;
- a PDEV inhibitor, such as 5-[2-ethoxy-5-(4-methyl-1-piperazinyl-sulphonyl)phenyl]-1-methyl-3-n-propyl-1,6-dihydro-7H-pyrazolo[4,3-d]pyrimidin-7-one (sildenafil), (6R,12aR)-2,3,6,7,12,12a-hexahydro-2-methyl-6-(3,4-methylenedioxyphenyl)-pyrazino[2',1':6,1]-pyrido[3,4-b]indole-1,4-dione (IC-351 or tadalafil), 2-[2-ethoxy-5-(4-ethyl-piperazin-1-yl-1-sulphonyl)-phenyl]-5-methyl-7-propyl-3H-imidazo[5,1-f][1,2,4]triazin-4-one (vardenafil), 5-(5-acetyl-2-butoxy-3-pyridinyl)-3-ethyl-2-(1-ethyl-3-azetidinyl)-2,6-dihydro-7H-pyrazolo[4,3-d]pyrimidin-7-one, 5-(5-acetyl-2-propoxy-3-pyridinyl)-3-ethyl-2-(1-isopropyl-3-azetidinyl)-2,6-dihydro-7H-pyrazolo[4,3-d]pyrimidin-7-one, 5-[2-ethoxy-5-(4-ethylpiperazin-1-ylsulphonyl)pyridin-3-yl]-3-ethyl-2-[2-methoxyethyl]-2,6-dihydro-7H-pyrazolo[4,3-d]pyrimidin-7-one, 4-[(3-chloro-4-methoxybenzyl)amino]-2-[(2S)-2-(hydroxymethyl)pyrrolidin-1-yl]-N-(pyrimidin-2-ylmethyl)pyrimidine-5-carboxamide, 3-(1-methyl-7-oxo-3-propyl-6,7-dihydro-1H-pyrazolo[4,3-d]pyrimidin-5-yl)-N-[2-(1-methylpyrrolidin-2-yl)ethyl]-4-propoxybenzenesulfonamide;
- a cannabinoid;
- metabotropic glutamate subtype 1 receptor (mGluR1) antagonist;
- a serotonin reuptake inhibitor such as sertraline, sertraline metabolite demethylsertraline, fluoxetine, norfluoxetine (fluoxetine desmethyl metabolite), fluvoxamine, paroxetine, citalopram, citalopram metabolite desmethylcitalopram, escitalopram, d,l-fenfluramine, femoxetine, ifoxetine, cyanodothiepin, litoxetine, dapoxetine, nefazodone, cericlamine and trazodone;
- a noradrenaline (norepinephrine) reuptake inhibitor, such as maprotiline, lofepramine, mirtazepine, oxaprotiline, fezolamine, tomoxetine, mianserin, buproprion, buproprion metabolite hydroxybuproprion, nomifensine and viloxazine (Vivalan®), especially a selective noradrenaline reuptake inhibitor such as reboxetine, in particular (S,S)-reboxetine;
- a dual serotonin-noradrenaline reuptake inhibitor, such as venlafaxine, venlafaxine metabolite O-desmethylvenlafaxine, clomipramine, clomipramine metabolite desmethylclomipramine, duloxetine, milnacipran and imipramine;
- an inducible nitric oxide synthase (iNOS) inhibitor such as S-[2-[(1-iminoethyl)amino]ethyl]-L-homocysteine, S-[2-[(1-iminoethyl)-amino]ethyl]-4,4-dioxo-L-cysteine, S-[2-[(1-iminoethyl)amino]ethyl]-2-methyl-L-cysteine, (2S,5Z)-2-amino-2-methyl-7-[(1-iminoethyl)amino]-5-heptenoic acid, 2-[[(1R,3S)-3-amino-4- hydroxy-1-(5-thiazolyl)-butyl]thio]-5-chloro-3-pyridinecarbonitrile; 2-[[(1R,3S)-3-amino-4-hydroxy-1-(5-

thiazolyl)butyl]thio]-4-chlorobenzonitrile, (2S,4R)-2-amino-4-[[2-chloro-5-(trifluoromethyl)phenyl]thio]-5-thiazolebutanol, 2-[[(1R,3S)-3-amino-4-hydroxy-1-(5-thiazolyl) butyl]thio]-6-(trifluoromethyl)-3 pyridinecarbonitrile, 2-[[(1R,3S)-3- amino-4-hydroxy- 1 -(5-thiazolyl)butyl]thio]-5-chlorobenzonitrile, N-[4-[2-(3-chlorobenzylamino)ethyl]phenyl]thiophene-2-carboxamidine, or guanidinoethyldisulfide;

- an acetylcholinesterase inhibitor such as donepezil;
- a prostaglandin E_2 subtype 4 (EP4) antagonist such as N-[({2-[4-(2-ethyl-4,6-dimethyl-1H-imidazo[4,5-c]pyridin-1-yl)phenyl]ethyl}amino)-carbonyl]-4-methylbenzenesulfonamide or 4-[(1S)-1-({[5-chloro-2-(3-fluorophenoxy)pyridin-3-yl]carbonyl}amino)ethyl]benzoic acid;
- a leukotriene B4 antagonist; such as 1-(3-biphenyl-4-ylmethyl-4-hydroxy-chroman-7-yl)-cyclopentanecarboxylic acid (CP-105696), 5-[2-(2-Carboxyethyl)-3-[6-(4-methoxyphenyl)-5E- hexenyl]oxyphenoxy]-valeric acid (ONO-4057) or DPC-11870,
- a 5-lipoxygenase inhibitor, such as zileuton, 6-[(3-fluoro-5-[4-methoxy-3,4,5,6-tetrahydro-2H-pyran-4-yl])phenoxy-methyl]-1-methyl-2-quinolone (ZD-2138), or 2,3,5-trimethyl-6-(3-pyridylmethyl),1,4-benzoquinone (CV-6504);
- a sodium channel blocker, such as lidocaine; or
- a 5-HT3 antagonist, such as ondansetron;
- a JAK inhibitor

and the pharmaceutically acceptable salts and solvates thereof.

According to a further aspect of the present disclosure there is provided a pharmaceutical composition for any one or more of treating, preventing, ameliorating, controlling, reducing incidence of, or delaying the development or progression of eczema, psoriasis, dermatitis, prurigo lesions of atopic dermatitis, obsessive-compulsive disorder, depression, schizophrenia, anorexia nervosa, bulimia nervosa, cardiovascular diseases, coronary atherosclerosis, acute coronary syndromes, obesity, type 2 diabetes, metabolic syndrome, multiple sclerosis, accelerating wound healing, treatment of skin ulcers and corneal ulcers, neurodegeneration, neurodevelopmental or neurological conditions, Huntington's disease, Rett syndrome, dementia, Alzheimer's disease, autism, development neurodegenerative disorders, primary open angle glaucoma, to reduce neural degeneration and to promote peripheral nerve regeneration, cancer, breast cancer, rheumatoid arthritis, osteoarthritis, cystitis and endometriosis, comprising the glycosylated protein described herein and a pharmaceutically acceptable carrier and/or an excipient.

Suitably the glycosylated protein of the present disclosure is prepared for or suitable for oral, sublingual, buccal, topical, rectal, inhalation, transdermal, subcutaneous, intravenous, intra-arterial, intramuscular, intracardiac, intraosseous, intradermal, intraperitoneal, transmucosal, vaginal, intravitreal, intra-articular, peri-articular, local or epicutaneous administration.

Suitably the glycosylated protein of the present disclosure or the pharmaceutical composition thereof is for, or prepared for administration between once to 7 times per week, further suitably between once to four times per month, further suitably between once to six times per 6 month period, further suitably once to twelve times per year. Suitably the medicament is to be or prepared to be peripherally administered in a period including but not limited to: once daily, once every two, three, four, five or six days, weekly, once every two weeks, once every three weeks, monthly, once every

two months, once every three months, once every four months, once every five months, once every six months, once every seven months, once every eight months, once every nine months, once every ten months, once every eleven months or yearly.

Further suitably the glycosylated protein or the pharmaceutical composition of this aspect is to be or prepared to be peripherally administered via a route including but not limited to one or more of; orally, sublingually, buccally, topically, rectally, via inhalation, transdermally, subcutaneously, intravenously, intra-arterially or intramuscularly, via intracardiac administration, intraosseously, intradermally, intraperitoneally, transmucosally, vaginally, intravitreally, epicutaneously, intra-articularly, peri-articularly or locally.

Suitably the glycosylated protein or the pharmaceutical composition is for, or is prepared for, administration at a concentration of between about 0.05 to about 200 mg/ml; suitably at any one of about 0.05, 0.1, 0.5, 1, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190 or 200 mg/ml +/- about 10% error, most suitably at about 3 mg/ml in veterinary applications and 0.1 in humans.

Suitably the glycosylated protein or the pharmaceutical composition is for, or is prepared for, administration at a concentration of between about 0.1 to about 200 mg/kg of body weight; suitably at any one of about 0.5, 1, 5, 10,15 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190 or about 200 mg/kg of body weight +/- about 10% error, most suitably at about 10 mg/kg in veterinary applications and 0.3 in humans.

Pharmaceutical compositions of this disclosure suitable for parenteral administration comprise one or more compounds of the disclosure in combination with one or more pharmaceutically-acceptable sterile isotonic aqueous or nonaqueous solutions, dispersions, suspensions or emulsions, or sterile powders which may be reconstituted into sterile injectable solutions or dispersions just prior to use, which may contain sugars, alcohols, antioxidants, buffers, bacteriostats, solutes which render the formulation isotonic with the blood of the intended recipient or suspending or thickening agents.

Examples of suitable aqueous and nonaqueous carriers which may be employed in the pharmaceutical compositions of the disclosure include water, ethanol, polyols (such as glycerol, propylene glycol, polyethylene glycol, and the like), and suitable mixtures thereof, vegetable oils, such as olive oil, and injectable organic esters, such as ethyl oleate. Proper fluidity can be maintained, for example, by the use of coating materials, such as lecithin, by the maintenance of the required particle size in the case of dispersions, and by the use of surfactants.

These compositions may also contain adjuvants such as preservatives, wetting agents, emulsifying agents and dispersing agents. Prevention of the action of microorganisms upon the subject compounds may be ensured by the inclusion of various antibacterial and antifungal agents, for example, paraben, chlorobutanol, phenol sorbic acid, and the like. It may also be desirable to include isotonic agents, such as sugars, sodium chloride, and the like into the compositions. In addition, prolonged absorption of the injectable pharmaceutical form may be brought about by the inclusion of agents which delay absorption such as aluminum monostearate and gelatin.

In some cases, in order to prolong the effect of a drug, it is desirable to slow the absorption of the drug from subcutaneous or intramuscular injection. This may be accomplished by the use of a liquid suspension of crystalline or amorphous material having poor water solubility. The rate of absorption of the drug then depends upon its rate of dissolution which, in turn, may depend upon crystal size and crystalline form. Alternatively, delayed absorption of a parenterally-administered drug form is accomplished by dissolving or suspending the drug in an oil vehicle.

Injectable depot forms are made by forming microencapsule matrices of the subject compounds in biodegradable polymers such as polylactide-polyglycolide. Depending on the ratio of drug to polymer, and the nature of the particular polymer employed, the rate of drug release can be controlled.

Depot injectable formulations are also prepared by entrapping the drug in liposomes or microemulsions which are compatible with body tissue.

A suitable formulation for injection, including transdermally, subcutaneously, intravenously, intra-arterially or intramuscularly administration would include a saline or oil solution, suitably buffered.

A specific example would include:

25 mM Histidine, 50 mM NaCl, 200 mM Mannitol and the compound or mixture of compounds of the present disclosure, buffered to pH6.5.

According to another aspect of the present disclosure there is provided a kit comprising:
(a) the glycosylated protein or the pharmaceutical composition; and
(b) instructions for the administration of an effective amount of said glycosylated protein, or pharmaceutical composition to an individual for any one or more of the prevention or treatment of eczema, psoriasis, dermatitis, prurigo lesions of atopic dermatitis, obsessive-compulsive disorder, depression, schizophrenia, anorexia nervosa, bulimia nervosa, cardiovascular diseases, coronary atherosclerosis, acute coronary syndromes, obesity, type 2 diabetes, metabolic syndrome, multiple sclerosis, accelerating wound healing, treatment of skin ulcers and corneal ulcers, neurodegeneration, neurodevelopmental or neurological conditions, Huntington's disease, Rett syndrome, dementia, Alzheimer's disease, autism, development neurodegenerative disorders, primary open angle glaucoma, to reduce neural degeneration and to promote peripheral nerve regeneration, cancer, breast cancer, rheumatoid arthritis, osteoarthritis, cystitis and endometriosis.

The kit may include one or more containers containing the glycosylated protein or pharmaceutical composition described herein and instructions for use in accordance with any of the methods and uses of the disclosure. The kit may further comprise a description of selecting an individual suitable for treatment. The instructions for the administration of the pharmaceutical composition may include information as to dosage, dosing schedule and routes of administration for the intended treatment.

According to yet another aspect of the present disclosure there is provided the glycosylated protein or the pharmaceutical composition for use in any one or more of the prevention or treatment or for ameliorating, controlling, reducing incidence of, or delaying the development or progression of a condition or the symptoms of a condition associated with any one or more of the neurotrophins NGF, BDNF, NT-3, NT-4/5.

- NGF (Nerve growth factor) binds with at least two classes of receptors: the p75NTR and TrkA, a transmembrane tyrosine kinase, it is involved in axonal growth, branching and elongation. Conditions and symptoms associated with NGF are known. NGF is expressed in and associated with inflammatory conditions and pain [Protein Sequence NP_002497.2, NP_038637]. Also, NGF has been shown to play a role in number cardiovascular diseases, such as coronary atherosclerosis, obesity, type 2 diabetes, and metabolic syndrome as well as in Multiple Sclerosis. Reduced plasma levels of NGF (and also of BDNF) have been associated with acute coronary syndromes and metabolic syndromes. NGF is also related to various psychiatric disorders, such as dementia, depression, schizophrenia, autism, Rett syndrome, anorexia nervosa, and bulimia nervosa and has also been implicated in development of Alzheimer's disease and neurodegenerative disorders NGF has also been shown to accelerate wound healing and there is evidence that it could be useful in the treatment of skin ulcers and corneal ulcers, it has been shown to reduce neural degeneration and to promote peripheral nerve regeneration in rats.

- BDNF (brain-derived neurotrophic factor) is a neurotrophin which supports neuronal survival and growth during development of the nervous system [Protein Sequence NP_001137277.1, NP_001041604]. BDNF binds cell surface receptors TrkB and p75NTR and also modulates the

activity of Alpha-7 nicotinic receptor. Conditions and symptoms associated with BDNF are known. BDNF has been shown to play a significant role in the transmission of physiologic and pathologic pain, particularly in models of acute pain, inflammatory pain and neuropathic pain, where BDNF synthesis is found to be greatly increased; also BDNF has been shown to be up-regulated in conditions of chronic pain as well as further conditions such as eczema and psoriasis. Down-regulation of BDNF is seen in depression, schizophrenia, obsessive-compulsive disorder, Alzheimer's disease, Huntington's disease, Rett syndrome, and dementia, as well as anorexia nervosa and bulimia nervosa.

- Neurotrophin-4 (NT-4), also known as neurotrophin-5 (NT-5), is a neurotrophic factor that signals predominantly through the p75NTR and TrkB receptors and promotes the survival of peripheral sensory sympathetic neurons. The mature peptide of this protein is identical in all mammals examined including human, pig, rat and mouse. [Protein Sequence NP_006170, NP_937833]. NT-4 is synthesized by most neurons of the dorsal root ganglion (DRG) and those in the paravertebral and prevertebral sympathetic ganglia, spinal dorsal and ventral horn and is found expressed in many tissues including the prostate, thymus, placenta and skeletal muscle. Conditions and symptoms associated with NT-4/5 are known. Defects in NT4/5 are associated with susceptibility to primary open angle glaucoma. Neurotrophin 4 has also been shown to contribute to breast cancer cell survival and is a target to inhibit tumour growth. NT-4/5 is known to be involved in pain-signaling systems such as nociceptive pain, upregulation of NT-4/5 is also seen in chronic inflammatory conditions of the skin, such as dermatitis, eczema, prurigo lesions of atopic dermatitis. Down regulation of NT-4/5 is seen in Alzheimer's Disease, Huntington's disease.

- Neurotrophin-3 (NT-3), is a neurotrophin that is structurally related to beta-NGF, BDNF, and NT-4, and that controls survival and differentiation of mammalian neurons and the maintenance of the adult nervous system, and may affect development of neurons in the embryo when it is expressed in human placenta. Conditions and symptoms associated with NT3 are known. NTF3-deficient mice generated by gene targeting display severe movement defects of the limbs. NT-3 signals through the Trk receptors and promotes the growth and survival of nerve and glial cells [Protein Sequence NP_001096124.1 and NP_032768]. The amino acid sequences of human, Mouse and rat NT-3 are identical. NT3 and its cognate receptor, tyrosine kinase C (TrkC), are known to modulate neuropathic pain and nociceptive pain and the mechanism of nociception and proprioception, for example NT3 expression is increased in the small DRG cells of neuropathic animals. NT3 expression is also associated with neuropathies such as diabetic polyneuropathy and HIV-related neuropathy, large fiber neuropathy including atrophy, it is further involved in the development of hyperalgesia (a decrease in the threshold of a normally noxious stimuli), allodynia (a non-noxious stimulus becomes noxious), and spontaneous pain (pain in the apparent absence stimuli) and is a known modulator of muscle pain.

The disclosure will now be described by reference to the following examples which are provided to illustrate, but not to limit, the disclosure.

Examples

Example 1 - Manufacturing Process.

The CHO cells and fermentation medium are available from Lonza.

The materials necessary for MabSelect SuReTM Affinity Chromatography are available from Sigma Aldrich/GE Lifesciences.

The materials necessary for POROS XS Cation Exchange Chromatography are available from ThermoFisher Scientific.

Step 1: Inoculum Expansion

The molecule of the present disclosure is expressed from Chinese Hamster Ovary cell lines, in particular the cell line CHOK1SV GS-KO. The cells are grown in medium CM16 (UKSL-7212). The inoculum is grown then sequentially transferred via a series of containers of increasing volume:

a) 5-30ml
b) 30-50ml
c) 50-100ml
d) 100-200ml
e) 200-400ml

The containers are on a shaker platform and should be maintained at a temperature range of 34-38C.

Step 2: Fermentation

The grown inoculum material is transferred to 100L cell bag, in CN68 (UKSL-8689) The material should be maintained at a temperature range of 34-38C.

Step 3: Harvest/Clarification

The production fermenter is harvested and the cells and cell debris are removed by depth filtration. The clarified supernatant is aseptically filtered via 0.22 μm filters into sterile containers. The filled containers are transferred to the 5 ± 3°C product cold room pending further processing for a maximum hold time of 14 days prior to completion of first purification step.

Step 4: Purification by MabSelect SuRe ™ Affinity Chromatography

MabSelect SuRe ™ resin used for the purification is dedicated to bulk glycosylated protein product and can be used for multiple cycles.

The processed harvest supernatant is removed from 5 ± 3°C cold room storage and allowed to equilibrate to ambient temperature (15 to 25°C) and divided into aliquots and loaded onto the MabSelect SuRe™ column in successive cycles and may be processed individually or as a run consisting of multiple cycles. An aliquot contains sufficient product to load the column at up to 6 g glycosylated protein/litre of packed matrix.

The chromatography column is packed using 50mM Sodium Phosphate / 250 mM sodium chloride pH 7.0. The column is Height Equivalent to a Theoretical Plate (HETP) tested and the peak asymmetry is calculated.

Each cycle proceeds as follows:

- The column is cleaned with 0.1M Sodium Hydroxide before use and after each cycle.
- The chromatography column is equilibrated with 50 mM Sodium Phosphate/ 250 mM sodium chloride, pH 7.0 buffer.
- Unbound contaminants are removed by washing the column in three stages:
    - 2CV of 50 mM Sodium Phosphate/ 250 mM sodium chloride, pH 7.0 buffer.
    - 4 CV of 50 mM Sodium Phosphate/ 2M sodium chloride, pH 7.0 buffer
    - 2CV of 50 mM Sodium Phosphate/ 250 mM sodium chloride, pH 7.0 buffer.

- Bound antibody is eluted from the column as a single fraction using 100 mM Sodium Citrate pH 3.6
- The column is washed post elution using 100 mM Citric Acid pH 2.1 buffer.

The MabSelect SuRe™ eluate is (if required) pH adjusted with 2M Acetic Acid or 2.0 M Tris Base and held at pH 3.50 ± 0.05 for between 60 and 75 minutes as a viral inactivation step, before adjusting the pH to 7.0 ± 0.10 with 2.0 M Tris Base.

Step 5: Concentration and Diafiltration

Providing that the MabSelect SuRe™ chromatograms meet the defined acceptance criteria for comparability as detailed in the associated batch record documentation, the glycosylated protein purified from all cycles is pooled and concentrated to 10.0 g/litre ± 1.0 g/litre using an ultrafiltration unit containing 50 kDa molecular weight cut off cassettes which are dedicated to this product and this process step. The product is then diafiltered into 40mM Sodium Acetate / 10mM Sodium Chloride pH 5.5 in preparation for the next column step.

Step 6: Purification by POROS XS Cation Exchange Chromatography

POROS XS matrix is used for the purification of one product batch; up to three cycles are performed and the resin is them discarded. The chromatography column is packed using 0.1 M sodium chloride solution. The column is HETP tested and the peak asymmetry is calculated.

The concentrated / diafiltered MabSelect SuRe ™ eluates are removed from 5 ± 3°C cold room storage and allowed to equilibrate to ambient temperature (15 to 25°C)

Each cycle proceeds as follows:

- The column is cleaned with 3 CV 0.5M sodium hydroxide.
- The chromatography column is pre-equilibrated with 250mM Sodium acetate pH5.5 (3CV) and then equilibrated with 3 CV 40 mM Sodium Acetate / 10mM Sodium Chloride pH5.5.
- The concentrated/diafiltered Mabselect SuRe™ eluate is loaded onto the column at up to 20 g/L of resin.
- The unbound product is washed through the column and collected from the flow-through as a single fraction using a 40 mM Sodium Acetate / 10mM Sodium Chloride pH5.5.
- Impurities bound to the column are removed by a post elution wash with 2.0 M Sodium Chloride pH 7.0
- The resin is subjected to a clean with 0.5M Sodium hydroxide (3 CV) and is then prepared for the next load by equilibrated with ≥5 CVs 40 mM Sodium Acetate / 10mM Sodium Chloride pH5.5.
- Samples are compared for similarity before pooling ahead of the next step.

Step 7: POROS HQ Anion Exchange Chromatography

POROS HQ matrix is used for the purification of one product batch; up to three cycles are performed and the resin is them discarded. The resin binds the product, allowing impurities to flow through the packed column. The bound product is eluted from the resin by increasing the conductivity of the buffer. Aggregated product is retained until eluted by a further increase in buffer conductivity

The chromatography column is packed using 0.1 M sodium chloride solution. The column is HETP (Height equivalent to a theoretical plate) tested and the peak asymmetry is calculated.

The POROS XS eluates are removed from 5 ± 3°C cold room storage and allowed to equilibrate to ambient temperature (15 to 25°C)

Each cycle proceeds as follows:

- The column is cleaned with 3 CV 0.5M sodium hydroxide.
- The chromatography column is pre-equilibrated with 200mM Sodium Phosphate pH7.0 (3CV) and then equilibrated with ≥5 CV 10mM Sodium Phosphate/40mM Sodium Chloride pH7.0.
- The POROS XS eluate is loaded onto the column at up to 20 g/L of resin.
- The column is washed with 3 column volumes 10mM Sodium Phosphate / 40mM Sodium Chloride pH7.0
- Product is eluted from the column with ≥3 CV 10mM Sodium Phosphate / 350mM Sodium Chloride pH7.0.
- Impurities bound to the column are removed by a post elution wash with 3CV 2.0 M Sodium Chloride
- The resin is subjected to a clean with 0.5M Sodium hydroxide (3 CV)
- 200mM Sodium Phosphate pH7.0 and is then prepared for the next load by pre-equilibration with 3 CVs 200mM Sodium Phosphate pH7.0 followed by equilibration with 10mM Sodium Phosphate / 40mM Sodium Chloride pH7.0
- Samples are compared for similarity. No pooling is necessary as stage 8 allows for sequential loading of the virus reduction filter.

Step 8 Virus Reduction Filtration

The POROS HQ eluate is passed through a 0.1μm pre-filter followed by a Planova™ 20N virus reduction filter up to a specified maximum pressure and volume of product per filter. The virus reduction filter is then flushed in 10mM Sodium Phosphate / 350mM Sodium Chloride pH7.0.

Step 9: Concentration and Diafiltration

Following the virus reduction filtration step, the product is concentrated to a maximum product concentration of 20 mg/mL using an ultrafiltration unit containing 30kDa molecular weight cut off cassettes which are dedicated to this product and process step. The product is then diafiltered into 25mM Histidine / 50mM Sodium Chloride / 200mM Mannitol pH6.5. The glycosylated protein concentration is determined by A280nm. The final product concentration specified is 10.0 mg/L ± 0.8 mg/mL.

Step 10: Bulk filtration and Dispensing

The product is filtered through a 0.5 /0.2 μm pre-filter followed by a 0.22 μm final filter into sterile high density polyethylene (HDPE) containers. After use the filter is integrity tested. The product is labelled and stored in quarantine at both 5 ± 3°C and ≤65°C pending batch disposition.

Example 2 – Characterisation of glycosylated forms of glycosylated protein

Glycosylation heterogeneity was assessed by neutral and charged Nitrogen-linked (N-linked) oligosaccharide profiling, monosaccharide composition and sialic acid content. Putative glycan

structures were further confirmed by mass spectrometry (MS) of the released N-linked glycans and from protease digestions analysed by RP HPLC-MS and MS/MS reduced tryptic peptide mapping.

Overall, glycan analysis indicated the presence of both N-linked and Oxygen-linked (O-linked) glycans, which were present at multiple sites on the glycosylated protein chain. The glycans detected as part of reduced neutral N-linked oligosaccharide profiling were predominantly G0F (representing 22.4 % of the total glycan population), G1F (representing 21.8 % of the total glycan population), G2F (representing 30.0 % of the total glycan population), G3F (representing 7.6 % of the total glycan population), G4F (representing 6.5 % of the total glycan population) and G4F+GN+Hex (representing 3.8 % of the total glycan population).

The charged N-linked oligosaccharide profile showed predominantly neutral glycans (55.1 % mol/mol total glycan). Mono-sialylated glycans were detected at a total of 9.5 % mol/mol total glycan. Di-sialylated glycans were detected at a total of 25.0 % mol/mol total glycan. Tri-sialylated glycans were detected at a total of 10.4 % mol/mol total glycan).

O-linked glycan composition (from material generated by sodium borohydride reductive release) was measured using a hydrophobic interaction chromatography-HPLC with charged aerosol detection (HILIC-CAD). The abundance was measured as % peak intensity. The reference standard was shown to contain two main glycan structures: NeuNAc α(2-3) Gal β(1-3) [NeuNAc α(2-6)] GalNAc (48.8%) and NeuNAcα(2-3)Galβ(1- 3)GalNAc (36.26%). The remaining O-glycans were as follows: NeuNAcα(2-3)Gal (5.92%) NeuNAc α(2-3) Gal β(1-3) [NeuGc α(2-6)] GalNAc (4.02%), NeuGcα(2-3)Galβ(1- 3)GalNAc (2.74%) NeuNAcα(2-6)GalNAc (-H2O) (2.22%), Gal β(1-3) NeuGc α(2- 3) GalNAc (0.47%) and Gal β(1-3) NeuNAc α(2-3) GalNAc (0.22%).

Monosaccharide composition analysis was consistent with the presence of N-linked glycans and O-linked glycans. The monosaccharides detected were mannose, glucosamine, galactose, galactosamine and fucose. Total percentage glycosylation (w/w) was 9.3 %.

The molar content of the N-acetylneuraminic acid (Neu5Ac) form of sialic acid and the N-glycolylneuraminic acid (Neu5Gc) form of sialic acid was assessed

Analysis showed:

Batch	mol Neu5Ac/mol glycosylated protein	mol Neu5Gc/mol glycosylated protein	mol total sialic acid/mol glycosylated protein
1	33.294	0.45	33.744
2	32.692	0.522	33.214
3	32.622	0.521	33.143
Mean	**32.87**	**0.50**	**33.37**
Stddev	0.37	0.04	0.33
CV	1.12	8.30	0.98

Primary Structure

Primary structure was assessed by RP HPLC-UV reduced tryptic peptide mapping and RP HPLC-MS and MS/MS reduced tryptic peptide mapping.

Tryptic peptide mapping with ultraviolet (UV) detection showed a well resolved profile at both 210 and 280 nm.

RP HPLC-MS reduced tryptic peptide mapping confirmed 100 % of the detected sequence as the expected sequence. MS/MS fragmentation analysis confirmed the identity of peptides representing 66 % of the expected sequence.

The N-terminal tryptic peptides obtained were detected as the expected sequence The first 17 residues of Glycosylated protein of Seq ID No 1 were confirmed by MS/MS analysis.

C-terminal tryptic peptides were detected as the expected sequence. 7 terminal residues were confirmed by MS analysis.

Low levels of potential deamidated variants were detected for tryptic peptides T7 and T31 (each at 1 % relative % deamidation). An oxidised variant was detected for tryptic peptide T15 (3 % relative % oxidation) and was confirmed by MS/MS analysis to be due to oxidation of a methionine residue in that peptide.

100 % occupancy of the N-linked glycosylation sites on Asparagine$_{32}$ (Asn$_{32}$) and (Asn$_{294}$) was detected. Occupancy was determined from the ratio of glycosylated tryptic peptide to aglycosylated tryptic peptide. Samples were digested individually with trypsin, endoproteinase Glu-C (Glu-C) and chymotrypsin in order to provide representation of the glycopeptides for Seq ID No. 1.

Following trypsin digestion, G2F+NeuAc was the predominant glycan structure detected at the Asn$_{32}$ N-linked glycosylation site and G1F was the predominant glycan structure at the Asn$_{294}$ N-linked glycosylation site. Following Glu-C digestion, only the glycans at Asn$_{294}$ were detected, with the predominant glycan being G0F.

At least three O-linked glycosylation sites were identified from the combined trypsin, Glu-C and chymotrypsin digest data.

Mass Isoform Heterogeneity

The molecular weight and mass isoform profile were determined by ESI-MS. Seq ID no 1was denatured and reduced, followed by treatment with peptide-N-glycosidase F (PNGaseF) and O-glycanase to remove N-linked and O-linked glycans respectively. ESI-MS detected a predominant isoform at 48,310 Da, which is consistent with the theoretical deglycosylated single chain (reduced) mass of 48,312 Da. Two further single chain species were detected with masses of 48,675 Da and 49,042 Da. These were likely to represent single chain species that were incompletely deglycosylated.

Isoforms were also detected within the mass range of 96,621 Da to 97,352 Da. These species were comparable in mass to the monomeric deglycosylated product (theoretical mass 96,564 Da) and were likely to represent incompletely reduced and / or incompletely deglycosylated species.

The range of isoforms detected in reduced and non-reduced samples suggests O-linked glycans were present after O-glycanase treatment.

Secondary Structure

Secondary structure was assessed by Fourier transform infra-red spectroscopy (FTIR). Results produced spectra that consisted predominantly of β-sheet (35.8 %) with α-helix detected at 10.5 %.

Free Thiol

Free thiol (sulphydryl group) concentration, under native and denatured conditions, was determined. The free thiol content detected under native and denatured conditions was less than the limit of quantitation (LOQ) (194.02 mmol/mol).

Mass Heterogeneity

Mass heterogeneity was determined by size exclusion chromatography with light scatter detection (SEC-LS). The main peak had an apparent apex molecular weight of 109 kDa and represented 99.4 % relative peak area. This was consistent with monomeric, glycosylated protein comprising SEQ ID No. 1. A high molecular weight species was detected with an apparent apex molecular weight of 221 kDa (0.6 % relative peak area), consistent with a glycosylated protein comprising SEQ ID No. 1 dimer.

Example 3 – Identification of glycosylation positions and identified glycans.

The molecule of the present invention, known as ‘Levi-04’ was analysed to identify glycan site and characteristics

Mass spectrometry of Immunomodulating metalloprotease (IMPa) and trypsin digested LEVI-04 to detect sites of O-glycosylation

Prepare two samples for each experiment.
Prepare denaturation buffer by adding 1mL 1M Tris pH8.0 to a 10mL volumetric flask and make up to 10mL with 8M guanidine HCl.

Add 100uL of denaturation buffer to an Eppendorf tube
Add 200ug LEVI-04 (i.e 20µL of 10mg/mL LEVI-04) to the tube containing the denaturation solution.
Add 1µL of 0.5M tris (2-carboxyethyl) phosphine (TCEP) reducing agent to tube. Close cap and vortex for a few seconds. Spin briefly in a microfuge to collect solution from tube wall. Incubate for 30 minutes at 37°C.
Add 2uL of 0.5M iodoacetamide alkylating agent to reduced sample. Close cap and vortex for a few seconds. Spin briefly in a microfuge to collect solution from tube wall. Incubate for 30 minutes at 37°C in the dark.
Prepare spin filters (Zeba™ Spin Desalting Columns, 7K MWCO (Thermo Fisher Scientific, Part No. 89882) before use:

- Remove the bottom of the filters (cap closed) and place in tube.
- Loosen the cap but do not take off completely
- Centrifuge 1500g for 1 minute at ambient temperature to remove the storage solution/
- Add 300µL 0.1M Tris pH8.0 and then centrifuge for 1 minute Centrifuge 1500g for 1 minute at ambient temperature.
- Perform step above twice to ensure column is equilibrated.

Add 120µL denatured, reduced and alkylated LEVI-04 to the equilibrated spin filter in a clean tube. Centrifuge 1500g for 2 minutes at ambient temperature and collect the flow through for next stage.

Digestion / O-Glycoprotease treatment
Tube 1:
Add 5µL of O-Glycoprotease solution (IMPa) and incubate at 37°C for 5h. After incubation has completed add 20µL of 0.5µg/µL trypsin solution and incubate at 37°C for a further 16-18 h.
Stop the reaction by addition of 2.8µL of 10% trifluoroacetic acid (TFA) and then transfer to Waters polypropylene vials for analysis.
Tube 2:
Add 20µL of 0.5µg/µL trypsin solution and incubate at 37°C for 16-18 hours. After incubation has completed add 5µL of O-Glycoprotease solution (IMPa) and incubate at 37°C for a further 5h.
Stop the reaction by addition of 2.8µL of 10% TFA and then transfer to Waters polypropylene vials for analysis.
Chromatographic and MS acquisition parameter
Chromatographic separation method for peptide mapping analysis

UHPLC system Agilent 1290 Infinity II UHPLC with column heater and on-line UV detector
Mobile phase A (MPA): 0.1% formic acid (FA), in water (v:v)
Mobile phase B (MPB): 0.1% FA, in acetonitrile (v:v)
Run/stop time: 98 min
Injection volume: 10 µL
Column temperature: set to 60 °C
Autosampler temperature: set to 5 °C
Column: Waters Acquity UPLC Peptide BEH 300 C18, 1.7 µm, 2.1 × 150 mm (Part No.186003687)

Chromatographic gradient for peptide mapping setup.

Time [min]	Eluent A [%]	Eluent B [%]	Flow Rate [mL/min]
0.0	99.5	0.5	0.30
5.0	99.5	0.5	0.30
66.0	60.0	40.0	0.30
66.1	10.0	90.0	0.30
69.9	10.0	90.0	0.30

70.0	99.0	1.0	0.30
75.0	99.0	1.0	0.30
76.0	50.0	50.0	0.30
79.0	50.0	50.0	0.30
79.1	99.0	1.0	0.30
84.0	99.0	1.0	0.30
85.0	50.0	50.0	0.30
88.0	50.0	50.0	0.30
88.1	99.5	0.5	0.30
98.0	99.5	0.5	0.30

MS acquisition parameters for peptide mapping analysis

Mass spectrometer:	Thermo Q-Exactive plus Orbitrap MS equipped with ESI (Electro Spray Ionization) Ion Source.
Ionization:	Electrospray Ionization (ESI)
Detection mode:	Heated-ESI Positive
Spray voltage:	3.5 kV
Capillary temperature:	350 °C
Sheath gas:	35 units
Auxiliary gas:	10 units
Probe heater	350 °C
Full MS	Full scan m/z 200 – 3000; Resolution 140'000; 1 Microscan
Data dependent MS^2 (TopN):	Top 5 signals from Full MS; Resolution 17'500; 1 Microscan
Divert valve:	0.0 min to waste, 1.8 min to source, 80.0 min to waste
MS run time	98.0 minutes

- Data acquisition was carried out on Chromeleon 7.2 software (Thermo Fisher Scientific).
- The 5 most intense ions from the Full-MS scan were selected for MS/MS by using a data dependent scheme and dynamic exclusion.

Data evaluation

Chromatogram and isotope extraction were performed in Chromeleon 7.2 (Thermo Fisher Scientific). The O-glycosylation site evaluation as performed using BioPharma Finder v3.2.

Parameters for the databased protein search in the software Peaks v8.5.

Reference sequence:	LEVI-04
Selected host cell:	CHO
Mass error tolerance:	5 ppm
Allowed modifications per peptide	3
Protease selected:	Trypsin (K, R), O-Glycoprotease (IMPa) (S, T)

Mass spectrometry of O-endoprotease digested LEVI-04 co-treated with PNGaseF and IdeS to detect site specific O-glycosylation

Sample preparation, LC-MS/MS analysis and data processing were based on Riley, N. M., & Bertozzi, C. R. (2022). Deciphering O-glycoprotease substrate preferences with O-Pair Search. *Mol Omics, 18*(10), 908-922. doi:10.1039/d2mo00244b

Sample preparation:

Two different digestion conditions using different O-glycoproteases were tested (with sample prep triplicates for each), either using OpeRATOR® enzyme (G2-OP1-020, Genovis) combined with pan sialidase (SialEXO®, G1-SM1-020, Genovis) or using ImpaRATOR™ enzyme(G1-IR1-020, Genovis). All enzymes were reconstituted according to manufacturer recommendations.

Digestion Mix

A digestion mix in 20 mM Tris-HCl buffer (pH 7.5) and containing all required enzymes was prepared for each condition from stock solutions, to reach a final concentration of 2.5 U/µL of O-glycoproteases enzymes (OpeRATOR or ImpaRATOR), pan sialidase (in combination with OpeRATOR only) and IdeS (FabRICATOR®, A0-FR1-020, Genovis), and final concentration of 5 U/µL of PNGaseF (from a 100 U/µL solution in water, P0708S, New England Biolabs).

Digestion

For each condition, add 50 µg (5 µL of 10mg/mL) LEVI-04 to 20 µL of digestion mix, for a final 1:1 protease unit : protein weight ratio (2:1 for PNGaseF) as directed by the manufacturer. Incubate at 37°C for 5h under gentle agitation (500 rpm).

Denaturation, Alkylation and sample preparation

After digestion, add 23 µL of a 4.2 M guanidine-HCl,10.4 mM DTT solution to the digest (final concentration of 2 M guanidine-HCl, 5 mM DTT) and incubate samples at room temperature for 1h under gentle agitation (500 rpm). Then, perform alkylation by adding 2 µL of 250 mM iodoacetamide (final concentration of 10 mM iodoacetamide) to the reduced samples, followed by incubation at room temperature for 1h in the dark under gentle agitation (500 rpm).

Buffer exchange alkylated samples to 100 mM ammonium acetate using Biospin-6 columns (732-6227, Bio-Rad) according to manufacturer's instructions. Vacuum-dry samples and resuspend pellets in 25 µL (final sample concentration of 2 µg/µL) of 99:1 water : acetonitrile with 0.2% formic acid prior to injection.

LC-MS/MS analysis

Data were acquired using product-dependent triggering of electron-transfer/higher-energy collision dissociation (EThcD) scans as described in Riley, N. M., & Bertozzi, C. R. (2022). Deciphering O-glycoprotease substrate preferences with O-Pair Search. *Mol Omics, 18*(10), 908-922. doi:10.1039/d2mo00244b.

O-glycopeptides separation was performed on a Vanquish Flex ultrahigh pressure liquid chromatography (UHPLC) instrument consisting of a Quaternary Pump F (VF-P20-A), Split Sampler FT with 25 µL autosampler loop (VF-A10-A), and a Column Compartment H (VH-C10-A) coupled to an Orbitrap Eclipse Tribrid mass spectrometer. Instrument modules were controlled with Thermo Scientific Xcalibur software version 4.7.

Inject approximately 3 µg of O-glycopeptides on a Hypersil Gold™ C18 column (100 × 1.0 mm, 3 µm, 175 Å, Thermo Fisher Scientific) heated to 40°C. Perform separation at 100 µL/min using the following gradient of mobile phases A (0.2% FA in water) and B (0.2% FA in acetonitrile): 5% B was held for the first 4 min, followed by an increase from 5% to 45% B from 4 to 64 min, an increase from 45% to 95% B from 64 to 74 min, isocratic flow at 95% B for 5 min and a re-equilibration at 5% B for 10 min. Eluted O-glycopeptides were analysed on the mass spectrometer in peptide mode using a standard flow Ion MAX Source containing a heated electrospray ionisation (H-ESI) probe (Thermo Fisher Scientific, San Jose, CA, USA). Spray voltage was set to 3.5 kV, ion transfer tube and vaporizer temperatures to 275°C and 200°C, respectively, and sheath and auxiliary gas to 15 and 5 a.u., respectively. Survey scans of peptide precursors were acquired in the Orbitrap in the 400-1,800 m/z range using a 60,000 (at m/z 200) resolution, a maximum injection time of 50 ms and a normalized automatic gain control (AGC) target of 100% (400,000 charges). Monoisotopic precursor selection was enabled for peptide isotopic distributions, precursors of z = 2 to 8 were selected for data-dependent MS/MS scans for 3 s of cycle time, and dynamic exclusion was enabled with a repeat count of 2, repeat duration of 20 s, exclusion duration of 20 s, and mass tolerance of 10 ppm. Priority

filters were set to favour highest precursor charge states and lowest precursor m/z values. An isolation window of 2 m/z was used to select precursor ions with the quadrupole. EThcD scans were collected in product-dependent fashion, where the presence of oxonium ions (m/z 126.055, 138.0549, 144.0655, 168.0654, 186.076, 204.0865, 274.0921, 292.1027, and 366.1395) in a ''scouting'' high-energy collisional dissociation (HCD) MS/MS scan triggered acquisition of a second MS/MS scan. The ''scout HCD'' scan used an automated scan range determination and a first mass of 100 Th, a normalized collision energy of 36, a normalized AGC target value of 100% (50 000 charges), a maximum injection time setting of Auto (54 ms), and a 30,000 (at m/z 200) resolution. If at least four of the nine listed oxonium ions were present in the scout HCD scan within a ±15 ppm tolerance and were among the 20 most intense peaks, an EThcD MS/MS scan was triggered that used calibrated charge dependent parameters for calculating reagent AGC targets and ion–ion reaction times, a supplemental collision energy of 25, a scan range of *m/z* 120 to 4,000, a maximum injection time of 400 ms, a normalized AGC target of 200% (100,000 charges), and a resolution of 60,000 (at *m/z* 200).

Data processing

All raw data were searched using O-Pair Search implemented in MetaMorpheus v.1.0.5, which is available at https://github.com/smith-chem-wisc/MetaMorpheus (Lu, L., Riley, N. M., Shortreed, M. R., Bertozzi, C. R., & Smith, L. M. (2020). O-Pair Search with MetaMorpheus for O-glycopeptide characterization. *Nat Methods, 17*(11), 1133-1138. doi:10.1038/s41592-020-00985-5). A customized O-glycans database was created containing the following core 1 O-glycans: HexNAc(1) (203.0794 Da), HexNAc(1)Hex(1) (365.1322 Da), HexNAc(1)NeuAc(1) (494.1748 Da), HexNAc(1)Hex(1)NeuAc(1) (656.2276 Da) and HexNAc(1)Hex(1)NeuAc(2) (947.3230 Da). The Glyco Search was performed using the customized O-glycan database with a maximum of 4 O-glycans allowed, top N candidates was set to 50, dissociation type set to HCD, child scan dissociation to EThcD, and oxonium ion fit was enabled. For in-silico digestion parameters of the FASTA file containing the sample sequence, decoy proteins were generated using reversed decoys, initiator methionine was set as variable, protease was set to non-specific with a peptide length from 3 to 125, 124 max missed cleavages and a maximum of 5 modifications per peptides with cysteine set as fixed modification. For fragment ion search parameters, mass tolerance was set to 10 ppm and 20 ppm for precursors and products, respectively, and the minimum score allowed was 3. All other parameters were kept as default. Each replicate was searched individually.

Additional data filtering was operated after O-Pair Search. Briefly, all decoy peptides were removed, and only level 1 glycopeptides (location probability > 0.75) with a Q-value higher than 0.01 that were identified in 2 over 3 replicates of one condition were kept. Finally, all identified glycopeptides were manually checked to confirm cleavage sites from the enzymes, with first amino acid of the peptide bearing a O-glycan and last amino acid corresponding to a potential cleavage site (i.e., XXX/S or XXX/T).

Mass Spectrometry of de-sialylated and PNGaseF treated LEVI-04 to measure O-glycan occupancy

Sample preparation

100 µg of material was brought to a final concentration of 1 mg/mL with 20 mM TRIS HCl pH 6.8. Enzymatic treatment was started by addition of 2.5 µL of sialidases (SialEXO, Genovis) and 1 µL of PNGase F (CarboClip, Asparia Glycomics). Samples were incubated overnight at 37°C under agitation.

Following digestion, samples were transferred to HPLC vial and directly injected.

LC-MS analysis

Inject 20 µg of sample on a NativePac column (2.1 x 50 mm, Thermo Scientific). Isocratic gradient was delivered by a Thermo Scientific Vanquish Flex uHPLC at a flow rate of 0.2 mL/min. Buffer was 50 mM ammonium acetate and column temperature was kept at 25°C. Column was connected to a Thermo Scientific Orbitrap Exploris MX mass detector. Mass spectrometer was operated at Intact Protein mode, with High Pressure settings for HCD gas trapping. Tune settings were as follows: sheath and auxiliary gasses were set at 28 and 10 arbitrary units respectively, ion transfer and vaporizer temperatures were set at 275 and 250°C, respectively. Orbitrap resolution was kept at 30,000 (at 200 m/z) and scan range was set between 2,500 and 8,000 m/z. In-source fragmentation

was on and kept at 130 eV, while acquisition gain control was set to 100%, maximum injection time was 250 ms and microscan number was 10. Full method duration was 5 minutes.

Data analysis

For intact analysis, raw data were deconvoluted using BioPharma Finder software v.5.2. Elution peak (0.5-1.0 RT) was selected through the average over selected time range tool and deconvoluted using the ReSpect algorithm. Briefly, model and output mass range were 90,000 and 110,000 Da, charge state range was between 10 and 50, deconvolution mass tolerance was set to 20 ppm and the minimum number of adjacent charge states was set to 4. Identification was achieved with a mass accuracy tolerance of 30 ppm.

Site Specific N-linked glycan analysis of LEVI-04

FabRICATOR Digestion

20µl LEVI-04 (10mg/mL) added to 80 µl 50 mM Tris HCL pH 7.9 (final conc. 2mg/mL). Take 50µL (100µg) of this solution and add to 100units of FabRICATOR (Genovis AO-FR1-008). Mix contents via pipette action and incubate at 37 °C for 3 hours. Isolate the glycan peaks using charged variant analysis.

Charged Variant Analysis

Sample preparation: 10 µg
Injection volume: 10 µL
Column: Thermo Scientific ProPac 3R SCX column (2.0x50 mm)
Column temperature: 25 °C
System: Thermo Scientific Vanquish UHPLC
Software: XCalibur (Thermo Scientific)
Solvent A: 25mM Ammonium Bicarbonate, 30 mM Acetic Acid
Solvent B: 10mM ammonium Hydroxide
Gradient: 30 minute linear gradient with a flow rate of 0.2 mL/min: Starting at 20 % Solvent B, increasing to 100 % Solvent B over 15.0 minutes, flowing at 100% Solvent B for 4.0 minutes, reducing to 0 % Solvent B for 3 minutes, returning to 20% Solvent B and equilibrating for 8.0 minutes.
Wavelengths: 280 nm.
Sample Temperature: 5 °C
Fraction collection: N32 glycan site elutes between 0-1.5 minutes (1st peak). Peak collected in 1.5 mL tube.
N294 glycan site elutes between 7.0-8.2 minutes (2nd peak). Peak collected in 1.5 mL tube.

In-solution release of N-glycans with PNGase F

Add each sample fraction onto separate Nanosep® 10 K MWCO filters (PALL) filter and spin @13,000 rpm until all protein is loaded onto the filter. Once all protein is on the filter add 100µl 20mM NaHCO3 to each filter and spin at 13,000rpm. Repeat this wash step 3 times before continuing to reduction step.
Add 90 µL of 20mM NaHCO3; 10 µL of 1 % Rapigest solution in 20mM NaHCO3; 2 µL 400 mM DTT. Mix the contents by pipette action and incubate on the filter at 65 °C for 15 minutes.
Alkylate samples by adding 2 µL of 80 mM iodoacetamide and incubate in the dark at room temperature for 30 min.
De-*N*-glycosylate samples by adding 2 µL PNGase F (NEB, P0709L). Mix via pipette action and incubate on the filter at 37 °C overnight.
Remove deglycosylated material by centrifugation @13,000 rpm and collect filtrate. Wash the filter twice with 100uL distilled H_2O to wash all the released glycans through the filter. Dry samples in a vacuum centrifuge before adding 20 µL of 1 % formic acid and incubate at room temperature for 20 minutes.
Dry samples in a vacuum centrifuge prior to further processing.

2-AB Glycan labelling and clean-up

Samples were labelled by adding 5 µL of 2-AB labelling solution (LudgerTag 2-AB labelling kit, Ludger, Abingdon, UK) to dried pellet and mix by pipette action. Incubate for 2 h at 65 °C. Remove excess 2-AB using amide resin Phytips from Phynexus. Dry sample in vacuum centrifuge.

HILIC UPLC N-Glycan Method

The UPLC system was calibrated by running an external standard of 2-AB dextran ladder (2-AB labeled glucose homopolymer) alongside the sample runs. A fifth-order polynomial distribution curve was fitted to the dextran ladder and used to allocate glucose unit (GU) values from retention times, using Empower software (Waters).
Take the dried sample at the end of the previous step (2-AB Glycan labelling and clean-up) and resuspend pellet in 12.5µL 75% acetonitrile.

Sample preparation: 70 % acetonitrile
Injection volume: 10 µL
Column: 1.7 µm BEH Glycan column (2.1 X 150 mm)
Column temperature: 40ºC
System: Waters Acquity UPLC equipped with a fluorescence detector
Software: Empower 3 (Waters)
Solvent A: 50 mM ammonium formate pH 4.4
Solvent B: Acetonitrile
Gradient: 30 minute linear gradient with a flow rate of 0.561 mL/min (except for wash step): 30 % Solvent A for 1.47 minutes, increasing to 47 % Solvent A over 23.34 minutes, increasing to 70 % Solvent A over 0.69 minutes; 70 % Solvent A for 0.75 minutes and then for a further 0.3 minutes at a reduced flowrate of 0.4 mL/min, returning to 30 % Solvent A over 0.3 minutes at a flow rate of 0.4 mL/min, then equilibrating with 30 % Solvent A for 1.95 minutes with the flow rate returned to 0.561 mL/min.
Wavelengths: Excitation 330 nm and emission 420 nm. Data rate: 20 pts/sec and PMT gain: 20.
Weak Wash: 80 % Acetonitrile
Strong Wash: 20 % Acetonitrile
Sample Temperature: 5 ºC

Quantification of N-glycans
Released 2-AB labelled N-glycans for each sample are loaded in triplicate and analysed as per HILIC UPLC method described. Resulting chromatograms are integrated using Waters Empower software. The relative percent area is used to express the abundance of each peak as a percentage of the total chromatographic area. Relative % area is taken as an average across the triplicate release.

Liquid chromatography-mass spectrometry (LC-MS)
LC-MS analysis is performed for orthogonal confirmation of the N-glycan structural assignments. LEVI-04 is prepared as described above (from FabRICATOR digestion through to end of 2-AB Glycan labelling and clean-up). Sample is analysed as per LC-MS method described below and resulting MS1 data integrated using XCalibur software.
Take the dried sample at the end of the previous step (2-AB Glycan labelling and clean-up) and resuspend pellet in 12.5µL 75% acetonitrile.

LC-FLD-mass spectrometry
Sample Temperature: 5 ºC
Sample preparation: 75 % acetonitrile
Injection volume: 10 µL
Column: 1.7 µm Waters BEH Glycan column (1.0 x 150 mm).
Column temperature: 60 ºC
System: Thermo Scientific Q Exactive Plus
Software: XCalibur (Thermo Scientific)
Solvent A: 50 mM ammonium formate pH 4.4
Solvent B: Acetonitrile
Gradient: 40 minute linear gradient with a flow rate of 0.15 mL/min: 28 % Solvent A for 1.0 minute, increasing to 43 % Solvent A over 30.0 minutes, increasing to 45 % Solvent A over 1 minute; returning to 28 % Solvent A over 4.0 minutes, then equilibrating with 30 % Solvent A for 4.0 minutes.
Wavelengths: Excitation 320 nm and emission 420 nm.
MS: Negative mode, spray voltage 3.40kV, Capillary temperature 320 °C, Aux gas heater temperature 300 °C, Sheath and sweep gas flow rate 30 and 10 L/h respectively, Scan range 450 to 2,500 *m/z*, Resolution 70,000.

Example 4

NT3 Affinity

p75NTR-Fc for NT3
A Biacore chip was prepared in an experiment in which Protein A/Levi 04 was amine coupled to flow cells 1 and 2. Single cycle kinetics of NT3 binding to captured p75-Fc were measured.
The binding capacity (R_{max}) of a chip surface depends of the immobilised level of the ligand (fusion protein). For a kinetics study an R_{max} of 50-100 RU is advised. By using the molecular weights of the p75-Fc and NT-3 , a desired immobilisation level for the fusion protein can be calculated.
R_{max} = (NT3 molecular weight/fusion protein molecular weight) x immobilisation level x stoichiometric ratio: 50 = (13,500/102,000) x immobilisation level x 1.
Hence, the immobilisation level required = (102,000/13,500) x 50 = 378 RU Sequence 1 (SEQ ID No. 1) and Sequence 3 (SEQ ID No. 3) p75NTR-Fc and NTR-Fc were immobilised onto the Protein A chip prior to single cycle kinetics.
Using a manual run, p75-Fc was captured onto flow cell 2 of the Protein A chip until the desired level of approx. 380 RU was achieved. This was performed with a 22 second injection at a flow rate of 10 µl/min and p75-Fc concentration 10 µg/ml which resulted in 418 RU of the fusion protein captured onto the protein A surface.
In the first instance NT-3 concentrations of 10, 5 2.5, 1.25 and 0.625 nM were tested. These concentrations were tested as the K_D for the fusion protein was approximated to be within this range of NT-3 concentrations.
The single cycle kinetics method involved:
- injecting 0.625 nM of NT-3 onto the captured p75-Fc for 120 seconds at 30 µl/min
- this process was then repeated with an injection of NT-3 at 1.25 nM, followed by 2.5, 5 and 10 nM
- after the final concentration of NT-3 had been injected a 600 second dissociation phase was performed by flowing the running buffer (HBS-EP) over the chip.
Once completed the chip was regenerated back to its Protein A surface by injecting 10 mM Glycine HCl, pH 2 for 60 seconds at 30 µl/min.
p75-Fc was then captured onto the chip by performing a 38 second injection at a flow rate of 10µl/min at a concentration of 10 µg/ml. This achieved the desired level of 430 RU. The single cycle kinetics procedure described above was then repeated.

Data analysis
The fusion protein-NT-3 binding data was analysed in the following manner using the Biacore T200 evaluation software v1:
- Data is recorded for the binding of NT-3 to the fusion protein on flow cell 2 (Fc=2) and for NT-3 flowing over the control flow cell 1 (Fc=1; protein A alone).
- The data from Fc=1 is then subtracted from Fc=2 to give "2-1" binding data.
- The 2-1 binding data for an injection of 0 nM (HBS-EP running buffer alone) is then subtracted from all the 2-1 binding data to control for any drifts in baseline throughout the experiment.
- Finally, this data is then fitted to a 1:1 binding model to calculate binding characteristics including association rates (ka), dissociation rates (kd) and affinities (K_D).
The sequence showed suitable affinity for NT-3.

Example 5

Comparison with molecule lacking glycosylation.

A closely related peptide to the one of the present disclosure was created, varying at several positions to create a protein that would not possess the glycosylation of the present disclosure. The protein, disclosed in WO2016/146841 and in Seq ID No. 2, has the following sequence:

KEACPTGLYTHSGECCKACNLGEGVAQPCGANQTVCEPCLDSVTFSDVVSATEPCKPCTECV
GLQSMSAPCVEADDAVCRCAYGYYQDETTGRCEACRVCEAGSLGLVFSCQDKQNTVCEEC
PDGTYDEANHVDPCLPCTVCEDTERQLFECTRWADAECEEIPGGGGEPKSDKTHTCPPCPAP
ELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREE
QYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRD
ELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQ
QGNVFSCVMHEALHNHYTQKSLLSLSPG

With respect to histological outcomes, the new molecule was not associated with efficacy at any dose studied. Given the variation in pathology severity scores within each group, any data trends are likely to be the result of the additive effects of variable group size (due to sample rejections) and variation in sample blocking (section depth and orientation). However, efficacy profiles with the compound of the present disclosure in this model showed a differential efficacy profile (cartilage and bone pathology inhibition and potentiation of mesenchyme cell expansion and homing) which would have overcome these artefacts. It is unlikely that significant efficacy has been masked.

Claims

1) A glycosylated p75NTR neurotrophin binding protein (NBP)-Fc fusion protein, comprising:
a p75NTR(NBP) portion, having at least 85% sequence identity with Seq ID No. 3; and an immunoglobulin Fc portion. wherein, the p75NTR(NBP) and Fc portions are connected via a linker, the linker comprises a peptide of formula G_x, where x is 1, 2, 3, 4, 5 or 6 wherein the linker does not comprise or consist of the sequence GGGGS.
2) The glycosylated protein of claim 1 where the linker portion is GGG and the Fc is a human Fc
3) A glycosylated protein having 5% or more homology with the protein of Seq ID No. 1
4) A glycosylated protein according to claim 1 or 2, wherein the glycosylated protein binds to any of NGF, BDNF, NT3 or NT4/5 with a binding affinity (K d) of between about 0.001 nM to about 50 nM.
5) A molecule, having 90% or greater homology with sequence ID No. 1 with glycosylation at one or more of positions 32, 169, 171, 172, 179, 180, 183, 184, 198, 199, 205, 206, 216, 217, 222 & 294.
6) a molecule having 90% or greater homology with sequence ID No. 1, with glycosylation at one of more of positions 169, 171, 172, 179, 180, 183, 184, 198, 199, 205, 206.
7) A molecule as described in any preceding claim wherein the N glycan at position 32 is selected from F(6)A2G(4)2S(3)2, F(6)A2G(4)2S(3)1
8) A molecule as described in any preceding claim wherein the Glycan at position 169 is selected from HexNAc(1), HexNAc(1)Hex(1), HexNAc(1)NeuAc(1), HexNAc(1)Hex(1)NeuAc(1), HexNAc(1)Hex(1)NeuAc(2)
9) A molecule as described in any preceding claim wherein the Glycan at position 171 is selected from HexNAc(1) HexNAc(1)Hex(1) , HexNAc(1)Hex(1)NeuAc(1) , HexNAc(1)Hex(1)NeuAc(2)
10) A molecule as described in any preceding claim wherein the Glycan at position 172 is selected from HexNAc(1) , HexNAc(1)Hex(1) , HexNAc(1)Hex(1)NeuAc(1) , HexNAc(1)Hex(1)NeuAc(2)
11) A molecule as described in any preceding claim wherein the Glycan at position 179 is selected from HexNAc(1)Hex(1) or HexNAc(1)Hex(1)NeuAc(2)
12) A molecule as described in any preceding claim wherein the Glycan at position 180 is selected from HexNAc(1) , HexNAc(1)Hex(1) , HexNAc(1)NeuAc(1) (N1S1), HexNAc(1)Hex(1)NeuAc(1) , HexNAc(1)Hex(1)NeuAc(2)
13) A molecule as described in any preceding claim wherein he Glycan at position 183 is selected from HexNAc(1) , HexNAc(1)Hex(1) , HexNAc(1)Hex(1)NeuAc(1) , HexNAc(1)Hex(1)NeuAc(2)
14) A molecule as described in any preceding claim wherein the Glycan at position 184 is selected from HexNAc(1) , HexNAc(1)Hex(1) , N1S1, HexNAc(1)Hex(1)NeuAc(1) , HexNAc(1)Hex(1)NeuAc(2)
15) A molecule as described in any preceding claim wherein the Glycan at position 185 is selected from HexNAc(1) , HexNAc(1)Hex(1) , HexNAc(1)Hex(1), HexNAc(1)Hex(1)NeuAc(1) , HexNAc(1)Hex(1)NeuAc(2)
16) A molecule as described in any preceding claim wherein the Glycan at position 198 is selected from HexNAc(1), HexNAc(1)Hex(1)NeuAc(1) , HexNAc(1)Hex(1)NeuAc(2)
17) A molecule as described in any preceding claim wherein the Glycan at position 199 is selected from HexNAc(1), HexNAc(1)Hex(1)NeuAc(1) , HexNAc(1)Hex(1)NeuAc(2)

18) A molecule as described in any preceding claim wherein the Glycan at position 205 is selected from HexNAc(1) , HexNAc(1)Hex(1) , N1S1, HexNAc(1)Hex(1)NeuAc(1) , HexNAc(1)Hex(1)NeuAc(2)
19) A molecule as described in any preceding claim wherein the Glycan at position 206 is selected from HexNAc(1) , HexNAc(1)Hex(1) , N1S1, HexNAc(1)Hex(1)NeuAc(1) , HexNAc(1)Hex(1)NeuAc(2)
20) A molecule as described in any preceding claim wherein the Glycan at position 216 is selected from HexNAc(1) or HexNAc(1)Hex(1)
21) A molecule as described in any preceding claim wherein the Glycan at position 217 is selected from HexNAc(1) , HexNAc(1)Hex(1) , HexNAc(1)Hex(1)NeuAc(1) , HexNAc(1)Hex(1)NeuAc(2)
22) A molecule as described in any preceding claim wherein the Glycan at position 222 is selected from HexNAc(1) , HexNAc(1)Hex(1) , HexNAc(1)Hex(1)NeuAc(1) , HexNAc(1)Hex(1)NeuAc(2)
23) A molecule as described in any preceding claim wherein the N glycan at position 294 is selected from F(6)A2, F(6)A2[6}G(4)1 & F(6)A2[3}G(4)1, F(6)A2G(4)2
24) A molecule having 90% or greater homology with sequence ID No1, with glycosylation at position 32, 294 and at 1-11 of each of positions 169, 171, 172, 179, 180, 183, 184, 198, 199, 205, 206.

25) A molecule having 90% or greater homology with sequence ID No. 1, with glycosylation at each of positions 169, 171, 172, 177, 179, 180, 183, 184, 198, 199, 205, 206, wherein the Glycan at position 169 is HexNAc(1)Hex(1)NeuAc(2); the Glycan at position 171 is HexNAc(1)Hex(1)NeuAc(2); the Glycan at position 172 is HexNAc(1)Hex(1)NeuAc(1); the Glycan at position 177 is HexNAc(1)Hex(1); the Glycan at position 179 is HexNAc(1)Hex(1)NeuAc(2); the Glycan at position 180 is selected from HexNAc(1)Hex(1)NeuAc(2); the Glycans at position 183 are selected from HexNAc(1) HexNAc(1)Hex(1) and HexNAc(1)Hex(1)NeuAc(2); the Glycans at position 184 are selected from HexNAc(1) , H1N1, HexNAc(1)Hex(1)NeuAc(1) , HexNAc(1)Hex(1)NeuAc(2); the Glycans at position 185 is selected from HexNAc(1) , H1N1, HexNAc(1)Hex(1)NeuAC(1) , HexNAc(1)Hex(1)NeuAc(2); the Glycans at position 198 are selected from HexNAc(1) , HexNAc(1)Hex(1) , HexNAc(1)Hex(1)NeuAc(1) , HexNAc(1)Hex(1)NeuAc(2); The Glycans at position 199 are selected from HexNAc(1) , HexNAc(1)Hex(1) , HexNAc(1)Hex(1)NeuAc(1) , HexNAc(1)Hex(1)NeuAc(2); the Glycans at position 205 are selected from HexNAc(1) , HexNAc(1)Hex(1) , N1S1, HexNAc(1)Hex(1)NeuAc(1) , HexNAc(1)Hex(1)NeuAc(2);
the Glycans at position 206 are selected from HexNAc(1) , HexNAc(1)Hex(1) , N1S1, HexNAc(1)Hex(1)NeuAc(1) , HexNAc(1)Hex(1)NeuAc(2)
26) The glycosylated protein according to claim 6-8 where an O linked glycan can be sialylated with 1-2 sialic acid molecules.
27) The glycosylated protein of any preceding claim containing 16-17 moles of sialic acid molecules to every mole of the monomer of Seq ID No. 1.
28) The glycosylated protein of claim 27 where there are 16.55-16.65 sialic acid molecules on each glycosylated monomer of Seq ID No. 1.
29) The glycosylated protein of claims 26-28 wherein the sialic acids consist of N-Acetylneuraminic acid (Neu5Ac) and N-glycolyl neuraminic acid (Neu5Gc).
30) The glycosylated protein of claim 29 where there are 16.1-16.35 molecules of Neu5Ac for each monomer of Seq ID No. 1.

31) The glycosylated protein of claim 29 where there are 0.255-0.265 molecules of Neu5G for each monomer of Seq ID No. 1.