Effect of 10 nM Syntaxin compound on PHA-mediated IP-10 secretion by PBMC

<table>
<thead>
<tr>
<th>Treatment condition</th>
<th>IP-10 secretion (% of LPS control)</th>
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<tbody>
<tr>
<td>SXX100221</td>
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<tr>
<td>SXX100328</td>
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<tr>
<td>SXX100501</td>
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<tr>
<td>SXX100221 + PHA</td>
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<tr>
<td>SXX100328 + PHA</td>
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<tr>
<td>SXX100501 + PHA</td>
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<tr>
<td>EGF + PHA</td>
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<tr>
<td>PHA alone</td>
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<td>No treatment</td>
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The present invention relates to treatment of disease by inhibition of cellular secretory processes, to agents and compositions therefor, and to manufacture of those agents and compositions. The present invention relates particularly, to treatment of disease dependent upon the exocytic activity of endocrine cells, exocrine cells, inflammatory cells, cells of the immune system, cells of the cardiovascular system and bone cells.
FIG. 1
FIG. 2

Insulin release (ng/well) vs Concentration of WGA-LH₅/A (μg/ml)
FIG. 3
FIG. 4
Fig. 5.
Fig. 6
Fig. 7
Fig. 8

- Release of High Molecular Weight Material (dpm)
- pH 4.7 vs pH 4.7 BoNT/B Treatment
Fig. 9
Fig. 16

Effect of 10 nM Syntaxin compounds on LPS-mediated IL-8 secretion by THP-1 cells
Effect of 10 nM Syntaxin compounds on LPS-mediated IL-10 secretion by RPMI-8226 cells
Fig. 18

Effect of 10 nM Syntaxin compounds on LPS-mediated IL-8 secretion by PBMC
Fig. 19

Effect of 10 nM Syntaxin compound on PHA-mediated IP-10 secretion by PBMC

IP-10 secretion (% of LPS control)

SXXN 100221, SXXN 100228, SXXN 100501, EGF, SXXN 100221 + PHA, SXXN 100228 + PHA, SXXN 100501 + PHA, EGF + PHA, PHA alone, No treatment

Treatment condition
INHIBITION OF SECRETION FROM NON-NEURONAL CELLS

[0001] This application is a continuation-in-part of U.S. patent application Ser. No. 11/327,855, filed on Jan. 9, 2006, which is a continuation of U.S. patent application Ser. No. 10/088,665, filed Aug. 14, 2002, which is a national phase entry of PCT/GB00/03681, filed Sep. 25, 2000, which claims the benefit of priority of GB 9922558.3, filed Sep. 23, 1999. Each of these applications is hereby incorporated by reference in their entirety.

[0002] The present invention relates to treatment of disease by inhibition of cellular secretory processes, to agents and compositions therefor, and to manufacture of those agents and compositions. The present invention relates particularly, to treatment of diseases dependent upon the exocytic activity of endocrine cells, excocrine cells, inflammatory cells, cells of the immune system, cells of the cardiovascular system and bone cells.

[0003] Exocytosis is the fusion of secretory vesicles with the plasma membrane and results in the discharge of vesicle content—a process also known as cell secretion. Exocytosis can be constitutive or regulated. Constitutive exocytosis is thought to occur in every cell type whereas regulated exocytosis occurs from specialised cells.

[0004] The understanding of the mechanisms involved in exocytosis has increased rapidly, following the proposal of the SNARE hypothesis (Rothman, 1994, Nature 372, 55-63). This hypothesis describes protein markers on vesicles, which recognise target membrane markers. These so-called cognate SNARES (denoted v-SNARE for vesicle and t-SNARE for target) facilitate docking and fusion of vesicles with the correct membranes, thus directing discharge of the vesicular contents into the appropriate compartment. Key to the understanding of this process has been the identification of the proteins involved. Three SNARE protein families have been identified for exocytosis: SNAP-25 and SNAP-23, and syntaxins are the t-SNARE families in the membrane, and VAMPs (vesicle-associated membrane protein), including synaptobrevin and cellubrevin, are the v-SNARE family on secretory vesicles. Key components of the fusion machinery including SNARES are involved in both regulated and constitutive exocytosis (De Camilli, 1993, Nature, 364, 387-388).

[0005] The clostridial neurotoxins are proteins with molecular masses of the order of 150 kDa. They are produced by various species of the genus Clostridium, most importantly C. tetani and several strains of C. botulinum. There are at least eight different classes of the neurotoxins known: tetanus toxin and botulinum neurotoxin in its serotypes A, B, C,, D, E, F and G, and they all share similar structures and modes of action. The clostridial neurotoxins are synthesized by the bacterium as a single polypeptide that is modified post-translationally to form two polypeptide chains joined together by a disulphide bond. The two chains are termed the heavy chain (H) which has a molecular mass of approximately 100 kDa and the light chain (L) which has a molecular mass of approximately 50 kDa. The clostridial neurotoxins are highly selective for neuronal cells, and bind with high affinity thereto (see Black, J. D. and Dolly, J. O. (1987) Selective location of acceptors for BoNT/A in the central and peripheral nervous systems. Neuroscience, 23, pp. 767-779; Habermann, E. and Dreyer, F. (1986) Clostridial neurotoxins: handling and action at the cellular and molecular level. Curr. Top. Microbiol. Immunol. 129, pp. 93-179; and Sugiyama, H. (1980) Clostridium botulinum neurotoxin. Microbiol. Rev., 44, pp. 419-448 (and internally cited references]).

[0006] The functional requirements of neurointoxication by the clostridial neurotoxins can be assigned to specific domains within the neurotoxin structure. The clostridial neurotoxins bind to an acceptor site on the cell membrane of the motor neuron at the neuromuscular junction and, following binding to the highly specific receptor, are internalised by an endocytotic mechanism. The specific neuromuscular junction binding activity of clostridial neurotoxins is known to reside in the carboxy-terminal portion of the heavy chain component of the dichain neurotoxin molecule, a region known as Hc. The internalised clostridial neurotoxins possess a highly specific zinc-dependent endopeptidase activity that hydrolyses a specific peptide bond in at least one of three protein families, synaptobrevin, syntaxin or SNAP-25, which are crucial components of the neurosecretory machinery. The zinc-dependent endopeptidase activity of clostridial neurotoxins is found to reside in the L-chain (LC). The amino-terminal portion of the heavy chain component of the dichain neurotoxin molecule, a region known as Hn, is responsible for translocation of the neurotoxin, or a portion of it containing the endopeptidase activity, across the endosomal membrane following internalisation, thus allowing access of the endopeptidase to the neuronal cytosol and its substrate protein(s). The result of neurointoxication is inhibition of neurotransmitter release from the target neuron due to prevention of release of synaptic vesicle contents.

[0007] The mechanism by which the Hn domain effects translocation of the endopeptidase into the neuronal cytosol is not fully characterised but is believed to involve a conformational change, insertion into the endosomal membrane and formation of some form of channel or pore through which the endopeptidase can gain access to the neuronal cytosol. Following binding to its specific receptor at the neuronal surface pharmacological and morphologic evidence indicate that the clostridial neurotoxins enter the cell by endocytosis [Black & Dolly (1986) J. Cell Biol. 103, 535-44] and then have to pass through a low pH step for neuron intoxication to occur [Simpson et al (1994) J. Pharmacol Exp. Ther., 269, 256-62]. Acidic pH does not activate the toxin directly via a structural change, but is believed to trigger the process of LC membrane translocation from the endosomal endosomal vesicle lumen to the neuronal cytosol [Montecucco et al (1994) FEBS Lett. 346, 92-98]. There is a general consensus that toxin-determined channels are related to the translocation process into the cytosol [Sciavo & Montecucco (1997) in Bacterial Toxins (ed. K. Aktories)]. This model requires that the Hn domain forms a transmembrane hydrophobic pore across the acidic vesicle membrane that allows the partially unfolded LC passage through to the cytosol. The requisite conformational change is believed to be triggered by environmental factors in the neuronal endosomal compartment into which the neurotoxin is internalised, and a necessary feature of the binding domain of the Hn is to target binding sites which enable internalisation into the appropriate endosomal compartment. Therefore clostridial neurotoxins have evolved to target cell surface moieties that fulfill this requirement.
Hormones are chemical messengers that are secreted by the endocrine glands of the body. They exercise specific physiological actions on other organs to which they are carried by the blood. The range of processes regulated by hormones includes various aspects of homeostasis (e.g., insulin regulates the concentration of glucose in the blood), growth (e.g., growth hormone promotes growth and regulates fat, carbohydrate and protein metabolism), and maturation (e.g., sex hormones promote sexual maturation and reproduction). Endocrine hyperfunction results in disease conditions which are caused by excessive amounts of a hormone or hormones in the bloodstream. The causes of hyperfunction are classified as neoplastic, autoimmune, iatrogenic and inflammatory. The endocrine hyperfunction disorders are a complex group of diseases, not only because there is a large number of glands that can cause a pathology (e.g., anterior pituitary, posterior pituitary, thyroid, parathyroid, adrenal cortex, adrenal medulla, pancreas, ovaries, testis) but because many of the glands produce more than one hormone (e.g., the anterior pituitary produces corticotropin, prolactin, luteinizing hormone, follicle stimulating hormone, thyroid stimulating hormone and gonadotrophins). The majority of disorders that cause hormone excess are due to neoplastic growth of hormone producing cells. However, certain tumours of non-endocrine origin can synthesise hormones causing endocrine hyperfunction disease symptoms. The hormone production under these conditions is termed "ectopic". Surgical removal or radiation induced destruction of part or all of the hypersecreting tissue is frequently the treatment of choice. However, these approaches are not always applicable, result in complete loss of hormone production or have to be repeated due to re-growth of the secreting tissue.

A further level of complexity in endocrine hyperfunction disorders arises in a group of conditions termed multiple endocrine neoplasia (MEN) where two or more endocrine glands are involved. The multiple endocrine neoplasia syndromes (MEN1 and MEN2) are familial conditions with an autosomal dominant pattern of inheritance. MEN1 is characterised by the association of parathyroid hyperplasia, pancreatic endocrine tumours, and pituitary adenomas, and has a prevalence of about 1 in 10000. MEN2 is the association of medullary cell carcinoma of the thyroid and phaeochromocytoma, though parathyroid hyperplasia may also occur in some sufferers.

Most of the morbidity associated with MEN1 is due to the effects of pancreatic endocrine tumours. Often surgery is not possible and the therapeutic aim is to reduce hormone excess. Aside from reducing tumour bulk, which is often precluded, inhibition of hormone secretion is the preferred course of action. Current procedures include subcutaneous application of the somatostatin analogue, octreotide. However, this approach is only temporarily effective, and the success diminishes over a period of months.

Many further disease states are known that involve secretion from other non-endocrine, non-neuronal cells. It would accordingly be desirable to treat, reduce or prevent secretion by non-neuronal cells, such as hyperfunction of the endocrine cells that causes or leads to these disease conditions.

The activity of the botulinum neurotoxins is exclusively restricted to inhibition of neurotransmitter release from neurons. This is due to the exclusive expression of high affinity binding sites for clostridial neurotoxins on neuronal cells [see Daniels-Hogate, P. U. and Dolly, J. O. (1996) Productive and non-productive binding of botulinum neurotoxin to motor nerve endings are distinguished by its heavy chain. J. Neurosci. Res. 44, 263-271].

Non-neuronal cells do not possess the high affinity binding sites for clostridial neurotoxins, and are therefore refractory to the inhibitory effects of exogenously applied neurotoxin. Simple application of clostridial neurotoxins to the surface of non-neuronal cells does not therefore lead to inhibition of secretory vesicle exocytosis.

The productive binding or lack of productive binding of clostridial neurotoxins thereby defines neuronal and non-neuronal cells respectively.

In addition to lacking high affinity binding sites for clostridial neurotoxins, absence of the correct internalisation and intracellular routing mechanism, or additional factors that are not yet understood, would prevent clostridial neurotoxin action in non-neuronal cells.

It is known from WO96/33273 that hybrid clostridial neurotoxins endopeptidases can be prepared and that these hybrids effectively inhibit release of neurotransmitters from neuronal cells to which they are targeted, such as pain transmitting neurons. WO96/33273 describes the activity of hybrids only in neuronal systems where neuronal mechanisms of internalisation and vesicular routing are operational.

Non-neuronal cells are, however, refractory to the effects of clostridial neurotoxins, since simple application of clostridial neurotoxins to the surface of non-neuronal cells does not lead to inhibition of secretory vesicle exocytosis. This insensitivity of non-neuronal cells to clostridial neurotoxins may be due to absence of the requisite receptor, absence of the correct internalisation & intracellular routing mechanism, or additional factors that are not yet understood.

WO95/17904 describes the use of C. botulinum holotoxin in the treatment of various disorders such as excessive sweating, lacrimation and mucus secretion, and pain. WO95/17904 describes treatment by targeting neuronal cells.

It is an object of the present invention to provide methods and compositions for inhibition of secretion from non-neuronal cells.

Accordingly, the present invention is based upon the use of a composition which inhibits the exocytotic machinery in neuronal cells and which surprisingly has been found to be effective at inhibiting exocytotic processes in non-neuronal cells.

A first aspect of the invention thus provides a method of inhibiting secretion from a non-neuronal cell comprising administering an agent comprising at least first and second domains, wherein the first domain cleaves one or more proteins essential to exocytosis and the second domain translocates the first domain into the cell.

Advantageously, the invention provides for inhibition of non-neuronal secretion and enables treatment of disease caused, exacerbated or maintained by such secretion.
[0023] An agent for use in the invention is suitably prepared by replacement of the cell-binding Hc domain of a clostridial neurotoxin with a ligand capable of binding to the surface of non-neuronal cells. Surprisingly, this agent is capable of inhibiting the exocytosis of a variety of secreted substances from non-neuronal cells. By covalently linking a clostridial neurotoxin, or a hybrid of two clostridial neurotoxins, in which the Hc region of the H-chain has been removed or modified, to a new molecule or moiety, the targeting moiety (TM), an agent is produced that binds to a binding site (BS) on the surface of the relevant non-neuronal secretory cells. A further surprising aspect of the present invention is that if the L-chain of a clostridial neurotoxin, or a fragment, variant or derivative of the L-chain containing the endopeptidase activity, is covalently linked to a TM which can also effect internalisation of the L-chain, or a fragment of the endopeptidase activity, into the cytoplasm of a non-neuronal secretory cell, this also produces an agent capable of inhibiting secretion. Thus, the present invention overcomes the insusceptibility of non-neuronal cells to the inhibitory effects of clostridial neurotoxins.

[0024] An example of an agent of the invention is a polypeptide comprising first and second domains, wherein said first domain cleaves one or more vesicle or plasma-membrane associated proteins essential to neuronal exocytosis and wherein said second domain translocates the polypeptide into the cell or translocates at least that portion responsible for the inhibition of exocytosis into the non-neuronal cell. The polypeptide can be derived from a neurotoxin in which case the polypeptide is typically free of clostridial neurotoxin and free of any clostridial neurotoxin precursor that can be converted into toxin by proteolytic action, being accordingly substantially non-toxic and suitable for therapeutic use. Accordingly, the invention may thus use polypeptides containing a domain equivalent to a clostridial toxin light chain and a domain providing the translocation function of the Hc of a clostridial toxin heavy chain, whilst lacking the functional aspects of a clostridial toxin Hc domain.

[0025] In use of the invention, the polypeptide is administered in vivo to a patient, the first domain is translocated into a non-neuronal cell by action of the second domain and cleaves one or more vesicle or plasma-membrane associated proteins essential to the specific cellular process of exocytosis, and cleavage of these proteins results in inhibition of exocytosis, thereby resulting in inhibition of secretion, typically in a non-cytotoxic manner.

[0026] The polypeptide of the invention may be obtained by expression of a recombinant nucleic acid, preferably a DNA, and can be a single polypeptide, that is to say not cleaved into separate light and heavy chain domains or two polypeptides linked for example by a disulphide bond.

[0027] The first domain preferably comprises a clostridial toxin light chain or a functional fragment or variant of a clostridial toxin light chain. The fragment is optionally an N-terminal, or C-terminal fragment of the light chain, or is an internal fragment, so long as it substantially retains the ability to cleave the vesicle or plasma-membrane associated protein essential to exocytosis. The minimal domains necessary for the activity of the light chain of clostridial toxins are described in J. Biol. Chem., Vol. 267, No. 21, July 1992, pages 14721-14729. The variant has a different peptide sequence from the light chain or from the fragment, though it too is capable of cleaving the vesicle or plasma-membrane associated protein. It is conveniently obtained by insertion, deletion and/or substitution of a light chain or fragment thereof. A variety of variants are possible, including (i) an N-terminal extension to a clostridial toxin light chain or fragment (ii) a clostridial toxin light chain or fragment modified by alteration of at least one amino acid (iii) a C-terminal extension to a clostridial toxin light chain or fragment, or (iv) combinations of 2 or more of (i)-(iii). In further embodiments of the invention, the variant contains an amino acid sequence modified so that (a) there is no protease sensitive region between the LC and Hc components of the polypeptide, or (b) the protease sensitive region is specific for a particular protease. This latter embodiment is of use if it is desired to activate the endopeptidase activity of the light chain in a particular environment or cell, though, in general, the polypeptides of the invention are in an active form prior to administration.

[0028] The first domain preferably exhibits endopeptidase activity specific for a substrate selected from one or more of SNAP-25, synaptobrevin/VAMP and syntaxin. The clostridial toxin from which this domain can be obtained or derived is preferably botulinum toxin or tetanus toxin. The polypeptide can further comprise a light chain or fragment or variant of one toxin type and a heavy chain or fragment or variant of another toxin type.

[0029] The second domain preferably comprises a clostridial toxin heavy chain Hc portion or a fragment or variant of a clostridial toxin heavy chain Hc portion. The fragment is optionally an N-terminal or C-terminal or internal fragment, so long as it retains the function of the Hc domain. Teachings of regions within the Hc responsible for its function are provided for example in Biochemistry 1995, 34, pages 15175-15181 and Eur. J. Biochem, 1989, 185, pages 197-203. The variant has a different sequence from the Hc domain or fragment, though it too retains the function of the Hc domain. It is conveniently obtained by insertion, deletion and/or substitution of a Hc domain or fragment thereof, and examples of variants include (i) an N-terminal extension to a Hc domain or fragment, (ii) a C-terminal extension to a Hc domain or fragment, (iii) a modification to a Hc domain or fragment by alteration of at least one amino acid, or (iv) combinations of 2 or more of (i)-(iii). The clostridial toxin is preferably botulinum toxin or tetanus toxin.

[0030] In preparation of the polypeptides by recombinant means, methods employing fusion proteins can be employed, for example a fusion protein comprising a fusion of (a) a polypeptide of the invention as described above with (b) a second polypeptide adapted for binding to a chromatography matrix so as to enable purification of the fusion protein using said chromatography matrix. It is convenient for the second polypeptide to be adapted to bind to an affinity matrix, such as glutathione Sepharose, enabling rapid separation and purification of the fusion protein from an impure source, such as a cell extract or supernatant.

[0031] One second purification polypeptide is glutathione-S-transferase (GST), and others may be chosen so as to enable purification on a chromatography column according to conventional techniques.
[0032] In a second aspect of the invention there is provided a method of inhibiting secretion from selected non-neuronal cells responsible for regulated secretion by administering an agent of the invention.

[0033] In a third aspect of the invention there is provided a method of treatment of disease resulting, or caused or maintained by secretions from non-neuronal cells, comprising administering an agent of the invention.

[0034] In further aspects of the invention there are provided agents of the invention targeted to non-neuronal cells responsible for secretion.

[0035] In one embodiment of the invention, an agent is provided for the treatment of conditions resulting from hyperfunction of endocrine cells, for example endocrine neoplasia.

[0036] Accordingly, an agent of the invention is used in the treatment of endocrine hyperfunction, to inhibit secretion of endocrine cell-derived chemical messengers. An advantage of the invention is that effective treatment of endocrine hyperfunction and associated disease states is now provided, offering relief to sufferers where hitherto there was none and no such agent available.

[0037] A further advantage of the invention is that agents are made available which, in use, result in the inhibition of or decrease in hypersecretion of multiple hormones from a single endocrine gland. Thus, the multitude of disorders that result from hyperfunction of one gland (e.g. the anterior pituitary) will be simultaneously treated by a reduction in the function of the hypersecreting gland.

[0038] The agent preferably comprises a ligand or targeting domain which binds to an endocrine cell, and is thus rendered specific for these cell types. Examples of suitable ligands include iodine; thyroid stimulating hormone (TSH); TSH receptor antibodies; antibodies to the islet-specific monosialo-ganglioside GM2-1; insulin, insulin-like growth factor and antibodies to the receptors of both; TSH releasing hormone (proteotrin) and antibodies to its receptor; FSH/LH releasing hormone (gonadorelin) and antibodies to its receptor; corticotrophin releasing hormone (CRH) and antibodies to its receptor; and ACTH and antibodies to its receptor. According to the invention, an endocrine targeted agent may thus be suitable for the treatment of a disease selected from: endocrine neoplasia including MEN; thyrotoxicosis and other diseases dependent on hypersecretions from the thyroid; acromegaly, hyperprolactinemia, Cushings disease and other diseases dependent on anterior pituitary hypersecretion; hyperandrogenism, chronic anovulation and other diseases associated with poly cystic ovarian syndrome.

[0039] In a further embodiment, an agent of the invention is used for the treatment of conditions resulting from secretions of inflammatory cells, for example allergies. Ligands suitable to target agent to these cells include (i) for mast cells, complement receptors in general, including C4 domain of the Fc IgE, and antibodies/ligands to the C3a/C4a-R complement receptor; (ii) for eosinophils, antibodies/ligands to the C3a/C4a-R complement receptor, anti VLA-4 monoclonal antibody, anti-IL5 receptor, antibodies or antibodies reactive toward CR4 complement receptor; (iii) for macrophages and monocytes, macrophage stimulating factor, (iv) for macrophages, monocytes and neutrophils, bacterial LPS and yeast B-glucans which bind to CR3, (v) for neutrophils, antibody to OX42, an antigen associated with the iC3b complement receptor, or IL8; (vi) for fibroblasts, mannose 6-phosphate/insulin-like growth factor-b (M6P/IGF-II) receptor and PA2.26, antibody to a cell-surface receptor for active fibroblasts in mice.

[0040] According to a preferred embodiment of the present invention, the TM is a growth factor, preferably an epidermal growth factor (EGF), vascular endothelial growth factor, platelet-derived growth factor, keratinocyte growth factor, hepatocyte growth factor, transforming growth factor alpha, transforming growth factor beta.

[0041] According to another preferred embodiment of the present invention, the TM is a peptide or protein that binds to an inflammatory cell. A preferred example of such a TM is an integrin-binding protein.

[0042] Integrins are obligate heterodimer transmembrane proteins containing two distinct chains a (alpha) and D (beta) subunits. In mammals, 19 alpha and 8 beta subunits have been characterised—see Humphries, M. J. (2000), Integrin structure. Biochem Soc Trans. 28: 311-339, which is herein incorporated by reference thereto. Integrin subunits span through the plasma membrane, and in general have very short cytoplasmic domains of about 40-70 amino acids. Outside the cell plasma membrane, the alpha and beta chains lie close together along a length of about 23 nm, the final 5 nm NH2-termini of each chain forming a ligand-binding region to which an agent of the present invention binds.

[0043] Preferred integrin-binding proteins of the present invention comprise the amino sequence Arg-Gly-Asp ("RGD"), which binds to the above-described ligand-binding region—see Craig, D et al. (2004), Structural insights into how the MIDAS ion stabilizes integrin binding to an RGD peptide under force. Structure, vol. 12, pp 2049-2058, which is herein incorporated by reference thereto.

[0044] In one embodiment, the integrin-binding protein TMs of the present invention have an amino acid length of between 3 and 100, preferably between 3 and 50, more preferably between 5 and 25, and particularly preferably between 5 and 15 amino acid residues.

[0045] The TMs of the present invention may form linear or cyclic structures.

[0046] Preferred integrin-binding TMs of the present invention include actin, alpha-actinin, focal contact adhesion kinase, paxillin, talin, RACK1, collagen, laminin, fibrinogen, heparin, phylohaemagglutinin, fibronectin, vitronectin, VCAM-1, ICAM-1, ICAM-2 and serum protein. Many integrins recognise the triple Arg-Gly-Asp (RGD) peptide sequence (Ruoslahti, 1996). The RGD motif is found in over 100 proteins including fibronectin, tenascin, fibrinogen and vitronectin. The RGD-integrin interaction is exploited as a conserved mechanism of cell entry by many pathogens including cossackievirus (Roovaninen et al., 1991) and adenovirus (Mathias et al., 1994).

[0047] Alternatively preferred integrin-binding TMs of the present invention include proteins selected from the following sequences: Arg-Gly-Asp-Phe-Val (SEQ ID NO:23); Arg-Gly-Asp-[D-Phe]-[N-methyl-Val] (SEQ ID NO:23); RGDFV (SEQ ID NO:23); RGDFMeV (SEQ ID NO:23); GGRGDMFGA (SEQ ID NO:21); GGRGDMPFGA (SEQ ID NO:22); GRGDSP (SEQ ID NO:26); GRGDSP (SEQ ID
Diseases thus treatable according to the invention include diseases selected from allergies (seasonal allergic rhinitis (hay fever), allergic conjunctivitis, vasomotor rhinitis and food allergy), eosinophilia, asthma, rheumatoid arthritis, systemic lupus erythematosus, discoid lupus erythematosus, ulcerative colitis, Crohn’s disease, hemorrhoids, puritus, glomerulonephritis, hepatitis, pancreatitis, gastritis, vasculitis, myocarditis, psoriasis, eczema, chronic radiation-induced fibrosis, lung scarring and other fibrotic disorders.

VAMP expression has been demonstrated in B-lymphocytes [see Olken, S. K. and Corley, R. B. 1998, Mol. Biol. Cell. 9, 207a]. Thus, an agent according to the present invention, when targeted to a B-lymphocyte and following internalisation and retrograde transport, may exert its inhibitory effect on such target cells.

In a further embodiment, an agent of the invention is provided for the treatment of conditions resulting from secretions of the exocrine cells, for example acute pancreatitis (Hansen et al, 1999, J. Biol. Chem. 274, 22871-22876). Ligands suitable to target agent to these cells include pituitary adenyl cyclase activating peptide (PACAP-38) or an antibody to its receptor. The present invention also concerns treatment of mucus hypersecretion from mucous-secreting cells located in the alimentary tract, in particular in the colon.

Gaisano, H. Y. et al. (1994) J. Biol. Chem. 269, pp. 17062-17066 has demonstrated that, following in vitro membrane permeabilisation to permit cellular entry, tetanus toxin light chain cleaves a vesicle- and B-lymphocyte protein (VAMP) isoform 2 in rat pancreatic zymogen granules, and inhibits enzyme secretion. Thus, an agent according to the present invention, when targeted to a pancreatic cell and following internalisation and retrograde transport, may exert its inhibitory effect on such target cells.

In a further embodiment, an agent of the invention is used for the treatment of conditions resulting from secretions of immunological cells, for example autoimmune disorders where B lymphocytes are to be targeted (immunosuppression). Ligands suitable to target agent to these cells include Epstein Barr virus fragment/surface feature or idiotypic antibody (binds to CR2 receptor on B-lymphocytes and lymph node follicular dendritic cells). Diseases treatable include myasthenia gravis, rheumatoid arthritis, systemic lupus erythematosus, discoid lupus erythematosus, organ transplant, tissue transplant, fluid transplant, Graves disease, thyrotoxicosis, autoimmune diabetes, hemolytic anemia, thymobocytopenic purpura, neutropenia, chronic autoimmune hepatitis, autoimmune gastritis, panniculitis anemia, Hashimoto’s thyroiditis, Addison’s disease, Sjogren’s syndrome, primary biliary cirrhosis, polymyositis, scleroderma, systemic sclerosis, pemphigus vulgaris, bullous pemphigoid, myocarditis, rheumatic carditis, glomerulonephritis (Goodpasture type), uveitis, orchitis, ulcerative colitis, vasculitis, atrophic gastritis, panniculitis anemia, type 1 diabetes mellitus.

By using cell permeabilisation techniques it has been possible to internalise BoNT/C into eosinophils [see Pinxteren J A., et al (2000) Biochimie, April; 82(4):385-93 Thirty years of stimulus-secretion coupling: from Ca(2+)+ to GTP in the regulation of exocytosis]. Following internalisation, BoNT/C exerted an inhibitory effect on exocytosis in eosinophils. Thus, an agent according to the present invention, when targeted to an eosinophil and following internalisation and retrograde transport, may exert its inhibitory effect on such target cells.

In a further embodiment of the invention, an agent is provided for the treatment of conditions resulting from secretions of cells of the cardiovascular system. Suitable ligands for targeting platelets for the treatment of disease states involving inappropriate platelet activation and thrombus formation include thrombin and TRAP (thrombin receptor agonist peptide) or antibodies to CD31/PECAM-1, CD24 or CD106/NCAM-1, and ligands for targeting cardiovascular endothelial cells for the treatment of hypertension include GP1b surface antigen recognising antibodies.

In a further embodiment of the invention, an agent is provided for the treatment of bone disorders. Suitable ligands for targeting osteoblasts for the treatment of a disease selected from osteoporosis and osteopenia include calcitonin, and for targeting an agent to osteoclasts include osteoclast differentiation factors (eg. TRANCE, or RANKL or OPGL), and an antibody to the receptor RANK.

In use of the invention, a Targeting moiety (TM) provides specificity for the BS on the relevant non-neuronal secretory cells. The TM component of the agent can comprise one of many cell binding molecules, including, but not limited to, antibodies, monoclonal antibodies, antibody fragments (Fab, Fab′, Fv, ScFv, etc.), lectins, hormones, cytokines, growth factors, peptides, carbohydrates, lipids, glycosins, nucleic acids or complement components.

The TM is selected in accordance with the desired cell-type to which the agent of the present invention is to be targeted, and preferably has a high specificity and/or affinity for non-neuronal target cells. Preferably, the TM does not substantially bind to neuronal cells of the presynaptic muscular junction and, thus the agent is substantially non-toxic in that it is not capable of effecting muscular paralysis. This is in contrast to clostridial holotoxin which targets the postsynaptic muscular junction and effect muscular paralysis. In addition, preferably the TM does not substantially bind to neuronal peripheral sensory cells, and thus the agent does not exert any substantial analgesic effect. Preferably, the TM does not substantially bind to neuronal cells, and does not therefore permit the agent to exert an inhibitory effect on secretion in a neuronal cell.

It is known in the art that the H₄ portion of the neurotoxin molecule can be removed from the other portion of the H-chain, known as H₄ₓ, such that the H₄ₓ fragment remains disulphide linked to the L-chain of the neurotoxin providing a fragment known as LH₄ₓ. Thus, in one embodiment of the present invention the LH₄ₓ fragment of a clostridial neurotoxin is covalently linked, using linkages which may include one or more spacer regions, to a TM.

In another embodiment of the invention, the H₄ domain of a clostridial neurotoxin is mutated, blocked or modified, e.g. by chemical modification, to reduce or pref-
erably incapacitate its ability to bind the neurotoxin to receptors at the neuromuscular junction. This modified clostridial neurotoxin is then covalently linked, using linkages which may include one or more spacer regions, to a TM.

In another embodiment of the invention, the heavy chain of a clostridial neurotoxin, in which the H$_2$ domain is mutated, blocked or modified, e.g. by chemical modification, to reduce or preferably incapacitate its ability to bind the neurotoxin to receptors at the neuromuscular junction, is combined with the L-chain of a different clostridial neurotoxin. This hybrid, modified clostridial neurotoxin is then covalently linked, using linkages which may include one or more spacer regions, to a TM.

In another embodiment of the invention, the H$_3$ domain of a clostridial neurotoxin is combined with the L-chain of a different clostridial neurotoxin. This hybrid L/H$_3$ is then covalently linked, using linkages which may include one or more spacer regions, to a TM.

In another embodiment of the invention, the light chain of a clostridial neurotoxin, or a fragment of the light chain containing the endopeptidase activity, is covalently linked, using linkages which may include one or more spacer regions, to a TM which can also affect the internalisation of the L-chain, or a fragment of the L-chain containing the endopeptidase activity, into the cytoplasm of the relevant non-neuronal cells responsible for secretion.

In another embodiment of the invention, the light chain of a clostridial neurotoxin, or a fragment of the light chain containing the endopeptidase activity, is covalently linked, using linkages which may include one or more spacer regions, to a translocation domain to effect transport of the endopeptidase fragment into the cytosol. Examples of translocation domains derived from bacterial neurotoxins are as follows:

- Botulinum type A neurotoxin—amino acid residues (449-871)
- Botulinum type B neurotoxin—amino acid residues (441-858)
- Botulinum type C neurotoxin—amino acid residues (442-866)
- Botulinum type D neurotoxin—amino acid residues (446-862)
- Botulinum type E neurotoxin—amino acid residues (423-845)
- Botulinum type F neurotoxin—amino acid residues (440-864)
- Botulinum type G neurotoxin—amino acid residues (442-863)
- Tetanus neurotoxin—amino acid residues (458-879)

Other clostridial sources include—C. butyricum, and C. argentinense.


In use, the domains of an agent according to the present invention are associated with each other. In one embodiment, two or more of the Domains may be joined together either directly (e.g. by a covalent linkage), or via a linker molecule. Conjugation techniques suitable for use in the present invention have been well documented:—Chemistry of protein conjugation and cross-linking, Edited by Wong, S. S. 1993, CRC Press Inc., Florida; and Bioconjugate techniques, Edited by Hermanson, G. T. 1996, Academic Press, London, UK.

Direct linkage of two or more of Domains is now described with reference to clostridial neurotoxins and to the present Applicant’s nomenclature of clostridial neurotoxin domains, namely Domain B (contains the binding domain), Domain T (contains the translocation domain) and Domain E (contains the protease domain), although no limitation thereto is intended.

In one embodiment of the present invention, Domains E and T may be mixed together in equimolar quantities under reducing conditions and covalently coupled by repeated dialysis (e.g. at 4°C, with agitation), into physiological salt solution in the absence of reducing agents. At this stage, in contrast to Example 6 of WO94/21300, the E-T complex is not blocked by iodoacetamide, therefore any remaining free —SH groups are retained.

Domain B is then modified, for example, by derivatisation with SPDP followed by subsequent reduction. In this reaction, SPDP does not remain attached as a spacer molecule to Domain B, but simply increases the efficiency of this reduction reaction.

Reduced domain B and the E-T complex may then be mixed under non-reducing conditions (e.g. at 4°C) to form a disulphide-linked E-T-B “agent”.

In another embodiment, a coupled E-T complex may be prepared according to Example 6 of WO94/21300, including the addition of iodoacetamide to block free sulphhydryl groups. However, the E-T complex is not further derivatised, and the remaining chemistry makes use of the free amino (—NH$_2$) groups on amino acid side chains (e.g. lysine, and arginine amino acids).

Domain B may be derivatised using carboxidiimide chemistry (e.g. using EDC) to activate carboxyl groups on amino acid side chains (e.g. glutamate, and aspartate amino acids), and the E-T complex mixed with the derivatised Domain B to result in a covalently coupled (amide bond) E-T-B complex.

Suitable methodology for the creation of such an agent is, for example, as follows:—

Domain B was dialysed into MES buffer (0.1 M MES, 0.1 M sodium chloride, pH 5.0) to a final concentra-
tion of 0.5 mg/ml. EDAC (1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride) was added to final concentrations of 0.2 mg/ml and reacted for 30 min at room temperature. Excess EDAC was removed by desalting over a MIEX buffer equilibrated PD-10 column (Pharmacia). The derivatised domain B was concentrated (to >2 mg/ml) using Millipore Biomax 10 concentrators. The E-T complex (1 mg/ml) was mixed for 16 hours at 4°C, and the E-T-B complex purified by size-exclusion chromatography over a Superose 12 HR10/30 column (Pharmacia) to remove unreacted Domain B (column buffer: 50 mM sodium phosphate pH 6.5+20 mM NaCl).

[0084] As an alternative to direct covalent linkage of the various Domains of an agent according to the present invention, suitable spacer molecules may be employed. The term linker molecule is used synonymously with spacer molecule. Spacer technology was readily available prior to the present application.

[0085] For example, one particular coupling agent (SPDP) is described in Example 6 of WO94/21300 (see lines 3-5 on page 16). In Example 6, SPDP is linked to an E-T complex, thereby providing an E-T complex including a linker molecule. This complex is then reacted a Domain B, which becomes attached to the E-T complex via the linker molecule. In this method, SPDP results in a spacing region of approximately 6.8 Angstroms between different Domains of the “agent” of the present invention.

[0086] A variant of SPDP known as LC-SPDP is identical in all respects to SPDP but for an increased chain length. LC-SPDP may be used to covalently link two Domains of the “agent” of the present invention resulting in a 15.6 Angstrom spacing between these Domains.

[0087] Examples of spacer molecules include, but are not limited to:—

[0088] (GGGGS)2, (SEQ ID NO:28), elbow regions of Fab—[see Anand et al., (1991) J. Biol. Chem. 266, 21874-9];


[0090] the interdomain linker of cellulose—[see Takkinen et al. (1991) Protein Eng. 4, 837-841];

[0091] PPPHEGR (SEQ ID NO:29)—[see Kim (1993) Protein Science, 2, 348-356];

[0092] Collagen-like spacer—[see Rock (1992) Protein Engineering, vol 5, No 6, pp 583-591]; and

[0093] Trypsin-sensitive diphtheria toxin peptide—[see O’Hare (1990) FEBS, vol 273, No 1, 2, pp 200-204].

[0094] In a further embodiment of the present invention, an agent having the structure E-X-T-X-B, where “X” is a spacer molecule between each domain, may be prepared, for example, as follows:—

[0095] Domain E is derivatised with SPDP, but not subsequently reduced. This results in an SPDP-derivatised Domain E.

[0096] Domain T is similarly prepared, but subsequently reduced with 10 mM dithiothreitol (DTT). The 10 mM DTT present in the Domain T preparation, following elution from the QAE column (see Example 6 in WO94/21300), is removed by passage of Domain T through a sephadex G-25 column equilibrated in PBS.

[0097] Domain T free of reducing agent is then mixed with the SPDP-derivatised Domain E, with agitation at 4°C, for 16 hours. E-T complex is isolated from free Domain E and from free Domain T by size-exclusion chromatography (Sephadex G-150). Whereafter, the same procedure can be followed as described in Example 6 of WO94/21300 for recrystallisation of the E-T complex with SPDP and subsequent coupling thereof to the free sulphhydril on Domain B.

[0098] The agents according to the present invention may be prepared recombinantly.

[0099] In one embodiment, the preparation of a recombinant agent may involve arrangement of the coding sequences of the selected TM and clostridial neurotoxin component in a single genetic construct. These coding sequences may be arranged in-frame so that subsequent transcription and translation is continuous through both coding sequences and results in a fusion protein. All constructs would have a 5' ATG codon to encode an N-terminal methionine, and a C-terminal translational stop codon.

[0100] Thus, a the light chain of a clostridial neurotoxin (or a fragment of the light chain containing the endopeptidase activity) may be expressed recombinantly as a fusion protein with a TM which can also effect the internalisation of the L-chain (or a fragment thereof) into the cytoplasm of the relevant non-neuronal cells responsible for secretion. The expressed fusion protein may also include one or more spacer regions.

[0101] In the case of an agent based on clostridial neurotoxin, the following information would be required to produce said agent recombinantly:—(i) DNA sequence data relating to a selected TM; (ii) DNA sequence data relating to the clostridial neurotoxin component; and (iii) a protocol to permit construction and expression of the construct comprising (i) and (ii).

[0102] All of the above basic information (i)-(iii) are either readily available, or are readily determinable by conventional methods. For example, both WO98/07864 and WO99/17806 exemplify clostridial neurotoxin recombinant technology suitable for use in the present application.

[0103] In addition, methods for the construction and expression of the constructs of the present invention may employ information from the following references and others:


[0111] Similarly, suitable TM sequence data are widely available in the art. Alternatively, any necessary sequence data may be obtained by techniques which were well-known to the skilled person.

[0112] For example, DNA encoding the TM component may be cloned from a source organism by screening a cDNA library for the correct coding region (for example by using specific oligonucleotides based on the known sequence information to probe the library), isolating the TM DNA, sequencing this DNA for confirmation purposes, and then placing the isolated DNA in an appropriate expression vector for expression in the chosen host.

[0113] As an alternative to isolation of the sequence from a library, the available sequence information may be employed to prepare specific primers for use in PCR, whereby the coding sequence is then amplified directly from the source material and, by suitable use of primers, may be cloned directly into an expression vector.

[0114] Another alternative method for isolation of the coding sequence is to use the existing sequence information and synthesise a copy, possibly incorporating alterations, using DNA synthesis technology. For example, DNA sequence data may be generated from existing protein and/or RNA sequence information. Using DNA synthesis technology to do this (and the alternative described above) enables the codon bias of the coding sequence to be modified to be optimal for the chosen expression host. This may give rise to superior expression levels of the fusion protein.

[0115] Optimisation of the codon bias for the expression host may be applied to the DNA sequence encoding the TM and clostridial components of the construct. Optimisation of the codon bias is possible by application of the protein sequence into freely available DNA/protein database software, e.g. programs available from Genecodes Computer Group, Inc.

[0116] According to a further aspect of the present invention, nucleic acid encoding the light chain of a clostridial neurotoxin (or a fragment of the light chain containing the endopeptidase activity), may be associated with a TM which can also effect the internalisation of the nucleic acid encoding the L-chain (or a fragment thereof) into the cytoplasm of the relevant non-neuronal cells responsible for secretion. The nucleic acid sequence may be coupled to a translocation domain, and optionally to a targeting moiety, for example direct covalent linkage or via spacer molecule technology. Ideally, the coding sequence will be expressed in the target cell.

[0117] Thus, the agent of the present invention may be the expression product of a recombinant gene delivered independently to the preferred site of action of the agent. Gene delivery technologies are widely reported in the literature [reviewed in “Advanced Drug Delivery Reviews” Vol. 27, (1997), Elsevier Science Ireland Ltd.]

[0118] According to another aspect, the present invention therefore provides a method of treating a condition or disease which is susceptible of treatment with a nucleic acid in a mammal e.g. a human which comprises administering to the sufferer an effective, non-toxic amount of a compound of the invention. A condition or disease which is susceptible of treatment with a nucleic acid may be for example a condition or disease which may be treated by or requiring gene therapy. The preferred conditions or diseases susceptible to treatment according to the present invention, together with the preferred TMs, have been described previously in this specification. Similarly, the preferred first domains which cleave one or more proteins (e.g. SNAP-25, synaptobrevin and syntaxin) essential to exocytosis have been described previously in this specification. The various domains of an agent for use in gene therapy may be directly linked (e.g. via a covalent bond) or indirectly linked (e.g. via a spacer molecule), as for example previously described in this specification.

[0119] The invention further provides a compound of the invention for use as an active therapeutic substance, in particular for use in treating a condition or disease as set forth in the present claims.

[0120] The invention further provides pharmaceutical compositions comprising an agent or a conjugate of the invention and a pharmaceutically acceptable carrier.

[0121] In use the agent or conjugate will normally be employed in the form of a pharmaceutical composition in association with a human pharmaceutical carrier, diluent and/or excipient, although the exact form of the composition will depend on the mode of administration.

[0122] The conjugate may, for example, be employed in the form of an aerosol or nebulisable solution for inhalation or a sterile solution for parenteral administration, intra-articular administration or intra-cranial administration.

[0123] For treating endocrine targets, i.e. injection, direct injection into gland, or aerosolisation for lung delivery are preferred; for treating inflammatory cell targets, i.e. injection, sub-cutaneous injection, or surface patch administration are preferred; for treating exocrine targets, i.e. injection, or direct injection into the gland are preferred; for treating immunological targets, i.e. injection, or injection into specific tissues e.g. thymus, bone marrow, or lymph tissue are preferred; for treatment of cardiovascular targets, i.e. injection, or direct injection is preferred. In cases of i.v. injection, this should also include the use of pump-systems.
[0124] The dosage ranges for administration of the compounds of the present invention are those to produce the desired therapeutic effect. It will be appreciated that the dosage range required depends on the precise nature of the conjugate, the route of administration, the nature of the formulation, the age of the patient, the nature, extent or severity of the patient’s condition, contraindications, if any, and the judgement of the attending physician.

[0125] Suitable daily dosages are in the range 0.0001-1 mg/kg, preferably 0.0001-0.5 mg/kg, more preferably 0.002-0.5 mg/kg, and particularly preferably 0.004-0.5 mg/kg. The unit dosage can vary from less than 1 microgram to 30 mg, but typically will be in the region of 0.01 to 1 mg per dose, which may be administered daily or less frequently, such as weekly or six monthly.

[0126] Wide variations in the required dosage, however, are to be expected depending on the precise nature of the conjugate, and the differing efficiencies of various routes of administration. For example, oral administration would be expected to require higher dosages than administration by intravenous injection.

[0127] Variations in these dosage levels can be adjusted using standard empirical routines for optimisation, as is well understood in the art.

[0128] Compositions suitable for injection may be in the form of solutions, suspensions or emulsions, or dry powders which are dissolved or suspended in a suitable vehicle prior to use.

[0129] Fluid unit dosage forms are typically prepared utilising a pyrogen-free sterile vehicle. The active ingredients, depending on the vehicle and concentration used, can be either dissolved or suspended in the vehicle.

[0130] Solutions may be used for all forms of parenteral administration, and are particularly used for intravenous injection. In preparing solutions the compound can be dissolved in the vehicle, the solution being made isotonic if necessary by addition of sodium chloride and sterilised by filtration through a sterile filter using aseptic techniques before filling into suitable sterile vials or ampoules and sealing. Alternatively, if solution stability is adequate, the solution in its sealed containers may be sterilised by autoclaving.

[0131] Advantageously additives such as buffering, solubilising, stabilising, preservative or bactericidal, suspending or emulsifying agents and/or local anaesthetic agents may be dissolved in the vehicle.

[0132] Dry powders which are dissolved or suspended in a suitable vehicle prior to use may be prepared by filling pre-sterilised drug substance and other ingredients into a sterile container using aseptic technique in a sterile area.

[0133] Alternatively the agent and other ingredients may be dissolved in an aqueous vehicle, the solution is sterilized by filtration and distributed into suitable containers using aseptic technique in a sterile area. The product is then freeze dried and the containers are sealed aseptically.

[0134] Parenteral suspensions, suitable for intramuscular, subcutaneous or intradermal injection, are prepared in substantially the same manner, except that the sterile compound is suspended in the sterile vehicle, instead of being dissolved and sterilisation cannot be accomplished by filtration. The compound may be isolated in a sterile state or alternatively it may be sterilised after isolation, e.g. by gamma irradiation.

[0135] Advantageously, a suspending agent for example polyvinylpyrrolidone is included in the composition to facilitate uniform distribution of the compound.

[0136] Compositions suitable for administration via the respiratory tract include aerosols, nebulisable solutions or microfine powders for insufflation. In the latter case, particle size of less than 50 microns, especially less than 10 microns, is preferred. Such compositions may be made up in a conventional manner and employed in conjunction with conventional administration devices.

[0137] The agent described in this invention can be used in vivo, either directly or as a pharmaceutically acceptable salt, for the treatment of conditions involving secretion from non-neuronal cells, such as hyposesecretion of endocrine cell derived chemical messengers, hypersecretion from exocrine cells, secretions from the cells of the immune system, the cardiovascular system and from bone cells.

[0138] The present invention will now be described by reference to the following examples illustrated by the accompanying drawings in which:

[0139] FIG. 1 shows SDS-PAGE analysis of WGA-LH/α interaction scheme;

[0140] FIG. 2 shows activity of WGA-LH/α on release of transmitter from HIT-T15 cells;

[0141] FIG. 3 shows correlation of SNAP-25 cleavage with inhibition of neurotransmitter release following application of WGA-LH/α to HIT-T15 cells;

[0142] FIG. 4 shows activity of WGA-LH/α on release of [3H]-noradrenaline from undifferentiated PC12 cells;

[0143] FIG. 5 shows a Western blot indicating expression of rece.LH/α in E. coli;

[0144] FIG. 6 shows in vitro cleavage of synthetic VAMP peptide by rece.LH/α;

[0145] FIG. 7 shows the effect of low pH and BoNT/B treatment on stimulated von Willebrands Factor (vWF) release from human umbilical vein endothelial cells;

[0146] FIG. 8 shows release of [3H]-glucosamine labelled high molecular weight material from LS180 cells;

[0147] FIG. 9 shows the effect of low pH and BoNT/B treatment on stimulated beta-glucuronidase release from differentiated HL.60 cells;

[0148] FIG. 10 shows purification of a LHN/C-EGF fusion protein;

[0149] FIG. 11 shows purification of a LHN/B-EGF fusion protein;

[0150] FIG. 12 shows purification of a LHN/C-RGD fusion protein;

[0151] FIG. 13 shows purification of a LHN/C-cyclic RGD fusion protein;

[0152] FIG. 14 shows purification of a LC/C-RGD-HN/C fusion protein;
FIG. 15 shows VAMP cleavage activity of LHN/B-EGF;

FIG. 16 shows effect of 10 nm Syntaxin compounds con LPS-mediated IL-8 secretion by THP-1 cells;

FIG. 17 shows effect of 10 nm Syntaxin compounds con LPS-mediated IL-10 secretion by RPMI-8226 cells;

FIG. 18 shows effect of EGF and fusions on IL-8 production and on LPS-stimulated IL-8 secretion; and

FIG. 19 shows effect of EGF and fusions on IP-10 production and on PHA-stimulated IP-10 secretion.

FIGS. 5-19 are now described in more detail.

Referring to FIG. 5, MBP-LH$_2$/B was expressed in E. coli as described in Example 4. Lane 1 represents the profile of the expressed fusion protein in E. coli. Lane 2 represents the profile of fusion protein expression in the crude E. coli lysate. Lane 3 represents the profile of the MBP-LH$_2$/B following purification by immobilised amylose. Molecular weights in kDa are indicated to the right side of the Figure.

Referring to FIG. 6, dilutions of recLH$_2$/B (prepared as described in Example 4) and BoNT/B were compared in an in vitro peptide cleavage assay. Data indicate that the recombinant product has similar catalytic activity to that of the native neurotoxin, indicating that the recombinant product has folded correctly into an active conformation.

Referring to FIG. 7, cells were exposed to pH 4.7 media with or without 500 nM BoNT/B (control cells received pH7.4 medium) for 2.5 hours then washed. 24 hours later release of vWF was stimulated using 1 mM histamine and the presented results are the net stimulated release with basal subtracted. Results are presented in mIU of vWF/ml and are the mean +/- SEM of three determinations apart from pH 4.7 alone which is two determinations. pH 4.7+BoNT/B has reduced vWF release by 27.4% compared to pH 4.7 controls.

Referring to FIG. 8, high molecular weight mucin synthesising colon carcinoma LS180 cells were treated with pH 4.7 medium and pH 4.7 medium containing 500 nM botulinum neurotoxin type B (BoNT/B) for four hours then labelled with $[^{1}H]$-glucosamine for 18 hours. Release of high molecular weight material was stimulated with 10 µM ionomycin and $[^{1}H]$-glucosamine labelled material recovered by ultrasentrifugation and centrifugal molecular weight sieving. Radiolabel of release of labelled high molecular weight weight material was determined by scintillation counting and net stimulated release calculated by subtracting non-stimulated basal values. Data are expressed as disintegrations per minute (dpm)/+/-SEM of three determinations. BoNT/B co-treatment clearly inhibits the release of high molecular weight material from these mucin synthesising cells and in this experiment a 74.5% reduction was seen.

Referring to FIG. 9, cells were exposed to pH 4.8 media with or without 500 nM BoNT/B (control cells received pH 7.4 medium) for 2.5 hours then washed and differentiated for 40 hours by the addition of 300 µM dibutryl cyclic AMP (dbcAMP). Cells were stimulated with Met-Leu-Phe (1 µM)+ATP (100 µM) in the presence of cytochalasin B (5 µM) for 10 minutes and released β-glucuronidase determined by colourimetric assay. Net stimulated release was calculated by subtraction of unstimulated basal release values from stimulated values and released activity is expressed as a percentage of the total activity present in the cells. Data are the mean +/- SEM of three determinations. BoNT/B treatment in low pH medium significantly inhibited stimulated release of β-glucuronidase compared to cells treated with low pH alone (p=0.0315 when subjected to a 2 tailed Student T test with groups of unequal variance).

Referring to FIG. 10, using the methodology outlined in Example 11, a LHN/C-EGF fusion protein was purified from E. coli BL21 cells. Briefly, the soluble products obtained following cell disruption were applied to a nickel-charged affinity capture column. Bound proteins were eluted with 100 mM imidazole, treated with Factor Xa to activate the fusion protein and remove the maltose-binding protein (MBP) tag, then re-applied to a second nickel-charged affinity capture column. Samples from the purification procedure were assessed by SDS-PAGE. Lane 1: Molecular mass markers (kDa), lane 2: Clarified crude cell lysate, lane 3: First nickel chelating Sepharose column eluant, lane 4: Factor Xa digested protein, lane 5: Purified LHN/C-EGF under non-reducing conditions, lane 7: Purified LHN/C-EGF under reduced conditions.

Referring to FIG. 11, using the methodology outlined in Example 12, a LHN/B-EGF fusion protein was purified from E. coli BL21 cells. Briefly, the soluble products obtained following cell disruption were applied to a nickel-charged affinity capture column. Bound proteins were eluted with 100 mM imidazole, treated with Factor Xa and enterokinase to activate the fusion protein and remove the maltose-binding protein (MBP) tag, then re-applied to a second nickel-charged affinity capture column. Samples from the purification procedure were assessed by SDS-PAGE. The final purified material in the absence and presence of reducing agent is identified in the lanes marked [-] and [+*] respectively.

Referring to FIG. 12, using the methodology outlined in Example 13, a LHN/C-RGD fusion protein was purified from E. coli BL21 cells. Briefly, the soluble products obtained following cell disruption were applied to a nickel-charged affinity capture column. Bound proteins were eluted with 100 mM imidazole, treated with Factor Xa to activate the fusion protein and remove the maltose-binding protein (MBP) tag, then re-applied to a second nickel-charged affinity capture column. Samples from the purification procedure were assessed by SDS-PAGE. The final purified material in the absence and presence of reducing agent is identified in the lanes marked [-] and [+*] respectively.

Referring to FIG. 13, using the methodology outlined in Example 14, a LHN/C-cyclic RGD fusion protein was purified from E. coli BL21 cells. Briefly, the soluble products obtained following cell disruption were applied to a nickel-charged affinity capture column. Bound proteins were eluted with 100 mM imidazole, treated with Factor Xa to activate the fusion protein and remove the maltose-binding protein (MBP) tag, then re-applied to a second nickel-charged affinity capture column. Samples from the purification procedure were assessed by SDS-PAGE. The
final purified material in the absence and presence of reducing agent is identified in the lanes marked [−] and [+] respectively.

[0168] Referring to FIG. 14, using the methodology outlined in Example 15, a LC-C-RGD-LHN/C fusion protein was purified from E. coli BL21 cells. Briefly, the soluble products obtained following cell disruption were applied to a nickel-charged affinity capture column. Bound proteins were eluted with 100 mM imidazole, treated with Factor Xa to activate the fusion protein and remove the maltose-binding protein (MBP) tag, then re-applied to a second nickel-charged affinity capture column. Samples from the purification procedure were assessed by SDS-PAGE. The final purified material in the absence and presence of reducing agent is identified in the lanes marked [−] and [+] respectively.

[0169] Referring to FIG. 15, using the methodology outlined in example 16, BoNT/B (○), LHN/B (■) and LHN/B-EGF (▲) were assayed for VAMP cleavage activity.

[0170] Referring to FIG. 16, using the methodology outlined in Example 17, the activity of EGF-LHN/C (SXXN100501) and EGF-LHN/B (SXXN100328) was assessed in THP-1 immune cells. The quantity of secreted IL-8 was determined by Luminex-based technology. Data are presented as % of LPS control.

[0171] Referring to FIG. 17, using the methodology outlined in Example 18, the activity of EGF-LHN/C (SXXN100501) and EGF-LHN/B (SXXN100328) was assessed in RPMI-8226 immune cells. The quantity of secreted IL-10 was determined by Luminex-based technology. Data are presented as % of LPS control.

[0172] Referring to FIG. 18, using the methodology outlined in Example 19, the activity of EGF-LHN/C (SXXN100501) and EGF-LHN/B (SXXN100328) and CP-RGD-LHN/C (SXXN100221) was assessed in PBMC immune cells. The quantity of secreted IL-8 was determined by Luminex-based technology. Data are presented as % of LPS control.

[0173] Referring to FIG. 19, using the methodology outlined in Example 20, the activity of EGF-LHN/C (SXXN100501) and EGF-LHN/B (SXXN100328) and CP-RGD-LHN/C (SXXN100221) was assessed in PBMC immune cells. The quantity of secreted IP-10 was determined by Luminex-based technology. Data are presented as % of PHA control.

EXAMPLES

Example 1

Production of a Conjugate of a Lectin from *Triticum vulgaris* and LHN/A

Materials

[0174] Lectin from *Triticum vulgaris* (Wheat Germ Agglutinin-WGA) was obtained from Sigma Ltd.

[0175] SPDP was from Pierce Chemical Co.

[0176] PD-10 desalting columns were from Pharmacia.

[0177] Dimethylsulphoxide (DMSO) was kept anhydrous by storage over a molecular sieve.

[0178] Denaturing sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and non-denaturing polyacrylamide gel electrophoresis was performed using gels and reagents from Novex.

[0179] Additional reagents were obtained from Sigma Ltd.

[0180] LHN/A was prepared according to a previous method (Shone, C. C. and Tranter, H. S. (1995) in “Clostridial Neurotoxins — The molecular pathogenesis of tetanus and botulism”, (Montecucco, C., Ed.), pp. 152-160, Springer). FPLC chromatography media and columns were obtained from Amersham Pharmacia Biotech, UK. Affi-gel® Hζ matrix and materials were from BioRad, UK.

Preparation of an Anti-BoNT/A Antibody-Affinity Column

[0181] An antibody-affinity column was prepared with specific monoclonal antibodies essentially as suggested by the manufacturers, quadrature, protocol. Briefly, monoclonal antibodies 5BA2.3 & 5BA9.3 which have different epitope recognition in the Hζ domain (Hallis, B., Fooks, S., Shone, C. and Hambleton, P. (1993) in “Botulimum and Tetanus Neurotoxins”, (Das Gupta, B. R., Ed.), pp. 433-436, Plenum Press, New York) were purified from mouse hybridoma tissue culture supernatant by Protein G (Amersham Pharmacia Biotech) chromatography. These antibodies represent a source of BoNT/A Hζ-specific binding molecules and can be immobilised to a matrix or used free in solution to bind BoNT/A. In the presence of partially purified LHN/A (which has no Hζ domain) these antibodies will only bind to BoNT/A. The antibodies 5BA2.3 & 5BA9.3 were pooled in a 3:1 ratio and two mg of the pooled antibody was oxidised by the addition of sodium periodate (final concentration of 0.2%) prior coupling to 1 ml Affi-Gel Hζ™ gel (16 hours at room temperature). Coupling efficiencies were routinely greater than 65%. The matrix was stored at 4°C. in the presence of 0.02% sodium azide.

Purification Strategy for the Preparation of Pure LHN/A

[0182] BoNT/A was treated with 17.5μg trypsin per mg BoNT/A for a period of 72-120 hours. After this time no material of 150 kDa was observed by SDS-PAGE and Coomassie blue staining. The trypsin digested sample was chromatographed (FPLC system, Amersham Pharmacia Biotech) on a Mono Q column (HR5/5) to remove trypsin and separate the majority of BoNT/A from LHN/A. The crude sample was loaded onto the column at pH 7 in 20 mM HEPES, 50 mM NaCl and 2 mM LHN/A fractions eluted in a NaCl gradient from 50 mM to 150 mM. The slightly greater pl of BoNT/A (6.3) relative to LHN/A (5.2) encouraged any BoNT/A remaining after trypsinisation to elute from the anion exchange column at a lower salt concentration than LHN/A. LHN/A containing fractions (as identified by SDS-PAGE) were pooled for application to the antibody column.

[0183] The semi-purified LHN/A mixture was applied and re-applied at least 3 times to a 1-2 ml immobilised monoclonal antibody matrix at 20°C. After a total of 3 hours in contact with the immobilised antibodies, the LHN/A-enriched supernatant was removed. Entrapment of the BoNT/A contaminant, rather than specifically binding the LHN/A, enables the elution conditions to be maintained at the optimum for LHN/A stability. The use of harsh elution conditions e.g. low pH, high salt, chaotropic ions, which may have detrimental effects on LHN/A polypeptide folding and enzymatic activity, are therefore avoided. Treatment of the
immobilised antibody column with 0.2M glycine/HCl pH2.5 resulted in regeneration of the column and elution of BoNT/A-reactive proteins of 150 kDa.

[0184] The L_{H_2}/A enriched sample was then applied 2 times to a 1 ml HiTrap™ Protein G column (Amersham Pharmacia Biotech) at 20°C. Protein G was selected since it has a high affinity for mouse monoclonal antibodies. This step was included to remove BoNT/A-antibody complexes that may leach from the immunocolumn. Antibody species bind to the Protein G matrix allowing purified L_{H_2}/A to elute, essentially by the method of Shone C. C., Hambleton, P., and Melling, J. 1987. Eur. J. Biochem. 167, 175-180, and as described in PCT/GB00/03519.

Methods

[0185] The lyophilised lectin was rehydrated in phosphate buffered saline (PBS) to a final concentration of 10 mg/ml. Aliquots of this solution were stored at ~20°C until use.

[0186] The WGA was reacted with an equal concentration of SPDP by the addition of a 10 mM stock solution of SPDP in DMSO with mixing. After one hour at room temperature the reaction was terminated by desalting into PBS over a PD-10 column.

[0187] The thiopyridone leaving group was removed from the product to release a free —SH group by reduction with dithiothreitol (DTT; 5 mM; 30 min). The thiopyridone and DTT were removed by once again desalting into PBS over a PD-10 column.

[0188] The L_{H_2}/A was desalted into PBSE (PBS containing 1 mM EDTA). The resulting solution (0.5-1.0 mg/ml) was reacted with a four-fold molar excess of SPDP by addition of a 10 mM stock solution of SPDP in DMSO. After 3 h at room temperature the reaction was terminated by desalting over a PD-10 column into PBSE.

[0189] A portion of the derivatized L_{H_2}/A was removed from the solution and reduced with DTT (5 mM, 30 min). This sample was analyzed spectrophotometrically at 280 nm and 343 nm to determine the degree of derivatisation. The degree of derivatisation achieved was 3.534/0.59 mol/mol.

[0190] The bulk of the derivatized L_{H_2}/A and the derivatized WGA were mixed in proportions such that the WGA was in greater than three-fold molar excess. The conjugation reaction was allowed to proceed for >16 h at 4°C.

[0191] The product mixture was centrifuged to clear any precipitate that had developed. The supernatant was concentrated by centrifugation through concentrators (with 10000 molecular weight exclusion limit) before application to a Superose 12 column on an FPLC chromatography system (Pharmacia). The column was eluted with PBS and the elution profile followed at 280 nm.

[0192] Fractions were analyzed by SDS-PAGE on 4-20% polyacrylamide gradient gels, followed by staining with Coomassie Blue. The major conjugate products appear to have an apparent molecular mass of between 106-150 kDa, these are separated from the bulk of the remaining unconjugated L_{H_2}/A and more completely from the unconjugated WGA.

contaminants (predominantly unconjugated L_{H_2}/A). WGA-L_{H_2}/A conjugate was eluted from the column by the addition of 0.3M N-acetylgalactosamine (in PBS) and the elution profile followed at 280 nm. See FIG. 1 for SDS-PAGE profile of the whole purification scheme.

[0193] The fractions containing conjugate were pooled, dialysed against PBS, and stored at 4°C until use.

Example 2

Activity of WGA-L_{H_2}/A in Cultured Endocrine Cells (HIT-T15)

[0194] The hamster pancreatic B cell line HIT-T15 is an example of a cell line of endocrine origin. It thus represents a model cell line for the investigation of inhibition of release effects of the agents. HIT-T15 cells possess surface moieties that allow for the binding, and internalisation, of WGA-L_{H_2}/A.

[0195] In contrast, HIT-T15 cells lack suitable receptors for clostridial neurotoxins and are therefore not susceptible to botulinum neurotoxins (BoNTs).

[0196] FIG. 2 illustrates the inhibition of release of insulin from HIT-T15 cells after prior incubation with WGA-L_{H_2}/A. It is clear that dose-dependent inhibition is observed, indicating that WGA-L_{H_2}/A can inhibit the release of insulin from an endocrine cell model.

[0197] Inhibition of insulin release was demonstrated to correlate with cleavage of the SNARE protein, SNAP-25 (FIG. 3). Thus, inhibition of release of chemical messenger is due to a clostridial endopeptidase-mediated effects of SNARE-protein cleavage.

Materials

[0198] Insulin radioimmunoassay kits were obtained from Linco Research Inc., USA. Western blotting reagents were obtained from Novex.

Methods

[0199] HIT-T15 cells were seeded onto 12 well plates and cultured in RPMI-1640 medium containing 5% foetal bovine serum, 2 mM L-glutamine for 5 days prior to use. WGA-L_{H_2}/A was applied for 4 hours on ice; the cells were washed to remove unbound WGA-L_{H_2}/A, and the release of insulin assayed 16 hours later. The release of insulin from HIT-T15 cells was assessed by radioimmunoassay exactly as indicated by the manufacturer's instructions.

[0200] Cells were lysed in 2M acetic acid/0.1% TFA. Lysates were dried then resuspended in 0.1 M Heps, pH 7.0. To extract the membrane proteins Triton-X-114 (10%, v/v) was added and incubated at 4°C for 60 min. The insoluble material was removed by centrifugation and the supernatants were warmed to 37°C for 30 min. The resulting two phases were separated by centrifugation and the upper phase discarded. The proteins in the lower phase were precipitated with chloroform/methanol for analysis by Western blotting.

[0201] The samples were separated by SDS-PAGE and transferred to nitrocellulose. Proteolysis of SNAP-25, a crucial component of the neurosecretory process and the substrate for the zinc-dependent endopeptidase activity of
BoNT/A, was then detected by probing with an antibody (SMI-81) that recognises both the intact and cleaved forms of SNAP-25.

Example 3
Activity of WGA-LH$_2$/A in Cultured Neuroendocrine Cells (PC12)

[0202] The rat pheochromocytoma PC12 cell line is an example of a cell line of neuroendocrine origin. In its undifferentiated form it has properties associated with the adrenal chromaffin cell [Greene and Tischler, in “Advances in Cellular Neurobiology” (Federoff and Hertz, eds), Vol. 3, p 373-414. Academic Press, New York, [982]]. It thus represents a model cell line for the investigation of inhibition release effects of the agents. PC12 cells possess surface moieties that allow for the binding, and internalisation, of WGA-LH$_2$/A. FIG. 4 illustrates the inhibition of release of noradrenaline from PC12 cells after prior incubation with WGA-LH$_2$/A. It is clear that dose-dependent inhibition is observed, indicating that WGA-LH$_2$/A can inhibit the release of a hormone from a neuroendocrine cell model. Comparison of the inhibition effects observed with conjugate and the untargeted LH$_2$/A demonstrate the requirement for a targeting moiety (TM) for efficient inhibition of transmitter release.

Methods

[0203] PC12 cells were cultured on 24 well plates in RPMI-1640 medium containing 10% horse serum, 5% foetal bovine serum, 1% L-glutamine. Cells were treated with a range of concentrations of WGA-LH$_2$/A for three days. Secretion of noradrenaline was measured by labelling cells with $[^3H]$noradrenaline (2 µCi/ml, 0.5 mU/ml) for 60 min. Cells were washed every 15 min for 1 hour then basal release determined by incubation with a balanced salt solution containing 5 mM KCl for 5 min. Secretion was stimulated by elevating the concentration of extracellular potassium (100 mM KCl) for 5 min. Radioactivity in basal and stimulated superfusates was determined by scintillation counting. Secretion was expressed as a percentage of the total uptake and stimulated secretion was calculated by subtracting basal. Inhibition of secretion was dose-dependent with an observed IC$_{50}$ of 0.63+/-0.15 µg/ml (n=3). Inhibition was significantly more potent when compared to untargeted endopeptidase (LH$_2}$/A in FIG. 4). Thus WGA-LH$_2$/A inhibits release of neurotransmitter from a model neuroendocrine cell type.

Example 4
Expression and Purification of Catalytically Active Recombinant LH$_2}$/B

[0204] The coding region for LH$_2}$/B was inserted in-frame to the 3' of the gene encoding maltose binding protein (MBP) in the expression vector pMAL (New England Biolabs). In this construct, the expressed MBP and LH$_2}$/B polypeptides are separated by a Factor Xa cleavage site.

[0205] Expression of the MBP-LH$_2}$/B in _E. coli_ TG1 was induced by addition of IPTG to the growing culture at an approximate OD$_{600}$ of 0.8. Expression was maintained for a further 3 hours in the presence of inducing agent prior to harvest by centrifugation. The recovered cell paste was stored at -20°C until required.

[0206] The cell paste was resuspended in resuspension buffer (50 mM Hepes pH 7.5+150 mM NaCl+ a variety of protease inhibitors) at 6 ml buffer per gram paste. To this suspension was added lysozyme to a final concentration of 1 mg/ml. After 10 min at 0°C, the suspension was sonicated for 6x30 seconds at 24°C at 0°C. The broken cell paste was then centrifuged to remove cell debris and the supernatant recovered for chromatography.

[0207] In some situations, the cell paste was disrupted by using proprietary disruption agents such as BugBuster™ Novagen as per the manufacturers protocol. These agents were satisfactory for disruption of the cells to provide supernatant material for affinity chromatography.

[0208] The supernatant was applied to an immobilised amylose matrix at 0.4 ml/min to facilitate binding of the fusion protein. After binding, the column was washed extensively with resuspension buffer to remove contaminating proteins. Bound proteins were eluted by the addition of elution buffer (resuspension buffer+10 mM maltose) and fractions collected. Eluted fractions containing protein were pooled for treatment with Factor Xa.

[0209] On some occasions a further purification step was incorporated into the scheme, prior to the addition of Factor Xa. In these instances, the eluted fractions were made to 5 mM DTT and applied to a Pharmacia Mono-Q HR5/5 column (equilibrated in resuspension buffer) as part of an FPLC system. Proteins were bound to the column at 150 mM NaCl, before increased to 500 mM NaCl over a gradient. Fractions were collected and analysed for the presence of MBP-LH$_2}$/B by Western blotting (prote antibody-guinea pig anti-BoNT/B or commercially obtained anti-MBP).

[0210] Cleavage of the fusion protein by Factor Xa was as described in the protocol supplied by the manufacturer (New England Biolabs). Cleavage of the fusion protein resulted in removal of the MBP fusion tag and separation of the LC and H$_N$ domains of LH$_2}$/B. Passage of the cleaved mixture through a second immobilised maltose column removed free MBP from the mixture to leave purified disulphide-linked LH$_2}$/B. This material was used for conjugation.

[0211] See FIG. 5 for an illustration of the purification of LH$_2}$/B.

[0212] See FIG. 6 for an illustration of the in vitro catalytic activity of LH$_2}$/B.

Example 5
Production of a Conjugate of a Lectin from _Triticum vulgaris_ and LH$_2}$/B

Materials

[0213] Lectin from _Triticum vulgaris_ (WGA) was obtained from Sigma Ltd.

[0214] LH$_2}$/B was prepared as described in Example 4.

[0215] SPDP was from Pierce Chemical Co.

[0216] PD-10 desalting columns were from Pharmacia.
Dimethylsulphoxide (DMSO) was kept anhydrous by storage over a molecular sieve.

Polyacrylamide gel electrophoresis was performed using gels and reagents from Novex.

Additional reagents were obtained from Sigma Ltd.

Methods

The lyophilised lectin was rehydrated in phosphate buffered saline (PBS) to a final concentration of 10 mg/ml. Aliquots of this solution were stored at -20°C until use.

The WGA was reacted with an equal concentration of SPDP by the addition of a 10 mM stock solution of SPDP in DMSO with mixing. After one hour at room temperature the reaction was terminated by desalting into PBS over a PD-10 column.

The thiopyridone leaving group was removed from the product to release a free ---SH group by reduction with dithiothreitol (DTT; 5 mM; 30 min). The thiopyridone and DTT were removed by once again desalting into PBS over a PD-10 column.

The recr.H$_2$B was desalted into PBS. The resulting solution (0.5-1.0 mg/ml) was reacted with a four-fold molar excess of SPDP by addition of a 10 mM stock solution of SPDP in DMSO. After 3 h at room temperature the reaction was terminated by desalting over a PD-10 column into PBS.

A portion of the derivatized recr.H$_2$B was removed from the solution and reduced with DTT (5 mM; 30 min). This sample was analysed spectrophotometrically at 280 nm and 343 nm to determine the degree of derivatisation.

The bulk of the derivatized recr.H$_2$B and the derivatized WGA were mixed in proportions such that the WGA was in greater than three-fold molar excess. The conjugation reaction was allowed to proceed for >16 h at 4°C.

The product mixture was centrifuged to clear any precipitate that had developed. The supernatant was concentrated by centrifugation through concentrators (with 10,000 molecular weight exclusion limit) before application to a Superdex G-200 column on an FPLC chromatography system (Pharmacia). The column was eluted with PBS and the elution profile followed at 280 nm.

Fractions were analysed by SDS-PAGE on 4-20% polycrylamide gradient gels, followed by staining with Coomassie Blue. The major conjugate products have an apparent molecular mass of between 106-150 kDa, these are separated from the bulk of the remaining unconjugated recr.H$_2$B and more completely from the unconjugated WGA. Fractions containing conjugate were pooled prior to addition to PBS-washed N-acetylgalactosamine-agarose. Lectin-containing proteins (i.e. WGA-recr.H$_2$B conjugate) remained bound to the agarose during washing with PBS to remove contaminants (predominantly unconjugated recr.H$_2$B). WGA-recr.H$_2$B conjugate was eluted from the column by the addition of 0.3 M N-acetylgalactosamine (in PBS) and the elution profile followed at 280 nm.

The fractions containing conjugate were pooled, dialysed against PBS, and stored at 4°C until use.

Human umbilical vein endothelial cells (HUVEC) secrete von Willebrand Factor (vWF) when stimulated with a variety of cell surface receptor agonists including histamine. These cells maintain this property when prepared from full term umbilical cords and grown in culture (Loesberg et al 1983, Biochim. Biophys. Acta. 763, 160-168). The release of vWF by HUVEC thus represents a secretory activity of a non-neuronal cell type derived from the cardiovascular system. FIG. 7 illustrates the inhibition of the histamine stimulated release of vWF by HUVEC when previously treated with BoNT/B in low pH medium. Treatment of cells with toxins in low pH can be used as a technique for facilitating toxin penetration of the plasmalemma of cells refractory to exogenously applied clostridial neurotoxins.

This result clearly shows the ability of botulinum neurotoxins to inhibit secretory activity of non-neuronal cells in the cardiovascular system (see FIG. 7).

Methods

HUVEC were prepared by the method of Jaffe et al 1973, J. Clin. Invest. 52, 2745-2756. Cells were passaged once onto 24 well plates in medium 199 supplemented with 10% foetal calf serum, 10% newborn calf serum, 5 mM L-glutamine, 100 units/ml penicillin, 100 units/ml streptomycin, 20 µg/ml endothelial cell growth factor (Sigma). Cells were treated with DMEM pH 7.4, DMEM pH 4.7 (pH lowered with HCl) or DMEM, pH 4.7 with 500 nM BoNT/B for 2.5 hours then washed three times with HUVEC medium. 24 hours later cells were washed with a balanced salt solution, pH 7.4 and exposed to this solution for 30 minutes for the establishment of basal release. This was removed and BSS containing 1 mM histamine applied for a further 30 minutes. Superfusates were centrifuged to remove any detached cells and the quantity of vWF determined using an ELISA assay as described by Paleolog et al 1990, Blood, 75, 688-695. Stimulated secretion was then calculated by subtracting basal from the histamine stimulated release. Inhibition by BoNT/B treatment at pH 4.7 was calculated at 27.4% when compared to pH 4.7 treatment alone.

Example 7

Activity of BoNT/B in Mucus Secreting Cells

The LS180 colon carcinoma cell line is recognised as a model of mucin secreting cells (McCool, D. J., Forstner, J. F. and Forstner, G. G. 1994 Biochem. J. 302, 111-118). These cells have been shown to adopt goblet cell morphology and release high molecular weight mucin when stimulated with muscarinic agonists (eg carbachol), phorbol esters (PMA) and Ca$^{2+}$ ionophores (eg A23187). (McCool, D. J., Forstner, J. F. and Forstner, G. G. 1995 Biochem. J. 312, 125-133). These cells thus represent a non-neuronal cell type derived from the colon which can undergo regulated mucin secretion. FIG. 8 illustrates the inhibition of the ionomycin stimulated release of high molecular weight, [U-14C]-glucosamine labelled material from LS180 cells by pretreatment with BoNT/B in low pH medium. Ionomycin is a Ca$^{2+}$ ionophore and treatment of cells with low pH medium has been previously shown to facilitate toxin entry into cells.
This result clearly shows the ability of botulinum neurotoxins to inhibit secretory activity of non-neuronal cells able to release mucin when stimulated with a secretagogue (see FIG. 8).

Methods

Mucin synthesising colon carcinoma LS180 cells were grown on Matrigel coated 24 well plates in minimum essential medium supplemented with 10% foetal calf serum, 2 mM L-glutamine and 1% non-essential amino acids (Sigma). Cells were treated with pH 7.4 medium, pH 4.7 medium and pH 4.7 medium containing 500 nM botulinum neurotoxin type B (BoNT/B) for four hours then labelled with $[^3]H$]-glucosamine (1 μCi/ml, 0.5 ml/well) for 18 hours in L15 glucose free medium. Cells were then washed twice with a balanced salt solution (BSS) pH 7.4 and then 0.5 ml of BSS was applied for 30 minutes. This material was removed and 0.5 ml of BSS containing 10 μM iomycin applied to stimulate mucin release. The stimulating solution was removed and all superflus centrifuged to remove any detached cells. Supernatants were then centrifuged at 100,000g for 1 hour. Supernatants were applied to Centricron centrifugal concentrators with a molecular weight cut-off of 100 kDa and centrifuged (2,500g) until all liquid had passed through the membrane. Membranes were washed with BSS by centrifugation three times and then the membrane scintillation counted for retained, $[^3]H$]-glucosamine labelled high molecular weight material.

Example 8

Activity of BoNT/B in Inflammatory Cells

The promyelocytic cell line HL.60 can be differentiated into neutrophil like cells by the addition of dibutyryl cyclic AMP to the culture medium. Upon differentiation these cells increase their expression of characteristic enzymes such as beta-glucuronidase. In this condition these cells therefore represent a model of a phagocytic cell type which contributes to the inflammatory response of certain disease states (eg rheumatoid arthritis). FIG. 9 illustrates the significant (p<0.05) inhibition of stimulated release of beta-glucuronidase from dbcAMP differentiated HL.60 cells by pre-treatment with BoNT/B in low pH medium.

This result clearly shows the ability of botulinum neurotoxins to inhibit the secretory activity of a non-neuronal cell type which is a model of the neutrophil a cell which participates in inflammation.

Methods

HL.60 cells were cultured in RPMI 1640 medium containing 10% foetal calf serum and 2 mM glutamine. Cells were exposed to low pH and toxin for 2.5 hours then washed 3 times and differentiated by the addition of dibutyryl cyclic AMP (dbcAMP) to a final concentration of 300 μM. Cells were differentiated for 40 hours and then stimulated release of beta-glucuronidase activity was determined. Cells were treated with cytochalasin B (5 μM) 5 minutes before stimulation. Cells were stimulated with 1 μM N-formyl-Met-Leu-Phe with 100 μM ATP for 10 minutes then centrifuged and the supernatant taken for assay of beta-glucuronidase activity. Activity was measured in cell lysates and the amount released expressed as a percentage of the total cellular content of enzyme.

β-glucuronidase activity was determined according to the method of Absolom D. R. 1986, (Methods in Enzymology, 132, 160) using p-Nitrophenyl-β-D-glucuronide as the substrate.

Example 9

Preparation of a LHN/B Backbone Construct

The following procedure creates a clone for use as an expression backbone for multidomain fusion expression. This example is based on preparation of a serotype B clone (SEQ ID NO:1).

Preparation of Cloning and Expression Vectors

pCR 4 (Invitrogen) is the chosen standard cloning vector chosen due to the lack of restriction sequences within the vector and adjacent sequencing primer sites for easy construct confirmation. The expression vector is based on the pMAM (NEB) expression vector which has the desired restriction sequences within the multiple cloning site in the correct orientation for construct insertion (BamHI/Sall/PstI-HindIII). A fragment of the expression vector has been removed to create a non-mobilisable plasmid and a variety of different fusion tags have been inserted to increase purification options.

Preparation of LC/B

The LC/B is created by one of two ways:

The DNA sequence is designed by back translation of the LC/B amino acid sequence (obtained from freely available database sources such as GenBank (accession number P10644) or Swissprot (accession locus BXB_CLOBO) using one of a variety of reverse translation software tools (for example EditSeq best E. coli reverse translation (DNASTAR Inc.), or Backtranslation tool v2.0 (Entelechon)). BamHI/Sall recognition sequences are incorporated at the 5' and 3' ends respectively of the sequence maintaining the correct reading frame. The DNA sequence is screened (using software such as MapDraw, DNASTAR Inc.) for restriction enzyme cleavage sequences incorporated during the back translation. Any cleavage sequences that are found to be common to those required by the cloning system are removed manually from the proposed coding sequence ensuring common E. coli codon usage is maintained. E. coli codon usage is assessed by reference to software programs such as Graphical Codon Usage Analyser (Geneart), and the % GC content and codon usage ratio assessed by reference to published codon usage tables (for example GenBank Release 143, Sep. 13, 2004). This optimised DNA sequence containing the LC/B open reading frame (ORF) is then commercially synthesised (for example by Entelechon, Geneart or Sigma-Genosys) and is provided in the pCR 4 vector.

The alternative method is to use PCR amplification from an existing DNA sequence with BamHI and Sall restriction enzyme sequences incorporated into the 5' and 3' PCR primers respectively. Complementary oligonucleotide primers are chemically synthesised by a Supplier (for example MWG or Sigma-Genosys) so that each pair has the ability to hybridize to the opposite strands (3' ends pointing “towards” each other) flanking the stretch of Choriridium target DNA, one oligonucleotide for each of the two DNA strands. To generate a PCR product the pair of short oligo-
nucleotide primers specific for the Clostridium DNA sequence are mixed with the Clostridium DNA template and other reaction components and placed in a machine (the "PCR machine") that can change the incubation temperature of the reaction tube automatically, cycling between approximately 94°C (for denaturation), 55°C (for oligonucleotide annealing), and 72°C (for synthesis). Other reagents required for amplification of a PCR product include a DNA polymerase (such as Taq or Plu polymerase), each of the four nucleotide dNTP building blocks of DNA in equimolar amounts (50-200 μM) and a buffer appropriate for the enzyme optimised for Mg2+ concentration (0.5-5 mM).

[0243] The amplification product is cloned into pCR 4 using either, TOPO TA cloning for Taq PCR products or Zero Blunt TOPO cloning for Plu PCR products (both kits commercially available from Invitrogen). The resultant clone is checked by sequencing. Any additional restriction sequences which are not compatible with the cloning system are then removed using site directed mutagenesis (for example using Quickchange (Stratagene Inc.)).

Preparation of HN/B Insert

The HN is created by one of two ways:

[0244] The DNA sequence is designed by back translation of the HN/B amino acid sequence (obtained from freely available database sources such as GenBank (accession number P10844) or Swissprot (accession locus BXBGLOBO) using one of a variety of reverse translation software tools (for example EditSeq best E. coli reverse translation (DNASTAR Inc.), or Back translation tool v2.0 (Entelechon)). A PstI restriction sequence added to the N-terminus and XbaI-stop codon-HindIII to the C-terminus ensuring the correct reading frame in maintained. The DNA sequence is screened (using software such as MapDraw, DNASTAR Inc.) for restriction enzyme cleavage sequences incorporated during the back translation. Any sequences that are found to be common to those required by the cloning system are removed manually from the proposed coding sequence ensuring common E. coli codon usage is maintained. E. coli codon usage is assessed by reference to software programs such as Graphical Codon Usage Analyser (Geneart), and the % GC content and codon usage ratio assessed by reference to published codon usage tables (for example GenBank Release 143, Sep. 13, 2004). This optimised DNA sequence is then commercially synthesised (for example by Entelechon, Geneart or Sigma-Genosys) and is provided in the pCR 4 vector.

[0245] The alternative method is to use PCR amplification from an existing DNA sequence with PstI and XbaI-stop codon-HindIII restriction enzyme sequences incorporated into the 5’ and 3’ PCR primers respectively. The PCR amplification is performed as described above. The PCR product is inserted into pCR 4 vector and checked by sequencing. Any additional restriction sequences which are not compatible with the cloning system are then removed using site directed mutagenesis (for example using Quickchange (Stratagene Inc.)).

Preparation of the Spacer (LC-HN Linker)

[0246] The LC-HN linker can be designed from first principle, using the existing sequence information for the linker as the template. For example, the serotype B linker (in this case defined as the inter-domain polypeptide region that exists between the cysteines of the disulphide bridge between LC and HN) has the sequence KSVKAPG (SEQ ID NO:30). This sequence information is freely available from available database sources such as GenBank (accession number P10844) or Swissprot (accession locus BXBGLOBO). For generation of a specific protease cleavage site, the recognition sequence for enterokinase is inserted into the activation loop to generate the sequence VDEEKLYDDDKRWRGSSVLQ (SEQ ID NO:31). Using one of a variety of reverse translation software tools (for example EditSeq best E. coli reverse translation (DNASTAR Inc.), or Backtranslation tool v2.0 (Entelechon)), the DNA sequence encoding the linker region is determined. BamHI/Sall and PstI/XbaI-stop codon/HindIII restriction enzyme sequences are incorporated at either end, in the correct reading frames. The DNA sequence is screened (using software such as MapDraw, DNASTAR Inc.) for restriction enzyme cleavage sequences incorporated during the back translation. Any sequences that are found to be common to those required by the cloning system are removed manually from the proposed coding sequence ensuring common E. coli codon usage is maintained. E. coli codon usage is assessed by reference to software programs such as Graphical Codon Usage Analyser (Geneart), and the % GC content and codon usage ratio assessed by reference to published codon usage tables (for example GenBank Release 143, Sep. 13, 2004). This optimised DNA sequence is then commercially synthesised (for example by Entelechon, Geneart or Sigma-Genosys) and is provided in the pCR 4 vector.

[0247] As an alternative to independent gene synthesis of the linker, the linker-encoding DNA can be included during the synthesis or PCR amplification of either the LC or HN.

Assembly and Confirmation of the Backbone Clone

[0248] The LC or the LC-linker is cut out from the pCR 4 cloning vector using BamHI/Sall or BamHI/PstI restriction enzymes digests. The pMAL expression vector is digested with the same enzymes but is also treated with calf intestinal protease (CIP) as an extra precaution to prevent re-circularisation. Both the LC or LC-linker region and the pMAL vector backbone are gel purified. The purified insert and vector backbone are ligated together using T4 DNA ligase and the product is transformed with TOP10 cells which are then screened for LC insertion using BamHI/Sall or BamHI/PstI restriction digestion. The process is then repeated for the HN or linker-HN insertion into the PstI/HindIII or Sall/HindIII sequences of the pMAL-LC construct.

[0249] Screening with restriction enzymes is sufficient to ensure the final backbone is correct as all components are already sequenced confirmed, either during synthesis or following PCR amplification. However, during the subcloning of some components into the backbone, where similar size fragments are being removed and inserted, sequencing of a small region to confirm correct insertion is required.
Example 10
Preparation of a LHN/C Backbone Construct

[0250] The following procedure creates a clone for use as an expression backbone for multidomain fusion expression. This example is based on preparation of a serotype C based clone (SEQ ID NO:2).

Preparation of Cloning and Expression Vectors

[0251] pCR 4 (Invitrogen) is the chosen standard cloning vector chosen due to the lack of restriction sequences within the vector and adjacent sequencing primer sites for easy construct confirmation. The expression vector is based on the pMAL (NEB) expression vector which has the desired restriction sequences within the multiple cloning site in the correct orientation for construct insertion (BamHI-Sall-PstI-HindIII). A fragment of the expression vector has been removed to create a non-mobilisable plasmid and a variety of different fusion tags have been inserted to increase purification options.

Preparation of L/C/C

The L/C/C is created by one of two ways:

[0252] The DNA sequence is designed by back translation of the L/C/C amino acid sequence (obtained from freely available database sources such as GenBank (accession number P18640) or Swissprot (accession locus BXC1 CLOBO) using one of a variety of reverse translation software tools (for example EditSeq best E. coli reverse translation (DNASTAR Inc.), or Backtranslation tool v2.0 (Entelechon)). BamHI/Sall recognition sequences are incorporated at the 5' and 3' ends respectively of the sequence maintaining the correct reading frame. The DNA sequence is screened (using software such as MapDraw, DNASTAR Inc.) for restriction enzyme cleavage sequences incorporated during the back translation. Any cleavage sequences that are found to be common to those required by the cloning system are removed manually from the proposed coding sequence ensuring common E. coli codon usage is maintained. E. coli codon usage is assessed by reference to software programs such as Graphical Codon Usage Analyser (Geneart), and the % GC content and codon usage ratio assessed by reference to published codon usage tables (for example Genbank Release 143, Sep. 13, 2004). This optimised DNA sequence containing the L/C/C open reading frame (ORF) is then commercially synthesised (for example by Entelechon, Geneart or Sigma-Genosys) and is provided in the pCR 4 vector.

[0253] The alternative method is to use PCR amplification from an existing DNA sequence with BamHI and Sall restriction enzyme sequences incorporated into the 5' and 3' PCR primers respectively. Complementary oligonucleotide primers are chemically synthesised by a supplier (for example MWG or Sigma-Genosys) so that each pair has the ability to hybridize to the opposite strands (3' ends pointing “towards” each other) flanking the stretch of Clostridium target DNA, one oligonucleotide for each of the two DNA strands. To generate a PCR product the pair of short oligonucleotide primers specific for the Clostridium DNA sequence are mixed with the Clostridium DNA template and other reaction components and placed in a machine (the “PCR machine”) that can change the incubation temperature of the reaction tube automatically, cycling between approximately 94°C (for denaturation), 55°C (for oligonucleotide annealing), and 72°C (for synthesis). Other reagents required for amplification of a PCR product include a DNA polymerase (such as Taq or Pfu polymerase), each of the four nucleotide dNTP building blocks of DNA in equimolar amounts (50-200 μM) and a buffer appropriate for the enzyme optimised for Mg2+ concentration (0.5-5 mM).

[0254] The amplification product is cloned into pCR 4 using either, TOPO TA cloning for Taq PCR products or Zero Blunt TOPO cloning for Pfu PCR products (both kits commercially available from Invitrogen). The resultant clone is checked by sequencing. Any additional restriction sequences which are not compatible with the cloning system are then removed using site directed mutagenesis (for example using Quickchange (Stratagene Inc.)).

Preparation of HN/C Insert

The HN is created by one of two ways:

[0255] The DNA sequence is designed by back translation of the HN/C amino acid sequence (obtained from freely available database sources such as GenBank (accession number P18640) or Swissprot (accession locus BXC1 CLOBO)) using one of a variety of reverse translation software tools (for example EditSeq best E. coli reverse translation (DNASTAR Inc.), or Backtranslation tool v2.0 (Entelechon)). A PstI restriction sequence added to the N-terminus and XbaI-stop codon-HindIII to the C-terminus ensuring the correct reading frame in maintained. The DNA sequence is screened (using software such as MapDraw, DNASTAR Inc.) for restriction enzyme cleavage sequences incorporated during the back translation. Any sequences that are found to be common to those required by the cloning system are removed manually from the proposed coding sequence ensuring common E. coli codon usage is maintained. E. coli codon usage is assessed by reference to software programs such as Graphical Codon Usage Analyser (Geneart), and the % GC content and codon usage ratio assessed by reference to published codon usage tables (for example Genbank Release 143, Sep. 13, 2004). This optimised DNA sequence is then commercially synthesized (for example by Entelechon, Geneart or Sigma-Genosys) and is provided in the pCR 4 vector.

[0256] The alternative method is to use PCR amplification from an existing DNA sequence with PstI and XbaI-stop codon-HindIII restriction enzyme sequences incorporated into the 5' and 3' PCR primers respectively. The PCR amplification is performed as described above. The PCR product is inserted into pCR 4 vector and checked by sequencing. Any additional restriction sequences which are not compatible with the cloning system are then removed using site directed mutagenesis (for example using Quickchange (Stratagene Inc.)).

Preparation of the Spacer (L-C-HN Linker)

[0257] The LC-HN linker can be designed from first principle, using the existing sequence information for the linker as the template. For example, the serotype C linker (in this case defined as the inter-domain polypeptide region that exists between the cysteines of the disulphide bridge between LC and HN) has the sequence HKAI DGRSLYNK- TLD (SEQ ID NO:32). This sequence information is freely available from available database sources such as GenBank (accession number P18640) or Swissprot (accession focus...
BXCl_CLOBO). For generation of a specific protease cleavage site, the recognition sequence for enterokinase is inserted into the activation loop to generate the sequence VDGHTSKTKSDDDDKKNKLNLQ (SEQ ID NO:33). Using one of a variety of reverse translation software tools (for example Editseq best E. coli reverse translation (DNASTAR Inc.), or Backtranslation tool v2.0 (Entelechon)), the DNA sequence encoding the linker region is determined. BamHI/Sall and PstI/XbaI/stop codon/HindIII restriction enzyme sequences are incorporated at either end, in the correct reading frames. The DNA sequence is screened (using software such as MapDraw, DNASTAR Inc.) for restriction enzyme cleavage sequences incorporated during the back translation. Any sequences that are found to be common to those required by the cloning system are removed manually from the proposed coding sequence ensuring common E. coli codon usage is maintained. E. coli codon usage is assessed by reference to software programs such as Graphical Codon Usage Analyser (Geneart), and the % GC content and codon usage ratio assessed by reference to published codon usage tables (for example GenBank Release 143, Sep. 13, 2004). This optimised DNA sequence is then commercially synthesized (for example by Entelechon, Geneart or Sigma-Genosys) and is provided in the pCR 4 vector. If it is desired to clone the linker out of the pCR 4 vector, the vector (encoding the linker) is cleaved with either BamHI/Sall or PstI/XbaI combination restriction enzymes. This cleaved vector then serves as the recipient vector for insertion and ligation of either the LC DNA (cleaved with BamHI/Sall) or HN DNA (cleaved with PstI/XbaI). Once the LC or the HN encoding DNA is inserted upstream or downstream of the linker DNA, the entire LC-linker or linker-HN DNA fragment can be isolated and transferred to the backbone clone.

[0258] As an alternative to independent gene synthesis of the linker, the linker-encoding DNA can be included during the synthesis or PCR amplification of either the LC or HN.

Assembly and Confirmation of the Backbone Clone

[0259] The LC or the LC-linker is cut out from the pCR 4 cloning vector using BamHI/Sall or BamHI/PstI restriction enzymes digests. The pMAL expression vector is digested with the same enzymes but is also treated with calf intestinal protease (CIP) as an extra precaution to prevent re-circularisation. Both the LC or LC-linker region and the pMAL vector backbone are gel purified. The purified insert and vector backbone are ligated together using T4 DNA ligase and the product is transformed with TOP10 cells which are then screened for LC insertion using BamHI/Sall or BamHI/PstI restriction digestion. The process is then repeated for the HN or linker-HN insertion into the PstI/HindIII or Sall/HindIII sequences of the pMAL-LC construct.

[0260] Screening with restriction enzymes is sufficient to ensure the final backbone is correct as all components are already sequenced confirmed, either during synthesis or following PCR amplification. However, during the subcloning of some components into the backbone, where similar size fragments are being removed and inserted, sequencing of a small region to confirm correct insertion is required.

Example 11

Construction, Expression, and Purification of a LHNC-EGF Fusion Protein

Preparation of Spacer-EGF Insert

[0261] For presentation of an EGF sequence at the C-terminus of the HN domain, a DNA sequence is designed to flank the spacer and targeting moiety (TM) regions allowing incorporation into the backbone clone (SEQ ID NO:2). The DNA sequence can be arranged as BamHI-Sall-PstI-XbaI-spacer-EGF-stop codon-HindIII (SEQ ID NO:3). The DNA sequence can be designed using one of a variety of reverse translation software tools (for example Editseq best E. coli reverse translation (DNASTAR Inc.), or Backtranslation tool v2.0 (Entelechon)). Once the TM DNA is designed, the additional DNA required to encode the preferred spacer is created in silico. It is important to ensure the correct reading frame is maintained for the spacer, EGF and restriction sequences and that the XbaI sequence is not preceded by the bases, TC which would result in DAM methylation. The DNA sequence is screened for restriction sequence incorporated and any additional sequences are removed manually from the remaining sequence ensuring common E. coli codon usage is maintained. E. coli codon usage is assessed by reference to software programs such as Graphical Codon Usage Analyser (Geneart), and the % GC content and codon usage ratio assessed by reference to published codon usage tables (for example GenBank Release 143, Sep. 13, 2004). This optimised DNA sequence is then commercially synthesized (for example by Entelechon, Geneart or Sigma-Genosys) and is provided in the pCR 4 vector.

Insertion of Spacer-EGF into Backbone

[0262] In order to create a LC-linker-HN spacer-EGF construct (SEQ ID NO:4) using the backbone construct (SEQ ID NO:2) and the newly synthesised pCR 4-spacer-TM vector encoding the EGF TM (SEQ ID NO:3), the following two-step method is employed. Firstly, the HN domain is excised from the backbone clone using restriction enzymes PstI and XbaI and ligated into similarly digested pCR 4-spacer-EGF vector. This creates an HN-spacer-EGF ORF in pCR 4 that can be excised from the vector using restriction enzymes PstI and HindIII for subsequent ligation into similarly cleaved backbone or expression construct. The final construct contains the LC-linker-HN spacer-EGF ORF (SEQ ID NO:4) for transfer into expression vectors for expression to result in a fusion protein of the sequence illustrated in SEQ ID NO:5.

[0263] Screening with restriction enzymes is sufficient to ensure the final backbone is correct as all components are already sequenced confirmed, either during synthesis or following PCR amplification. However, during the subcloning of some components into the backbone, where similar size fragments are being removed and inserted, sequencing of a small region to confirm correct insertion is required.

Alternative Construction Approach

[0264] As an alternative to the methodologies described above for the construction of LHNC-EGF, complete gene synthesis has been used to create a single DNA insert that encodes the LC, the HN, linkers, spacers and a protease activation site. The synthetic DNA is designed to have a
Expression of LHN/C-EGF Fusion Protein

[0265] Expression of the LHN/C-EGF fusion protein is achieved using the following protocol. Inoculate 100 ml of modified TB containing 0.2% glucose and 100 mg/ml ampicillin in a 250 ml flask with a single colony from the LHN/C-EGF expression strain. Grow the culture at 37°C, 225 rpm for 16 hours. Inoculate 1 L of modified TB containing 0.2% glucose and 100 μg/ml ampicillin in a 2 L flask with 10 ml of overnight culture. Grow cultures at 37°C until an approximate OD600 nm of 0.5 is reached at which point reduce the temperature to 16°C. After 1 hour induce the cultures with 1 mM IPTG and grow at 16°C for a further 16 hours.

Purification of LHN/C-EGF Fusion Protein

[0266] Defrost falkon tube containing 25 ml 50 mM HEPES pH 7.2 200 mM NaCl and approximately 10 g of E. coli BL21 cell paste. Sonicate the cell paste on ice 30 seconds on, 30 seconds off for 10 cycles at a power of 22 microns ensuring the sample remains cool. Spin the lysed cells at 18,000 rpm, 4°C for 30 minutes. Load the supernatant onto a 0.1 M NiSO4 charged Chelating column (20-30 ml column is sufficient) equilibrated with 50 mM HEPES pH 7.2 200 mM NaCl. Using a step gradient of 10 and 40 mM imidazole, wash away the non-specific bound protein and elute the fusion protein with 100 mM imidazole. Dialyse the eluted fusion protein against 5 L of 50 mM HEPES pH 7.2 200 mM NaCl at 4°C overnight and measure the OD of the dialysed fusion protein. Add 1 μl of factor Xa per 100 μg fusion protein and incubate at 25°C, static overnight. Load onto a 0.1 M NiSO4 charged Chelating column (20-30 ml column is sufficient) equilibrated with 50 mM HEPES pH 7.2 200 mM NaCl. Wash column to baseline with 50 mM HEPES pH 7.2 200 mM NaCl. Using a step gradient of 10 and 40 mM imidazole, wash away the non-specific bound protein and elute the fusion protein with 100 mM imidazole. Dialyse the eluted fusion protein against 5 L of 50 mM HEPES pH 7.2 200 mM NaCl at 4°C overnight and concentrate the fusion to about 2 mg/ml, aliquot sample and freeze at −20°C. Test purified protein using OD, BCA and purity analysis. FIG. 8 demonstrates the purified protein as analysed by SDS-PAGE.

Example 12

Construction, Expression and Purification of a LHN/B-EGF Fusion Protein

[0267] The LC-HN linker is designed using the methods described in example 11 using the B serotype linker arranged as BamHI-Sall-PstI-Xbal-spacer-EGF-stop codon-HindIII (SEQ ID NO:3). The LHN/B-EGF fusion is then assembled using the LHN/B backbone clone (SEQ ID NO:1) made using the methods described in example 9 and constructed using methods described in example 11. The final construct contains the LC-linker-HN-spacer-EGF ORF (SEQ ID NO:6) for transfer into expression vectors for expression to result in a fusion protein of the sequence illustrated in SEQ ID NO:7. The resultant expression plasmid, pMAL LHN/B-EGF is transformed into E. coli BL21 for recombinant protein expression. Expression and purification of the fusion protein was carried out as described in example 6 except that enterokinase replaced factor Xa in the activation of the fusion protein. FIG. 9 demonstrates the purified protein as analysed by SDS-PAGE.

Example 13

Preparation and Purification of a LHN/C-RGD Fusion Protein

Preparation of Spacer-RGD Insert

[0268] For presentation of an RGD sequence at the C-terminus of the HN domain, a DNA sequence is designed to flank the spacer and TM regions allowing incorporation into the backbone clone (SEQ ID NO:2). The DNA sequence can be arranged as BamHI-Sall-PstI-Xbal-spacer-Spel-RGD-stop codon-HindIII (SEQ ID NO:8). The DNA sequence can be designed using one of a variety of reverse translation software tools (for example EditSeq best E. coli reverse translation (DNAstar Inc.), or Backtranslation tool v2.0 (Entelechon)). Once the TM DNA is designed, the additional DNA required to encode the preferred spacer is created in silico. It is important to ensure the correct reading frame is maintained for the spacer, RGD and restriction sequences and that the Xbal sequence is not preceded by the bases, TC which would result in DAM methylation. The DNA sequence is screened for restriction sequence Incorporated and any additional sequences are removed manually from the remaining sequence ensuring common E. coli codon usage is maintained. E. coli codon usage is assessed by reference to software programs such as Graphical Codon Usage Analyzer (Geneart), and the % GC content and codon usage ratio assessed by reference to published codon usage tables (for example GenBank Release 143, Sep. 13, 2004). This optimised DNA sequence is then commercially synthesised (for example by Entelechon, Geneart or Sigma-Genosys) and is provided in the pCR 4 vector.

Insertion of Spacer-RGD into Backbone

[0269] In order to create a LC-linker-HN-spacer-RGD construct (SEQ ID NO:9) using the backbone construct (SEQ ID NO:2) and the newly synthesised pCR 4-spacer-TM vector encoding the RGD TM (SEQ ID NO:8), the following two-step method is employed. Firstly, the HN domain is excised from the backbone clone using restriction enzymes PstI and Xbal and ligated into similarly digested pCR 4-spacer-RGD vector. This creates an HN-spacer-RGD ORF in pCR 4 that can be excised from the vector using restriction enzymes PstI and HindIII for subsequent ligation into similarly cleaved backbone or expression construct. The final construct contains the LC-linker-HN-spacer-RGD ORF (SEQ ID NO:9) for transfer into expression vectors for expression to result in a fusion protein of the sequence illustrated in SEQ ID NO:10.

[0270] Screening with restriction enzymes is sufficient to ensure the final backbone is correct as all components are already sequenced confirmed, either during synthesis or following PCR amplification. However, during the subcloning of some components into the backbone, where
similar size fragments are being removed and inserted, sequencing of a small region to confirm correct insertion is required.

[0271] Expression and puriﬁcation of the fusion protein was carried out as described in example 11. FIG. 10 demonstrates the puriﬁed protein as analysed by SDS-PAGE.

Example 14
Preparation and Puriﬁcation of a LHN/C-cyclic RGD Fusion Protein

[0272] The LC-HN linker can be designed using the methods described in example 13 using the C serotype linker arranged as BamHI-SalI-PstI-XbaI-spacer-Spal-cyclic RGD-stop codon-HindIII (SEQ ID NO:11). The LHN/C-cyclic RGD fusion is then assembled using the LHN/C backbone clone (SEQ ID NO:2) made using the methods described in example 10 and constructed using methods described in example 13. The ﬁnal construct contains the LC-linker-HN-spacer-cyclic RGD ORF (SEQ ID NO:11) for transfer into expression vectors for expression to result in a fusion protein of the sequence illustrated in SEQ ID NO:13. The resulting expression plasmid, pMAL-LHN/C-cyclic RGD was transformed into E. coli BL21 for recombinant protein expression. Expression and puriﬁcation of the fusion protein was carried out as described in example 11. FIG. 11 demonstrates the puriﬁed protein as analysed by SDS-PAGE.

Example 15
Preparation and Puriﬁcation of a LC/C-RGD-HN/C Fusion Protein

[0273] In order to create the LC-linker-RGD-spacer-HN construct (SEQ ID NO:15), the PCR 4 vector encoding the linker (SEQ ID NO:14) is cleaved with BamHI-SalI restriction enzymes. This cleaved vector then serves as the recipient vector for insertion and ligation of the LC/C DNA (SEQ ID NO:2) cleaved with BamHI-SalI. The resulting plasmid DNA is then cleaved with PstI-XbaI restriction enzymes and serves as the recipient vector for the insertion and ligation of the HN/C DNA (SEQ ID NO:2) cleaved with PstI-XbaI. The ﬁnal construct contains the LC-linker-RGD-spacer-HN ORF (SEQ ID NO:15) for transfer into expression vectors for expression to result in a fusion protein of the sequence illustrated in SEQ ID NO:16. The resultant expression plasmid, pMAL LC/C-RGD-HN/C was transformed into E. coli BL21 for recombinant protein expression. Expression and puriﬁcation of the fusion protein was carried out as described in example 11. FIG. 12 demonstrates the puriﬁed protein as analysed by SDS-PAGE.

Alternative Construction Approach

[0274] As an alternative to the methodologies described above for the construction of LC-linker-RGD-spacer-HN, complete gene synthesis has been used to create a single DNA insert that encodes the L, C, HN, linkers, spacers and a protease activation site. The synthetic DNA is designed to have a NdeI restriction site at the 5' end and a HindIII restriction site at the 3' end to facilitate direct cloning into expression vectors. The sequence of the engineered coding region is subject to the same codon utilisation analysis as described above. The sequence of the synthetic DNA is illustrated in SEQ ID NO:17, and the protein that it encodes is illustrated in SEQ ID NO:18.

Example 16
VAMP Cleavage Activity Assay

[0275] A range of concentrations of LHN/B-EGF in cleavage buffer (50 mM HEPES pH 7.4, 10 mM DTT, 20 mM ZnCl2, 1% FBS) are incubated with biotinylated VAMP substrate (1 mg/ml) for two hours at 37°C in a shaking incubator. The cleavage reaction is transferred to a washed 96-well streptavidin coated plate and incubated at 37°C in a shaking incubator for 5 minutes. The plate is washed three times with PBS-0.1% tween-20 (PBS-T). The wells are blocked with blocking buffer (5% FCS in PBS-T) for 1 hour at 37°C. The primary antibody (anti-FESS) is added at a dilution of 1 in 500 in blocking buffer and the plate is incubated at 37°C for 1 hour. The plate is washed three times with PBS-T and the secondary antibody (anti guinea pig HRP conjugate) diluted 1 in 1000 in blocking buffer is applied. Following 1 hour incubation at 37°C, the plate is developed with biotin-FX TMB substrate. Colour development is allowed to proceed for 1-5 minutes and then stopped with stop solution. The absorbance is measured at 450 nm. FIG. 13 shows the VAMP cleavage activity of LHN/B-EGF fusion protein.

Example 17
Activity of EGF-LHN/C and EGF-LHN/B in THP-1 Immune Cells

[0276] The THP-1 cell line is a human-derived suspension (non-adherent) culture that is used frequently to provide a model system for primary monocytes. It is a well characterized model and over 2000 reviewed publications have utilized the THP-1 line to investigate molecular and cellular processes. Recent studies have demonstrated the utility of the THP-1 cell line as a model to assess the secretion of anti- and pro-inﬂammatory cytokines (Qu et al. 2007 J. Lipid Res. 48(2) 385-394, Prunet et al. 2006 Cytometry A. 69, 359-373 and Segura et al 2002 Clin. Exp. Immunol. 127(2) 243-254).

[0277] FIG. 14 illustrates the signiﬁcant inhibition of LPS-stimulated release of IL-8 from THP-1 cells in culture by pretreatment with either EGF-LHN/C (SXX 100501) or with EGF-LHN/B (SXX 100328).

[0278] This result shows clearly the ability of fusion proteins to inhibit the pro-inﬂammatory cytokine secretory activity of a non-neuronal immune cell type that is a model for the monocyte cell which participates in inﬂammation.

Methods

[0279] THP-1, cells were pre-incubated with 10 nM compound or vehicle control for 48 hours at 37°C/5% CO2. After the pre-incubation, LPS was added at a final concentration of 1 mg/ml and the cells incubated for a further 16 hours (overnight). For inhibitory controls; cells were treated with Staurosporine (1 µM) or Dexamethasone (1 µM) for 30 minutes prior to adding the LPS, and then incubated for 16 hours (overnight). Culture supernatant from each well was harvested and analyzed for cytokine by Luminex-based technology (BioSource). All estimations were performed in triplicate.
Example 18

Activity of EGF-LHN/C and EGF-LHN/B in RPMI Immune Cells

[0280] The RPMI-8226 cell line is a human-derived culture that is used frequently to provide a model system for primary B-lymphocytes. It is a well characterized model and over 250 reviewed publications have utilized the RPMI-8226 line to investigate molecular and cellular processes. Recent studies have demonstrated the utility of the RPMI-8226 cell line as a model to assess the secretion of cytokines (Xu et al. J. Leukoc. Biol. 2002, 72(2) 410-416 and Gupta et al. 2001, 15(12) 1950-1961).

[0281] FIG. 15 illustrates the significant inhibition of LPS-stimulated release of IL-10 from RPMI-8226 cells in culture by pretreatment with either EGF-LHN/C (SXXN 100501) or with EGF-LHN/B (SXXN 100328).

[0282] This result shows clearly the ability of fusion proteins to inhibit the cytokine secretory activity of a non-neuronal immune cell type that is a model for the B-lymphocyte cell which participates in immune responses.

[0283] Methods

[0284] RPMI-8226 cells were pre-incubated with 10 nM compound or vehicle control for 48 hours at 37°C/5% CO2. After the pre-incubation, LPS was added at a final concentration of 1 mg/ml and the cells incubated for a further 16 hours (overnight). For inhibitory controls; cells were treated with Staurospherine (1 µM) or Dexamethasone (1 µM) for 30 minutes prior to adding the LPS, and then incubated for 16 hours (overnight). Culture supernatant from each well was harvested and analyzed for cytokine by Luminescent-based technology (BioSource). All estimations were performed in triplicate.

Example 19

Activity of EGF-LHN/C, CP-RGD-LHN/C and EGF-LHN/B in Human PBMC Immune Cells

[0285] PBMC are peripheral blood mononuclear cells providing a primary culture that is highly diverse in constituent cell phenotype. It is a well characterized model and over 3000 reviewed publications have utilized human peripheral blood mononuclear cells to investigate molecular and cellular processes. Recent studies have demonstrated the utility of human PBMC as a model to assess the secretion of cytokines (Bachmann et al. Cell Microbiol. 2006, 8(2) 289-300, Siejka et al. Endocr. Regul. 2005, 39(1) 7-11, Reddy et al. 2004, 293(1-2) 127-142).

[0286] FIG. 16 illustrates the significant inhibition of LPS-stimulated release of IL-8 from human PBMC cells in culture by pretreatment with CP-RGD-LHN/C (SXXN 100221), EGF-LHN/C (SXXN 100501) or with EGF-LHN/B (SXXN 100328).

[0287] This result shows clearly the ability of fusion proteins to inhibit the cytokine secretory activity of non-neuronal human immune cells which participates in immune responses.

Methods

[0288] PBMC cells were pre-incubated with 10 nM compound or vehicle control for 24 hours at 37°C/5% CO2. After the pre-incubation, LPS was added at a final concentration of 1 mg/ml and the cells incubated for a further 16 hours (overnight). For inhibitory controls; cells were treated with Staurospherine (1 µM) or Dexamethasone (1 µM) for 30 minutes prior to adding the LPS, and then incubated for 16 hours (overnight). Culture supernatant from each well was harvested and analyzed for cytokine by Luminescent-based technology (BioSource). All estimations were performed in triplicate.

Example 20

Activity of EGF-LHN/C, CP-RGD-LHN/C and EGF-LHN/B in Human PBMC Immune Cells

[0289] PBMC are peripheral blood mononuclear cells providing a primary culture that is highly diverse in constituent cell phenotype. It is a well characterized model and over 3000 reviewed publications have utilized human peripheral blood mononuclear cells to investigate molecular and cellular processes. Recent studies have demonstrated the utility of human PBMC as a model to assess the secretion of cytokines (Bachmann et al. Cell Microbiol. 2006, 8(2) 289-300, Siejka et al. Endocr. Regul. 2005, 39(1) 7-11, Reddy et al. 2004, 293(1-2) 127-142).

[0290] FIG. 17 illustrates the significant inhibition of PHA-stimulated release of IP-10 from human PBMC cells in culture by pretreatment with CP-RGD-LHN/C (SXXN 100221), EGF-LHN/C (SXXN 100501) or with EGF-LHN/B (SXXN 100328).

[0291] This result shows clearly the ability of fusion proteins to inhibit the cytokine secretory activity of non-neuronal human immune cells which participates in immune responses.

Methods

[0292] PBMC cells were pre-incubated with 10 nM compound or vehicle control for 24 hours at 37°C/5% CO2. After the pre-incubation, PHA was added at a final concentration of 2 mg/ml and the cells incubated for a further 16 hours (overnight). For inhibitory controls; cells were treated with Staurospherine (1 µM) or Dexamethasone (1 µM) for 30 minutes prior to adding the PHA, and then incubated for 16 hours (overnight). Culture supernatant from each well was harvested and analyzed for cytokine by Luminescent-based technology (BioSource). All estimations were performed in triplicate.

Example 21

Clinical Example

[0293] A 54 year old male suffering from asthma presents at his GP. Despite daily treatment with his preventer inhaler, the use of his reliever inhaler has increased significantly. The patient presents with difficulty in performing everyday tasks due continued shortness of breath and frequent asthma attacks. The GP prescribes a 6-month course of SXXN100501 (as prepared in previous examples) in nebuliser form, 80 µg to be taken monthly. Following discussion with the physician, the patient selects the most appropriate nebuliser for
their personal situation from a range of suitable devices. After a single dose of SXN100501 the patient experiences a reduced frequency of attacks and a general improvement in FEV1. Further treatment enhances these parameters further and improves quality of life.

Example 22

Clinical Example

[0294] A 26 year old female suffering from seasonal allergic rhinitis (hay fever) presents at her GP. Despite completion of a course of preventer treatment (consisting of daily treatment with fluticasone for a period of 3 weeks) and subsequent treatment with OTC anti-histamines, the frequency and severity of rhinitis increases. The GP prescribes a 4-month course of SXN100328 (as prepared in previous examples), 80 µg to be taken monthly in the form of a nasal spray. After a single dose of SXN100328 the patient experiences a reduced frequency of rhinitis and generally improved quality of life. Further treatments continue to decrease the severity of the rhinitis.

SEQ ID List

[0295] SEQ ID NO:1 DNA sequence of LHN/B
[0296] SEQ ID NO:2 DNA sequence of LHN/C
[0297] SEQ ID NO:3 DNA sequence of the EGF linker
[0298] SEQ ID NO:4 DNA sequence of the EGF-C fusion
[0299] SEQ ID NO:5 Protein sequence of the EGF-C fusion
[0300] SEQ ID NO:6 DNA sequence of the EGF-B fusion
[0301] SEQ ID NO:7 Protein sequence of the EGF-B fusion
[0302] SEQ ID NO:8 DNA sequence of the RGD linker
[0303] SEQ ID NO:9 DNA sequence of the RGD-C fusion
[0304] SEQ ID NO:10 Protein sequence of the RGD-C fusion
[0305] SEQ ID NO:11 DNA sequence of the cyclic RGD linker
[0306] SEQ ID NO:12 DNA sequence of the cyclic RGD-C fusion
[0307] SEQ ID NO:13 Protein sequence of the cyclic RGD-C fusion
[0308] SEQ ID NO:14 DNA sequence of the LC/C-RGDS-HN/C linker
[0309] SEQ ID NO:15 DNA sequence of the LC/C-RGDS-HN/C fusion
[0310] SEQ ID NO:16 Protein sequence of the LC/C-RGDS-HN/C fusion
[0311] SEQ ID NO:17 DNA sequence of the fully synthesised LC/C-RGDS-HN/C fusion
[0312] SEQ ID NO:18 Protein sequence of the fully synthesised LC/C-RGDS-HN/C fusion
[0313] SEQ ID NO:19 DNA sequence of the fully synthesised EGF-LHN/C fusion
[0314] SEQ ID NO:20 Protein sequence of the fully synthesised EGF-LHN/C fusion
[0315] SEQ ID NO:21 Integrin binding peptide sequence
[0316] SEQ ID NO:22 Integrin binding peptide sequence
[0317] SEQ ID NO:23 Cyclic RGD peptide
[0318] SEQ ID NO:24 Linear integrin binding sequence
[0319] SEQ ID NO:25 Cyclic integrin binding sequence

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<213> ORGANISM: Unknown
<215> FEATURE: OTHER INFORMATION DNA sequence of EGF linker
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<210> SEQ ID NO: 4
<211> LENGTH: 2838
<212> TYPE: DNA
<213> ORGANISM: Unknown
<215> FEATURE: OTHER INFORMATION DNA sequence of EGF-C fusion
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<210> SEQ ID NO 5
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<212> TYPE: PRT
<213> ORGANISM: Unknown
<220> FEATURE:
<223> OTHER INFORMATION: Protein sequence of the EGF-C fusion
<400> SEQUENCE: 5

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35  40
Val Ile Pro Asp Arg Phe Ser Arg Asn Ser Asn Pro Gen Leu Asn Lys
50  55
Pro Pro Arg Val Thr Ser Pro Ser Gly Tyr Tyr Asp Pro Asn Tyr
65  70  75
Leu Ser Thr Asp Ser Asp Lys Asp Thr Phe Leu Lys Glu Ile Ile Lys
85  90  95
Leu Phe Lys Asp Arg Ile Asn Ser Arg Glu Ile Gly Glu Leu Ile Tyr
100 105 110
Arg Leu Ser Thr Asp Arg Pro Pro Gly Asn Asn Thr Pro Ile
115 120 125
Asn Thr Phe Asp Phe Asp Val Asp Phe Asp Ser Val Asp Val Lys Thr
130 135 140
Arg Gin Gly Asn Asn Thr Val Lys Thr Gly Ser Ile Asn Pro Ser Val
145 150 155 160
Ile Ile Thr Gly Pro Arg Glu Asn Ile Ile Asp Pro Glu Thr Ser Thr
165 170 175
Phe Lys Leu Thr Ser Asn Arg Thr Asp Ala Ala Gln Glu Gly Phe Gly Ala
180 185 190
Leu Ser Ile Ile Ser Ile Ser Pro Arg Phe Met Leu Thr Tyr Ser Asn
195 200 205
Ala Thr Asn Asp Val Gly Glu Gly Arg Phe Ser Lys Ser Glu Phe Cys
210 215 220
Met Asp Pro Ile Leu Ile Leu Met His Glu Leu Asn His Ala Met His
225 230 235 240
Asn Leu Tyr Gly Ile Ala Ile Pro Asn Gin Thr Ile Ser Ser Val
245 250 255
Thr Ser Asn Ile Phe Tyr Ser Gin Tyr Asn Val Lys Leu Gly Tyr Ala
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Glu Ile Tyr Ala Phe Gly Gly Pro Thr Ile Asp Leu Ile Pro Lys Ser
275 280 285
Ala Arg Lys Tyr Phe Glu Lys Ala Leu Asp Tyr Tyr Arg Ser Ile
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Ala Lys Arg Leu Asn Ser Ile Thr Thr Ala Asn Pro Ser Ser Ser Phe
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Lys Tyr Ile Gly Tyr Lys Gly Gly Lys Leu Ile Arg Lys Tyr Arg Phe
325 330 335
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Glu Leu Tyr Asn Glu Leu Thr Gin Ile Phe Thr Glu Phe Asn Tyr Ala
355 360 365
Lys Ile Tyr Asn Val Gin Asn Arg Lys Ile Tyr Leu Ser Asn Val Tyr
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Thr Pro Val Thr Ala Asn Ile Leu Asp Asp Asn Val Tyr Asp Ile Gin
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Gln Asn Leu Ser Arg Asn Pro Ala Leu Arg Lys Val Asn Pro Glu Asn
405 410 415
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Ser Leu Tyr Asn Lyu Thr Leu Gln Arg Glu Leu Leu Val Lys Asn
435 440 445
Thr Asp Leu Pro Phe Ile Gly Asp Ile Ser Asp Val Lys Thr Asp Ile
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Phe Leu Arg Lys Asp Ile Asn Glu Thr Glu Val Ile Tyr Tyr Pro
465 470 475 480
Asp Asn Val Ser Val Asp Gln Val Ile Leu Ser Lys Asn Thr Ser Glu
485 490 495
His Gly Gin Leu Asp Leu Tyr Pro Ser Ile Asp Ser Glu Ser Glu
500 505 510
Ile Leu Pro Gly Glu Asn Gln Val Phe Tyr Asp Arg Thr Gln Asn
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530 535 540
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545 550 555 560
Asp Asn Ser Ala Lys Val Tyr Thr Tyr Phe Pro Thr Leu Ala Asn Lys
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Val Val Glu Asp Phe Thr Thr Asn Ile Leu Arg Lys Asp Thr Leu Asp
595 600 605
Lys Ile Ser Asp Val Ser Ala Ile Ile Pro Tyr Ile Gly Pro Ala Leu
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Val Thr Gly Val Thr Ile Leu Leu Glu Ala Phe Pro Glu Phe Thr Ile
645 650 655
Pro Ala Leu Gly Ala Phe Val Ile Tyr Ser Lys Val Gin Glu Arg Asn
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Trp Lys Asp Ser Tyr Glu Trp Met Met Gly Thr Trp Leu Ser Arg Ile
690 695 700
Ile Thr Gln Phe Asn Asn Ile Ser Tyr Gin Met Tyr Asp Ser Leu Asn
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Tyr Gin Ala Gly Ala Ile Lys Ala Lys Ile Asp Leu Glu Tyr Lys Tyr
725 730 735
Tyr Ser Gly Ser Asp Lys Glu Asn Ile Lys Ser Gin Val Glu Asn Leu
740 745 750
Asn Ser Leu Asp Val Lys Ile Ser Glu Ala Met Asn Arg Ile Asn
755 760 765
Lys Phe Ile Arg Glu Cys Ser Val Thr Tyr Leu Phe Lys Asn Met Leu
770 775 780
Lys Phe Ile Arg Glu Cys Ser Val Thr Tyr Leu Phe Lys Asn Met Leu
785 790 795 800
Pro Lys Val Ile Asp Glu Leu Asn Glu Phe Asp Arg Arg Thr Lys Ala
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Lys Leu Ile Asn Leu Ile Asp Ser His Arg Ile Ile Thr Leu Val Gly Gly
  020     025     030
Val Asp Lys Leu Lys Ala Glu Val Leu Asn Ser Phe Glu Asn Thr Ile
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Pro Phe Arg Ile Phe Ser Tyr Thr Asn Arg Ser Leu Lys Asp Ile
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Ile Asn Glu Tyr Phe Arg Leu Gly Gly Gly Gly Ser Gly Gly Gly
  065     070     075     080
Gly Ser Gly Gly Gly Ser Ala Leu Asp Arg Ile Ser Leu Ser Glu Cys
  085     090     095
Pro Leu Ser His Arg Gly Tyr Cys Leu His Asp Gly Val Cys Met Tyr
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Ile Glu Ala Leu Asp Lys Tyr Ala Cys Asn Cys Val Val Gly Tyr Ile
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<210> SEQ ID NO 6
<211> LENGTH: 2850
<212> TYPE: DNA
<213> ORGANISM: Unknown
<220> FEATURE:
<223> OTHER INFORMATION: DNA sequence of the KIF-B fusion

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<210> SEQ ID NO 7
<211> LENGTH: 949
<212> TYPE: PRT
<213> ORGANISM: Unknown
<220> FEATURE: 
<223> OTHER INFORMATION: Protein sequence of the EGF-B fusion
<400> SEQUENCE: 7

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Pro Glu Arg Tyr Thr Phe Gly Tyr Lys Pro Glu Asp Phe Asn Lys Ser 50 55 60
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Leu Phe Asn Arg Ile Lys Ser Lys Pro Leu Gln Gln Lys Leu Leu Glu 100 105 110
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Thr Phe Gly Gly Asp Pro Ser Ile Ile Thr Pro Ser Thr Asp Lys 275 280 285
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Leu Ser Lys Asn Glu Arg Ile Glu Tyr Asn Thr Gin Ser Asn Tyr Ile 485 490 495
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Pro Leu Asp Ile Arg Asp Ile Ser Leu Thr Ser Ser Phe Asp Asp Ala 565 570 575 580
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Lys Thr Ala Asn Lys Val Val Glu Ala Gly Leu Phe Ala Gly Trp Val 595 600 605
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Met Asp Lys Ile Ala Asp Ile Ser Leu Ile Val Pro Tyr Ile Gly Leu 625 630 635 640
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Phe Glu Ile Ala Gly Ala Ser Ile Leu Leu Glu Phe Ile Pro Glu Leu 665 670
Leu Ile Pro Val Val Gly Ala Phe Leu Leu Glu Ser Tyr Ile Asp Asn 675 680 685 690
Lys Asn Lys Ile Ile Lys Thr Ile Asp Asn Ala Leu Thr Lys Arg Asn 690 695 700 705
Glu Lys Trp Ser Asp Met Tyr Gly Leu Ile Val Ala Gin Trp Leu Ser 710 715 720
Thr Val Asn Thr Gin Phe Tyr Thr Ile Lys Glu Gly Met Tyr Lys Ala 725 730 735
Leu Asn Tyr Gin Ala Gin Ala Leu Glu Glu Ile Lys Tyr Arg Tyr 740 745 750 755
Asn Ile Tyr Ser Glu Lys Glu Lys Ser Asn Ile Asn Asp Phe Asn 760 765 770 775
Asp Ile Asn Ser Lys Leu Asn Glu Gly Ile Asn Gin Ala Ile Asp Asn 780 785 790 795
Ile Asn Asn Phe Ile Asn Gly Cys Ser Val Ser Tyr Leu Met Lys Lys 800 805 810 815
Met Ile Pro Leu Ala Val Glu Leu Leu Asp Phe Asp Asn Thr Leu 820 825 830 835
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<210> SEQ ID NO: 8
<211> LENGTH: 120
<212> TYPE: DNA
<213> ORGANISM: Unknown
<220> FEATURE:
<223> OTHER INFORMATION: DNA sequence of the RGD linker

<400> SEQUENCE: 8

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<210> SEQ ID NO: 9
<211> LENGTH: 2709
<212> TYPE: DNA
<213> ORGANISM: Unknown
<220> FEATURE:
<223> OTHER INFORMATION: DNA sequence of the RGD-C fusion

<400> SEQUENCE: 9

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<210> SEQ ID NO 10
<211> LENGTH: 901
<212> TYPE: PRT
<213> ORGANISM: Unknown
<220> FEATURE:
<223> OTHER INFORMATION: Protein sequen eef RGD-C fusion

<400> SEQUENCE: 10

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<210> SEQ ID NO 11
<211> LENGTH: 126
<212> TYPE: DNA
<213> ORGANISM: Unknown
<220> FEATURE:
<223> OTHER INFORMATION: DNA sequence of cyclic RGD linker

<400> SEQUENCE: 11

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<210> SEQ ID NO 12
<211> LENGTH: 2715
<212> TYPE: DNA
<213> ORGANISM: Unknown
<220> FEATURE:
<223> OTHER INFORMATION: DNA sequence of cyclic RGD-C fusion

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<210> SEQ ID NO 13
<211> LENGTH: 903
<212> TYPE: PRT
<213> ORGANISM: Unknown
<220> FEATURE: 
<223> OTHER INFORMATION: Protein sequence of cyclic RGD-C fusion

<400> SEQUENCE: 13

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Ser Leu Tyr Asn Lys Thr Leu Gln Cys Arg Glu Leu Leu Val Lys Asn
450 460
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465 470 475 480
Phe Leu Arg Lys Asp Ile Asn Glu Thr Glu Val Ile Tyr Tyr Pro
485 490 495
Asp Asn Val Ser Val Asp Gln Val Ile Leu Ser Lys Asn Thr Ser Glu
500 505 510
His Gly Gln Leu Asp Leu Leu Tyr Pro Ser Ile Asp Ser Glu Ser Glu
515 520 525
Ile Leu Pro Gly Glu Asn Val Phe Tyr Asp Asn Arg Thr Gin Ann
530 535 540
Val Asp Tyr Leu Asn Ser Tyr Tyr Leu Glu Ser Gin Lys Leu Ser
545 550 555 560
Asp Asn Val Glu Asp Phe Thr Phe Thr Arg Ser Ile Glu Glu Ala Leu
565 570 575
Asp Asn Ser Ala Lys Val Tyr Thr Tyr Phe Pro Thr Leu Ala Asn Lys
580 585 590
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595 600 605
Val Val Glu Asp Phe Thr Asn Ile Leu Arg Lys Asp Thr Leu Asp
610 615 620
Lys Ile Ser Asp Val Ser Ala Ile Ile Pro Tyr Ile Gly Pro Ala Leu
625 630 635 640
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Val Thr Gly Val Thr Ile Leu Leu Glu Ala Phe Pro Glu Phe Thr Ile
660 665 670
Pro Ala Leu Gly Ala Phe Val Ile Tyr Ser Lys Val Gin Glu Gin Asn
675 680 685
Glu Ile Ile Lys Thr Ile Asp Asn Cys Leu Glu Gin Arg Ile Lys Arg
690 695 700
Trp Lys Asp Ser Tyr Glu Trp Met Met Gin Thr Trp Leu Ser Arg Ile
705 710 715 720
Ile Thr Gin Phe Asn Asn Ile Ser Tyr Gin Met Tyr Gin Gin Ser Gin Gin
725 730 735
Tyr Gin Ala Gly Ala Ile Lys Ala Lys Ile Gin Leu Gin Tyr Gin Lys Gin
740 745 750
Tyr Ser Gin Ser Gin Lys Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin
755 760 765
Lys Asn Ser Gin Asp Val Lys Ile Ser Gin Glu Gin Asn Gin Asn Ile Gin
770 775 780
Lys Pro Gin Ile Arg Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin
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Pro Lys Gin Val Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin
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Lys Leu Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin
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Val Asp Lys Leu Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin
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Met Phe Gly Cys Ala Lys Leu
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<210> SEQ ID NO 14
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<212> TYPE: DNA
<213> ORGANISM: Unknown
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<223> OTHER INFORMATION: DNA sequence of the LC/C-RGD-HN/C LINKER
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Leu Ile Pro Lys Ser Ala Arg Lys Tyr Phe Glu Glu Lys Ala Leu Asp
Tyr Tyr Arg Ser Ile Ala Lys Arg Leu Asn Ser Ile Thr Thr Ala Asn
Tyr Thr Arg Ser Ile Ala Lys Arg Leu Asn Ser Ile Thr Thr Ala Asn
Pro Ser Ser Phe Asn Lys Tyr Ile Gly Glu Tyr Lys Gln Lys Leu Ile
Arg Asn Tyr Arg Phe Val Val Glu Ser Ser Gly Glu Val Thr Val Asn
Arg Asn Tyr Leu Val Glu Leu Tyr Asn Glu Leu Thr Gln Ile Phe Thr
Glu Phe Asn Tyr Ala Lys Ile Tyr Asn Val Glu Asn Arg Lys Ile Tyr
Leu Ser Asn Val Tyr Thr Pro Val Thr Ala Asn Ile Leu Asp Asp Asn
Val Tyr Ile Gln Asn Gly Phe Asn Ile Pro Lys Ser Asn Leu Asn
Val Leu Phe Met Gly Glu Asn Leu Ser Arg Asn Pro Ala Leu Arg Lys
Val Asn Pro Glu Asn Met Leu Tyr Leu Phe Thr Lys Phe Cys Val Asp
Val Asn Pro Glu Asn Met Leu Tyr Leu Phe Thr Lys Phe Cys Val Asp
Val Asn Pro Glu Asn Met Leu Tyr Leu Phe Thr Lys Phe Cys Val Asp
Val Asn Pro Glu Asn Met Leu Tyr Leu Phe Thr Lys Phe Cys Val Asp
Ala Ile Asp Gly Arg Ser Leu Tyr Asn Lys Thr Leu Glu Cys Arg Glu
Leu Leu Val Lys Asn Thr Asp Leu Pro Phe Ile Gly Asp Ile Ser Asp
Val Lys Thr Asp Ile Phe Leu Arg Lys Ile Asn Glu Glu Thr Glu
Val Ile Tyr Tyr Pro Asp Val Asn Val Ser Val Asp Glu Val Ile Leu Ser
Lys Asn Thr Ser Glu His Gly Glu Leu Asp Leu Leu Tyr Pro Ser Ile
Asp Ser Glu Ser Glu Ile Leu Pro Gly Glu Asm Val Phe Tyr Asp
Asp Asp Lys Thr Asn Val Asp Tyr Leu Asn Ser Tyr Tyr Tyr Tyr Leu Glu
Ser Gln Lys Leu Ser Asp Asn Val Glu Asp Phe Thr Phe Thr Arg Ser
Ile Glu Glu Asp Leu Asp Ser Ala Lys Val Tyr Thr Tyr Phe Pro
Thr Leu Ala Asn Lys Val Asn Ala Gly Val Glu Gly Gly Leu Phe Leu
Met Trp Ala Asn Val Val Glu Asp Phe Thr Thr Asn Ile Leu Arg
Lys Asp Thr Leu Asp Lys Ile Ser Asp Val Ser Ala Ile Ile Pro Tyr
Ile Gly Pro Ala Leu Asn Ile Ser Asn Ser Val Arg Arg Gly Asn Phe
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Pro Glu Phe Thr Ile Pro Ala Leu Gly Ala Phe Val Ile Tyr Ser Lys
  675 680 685
Val Glu Glu Arg Asn Glu Ile Lys Thr Ile Asp Asn Cys Leu Glu
  690 695 700
Gln Arg Ile Lys Arg Trp Lys Asp Ser Tyr Glu Trp Met Met Gly Thr
  705 710 715 720
Trp Leu Ser Arg Ile Thr Glu Phe Asn Asn Ile Ser Tyr Glu Met
  725 730 735
Tyr Asp Ser Leu Asn Tyr Gln Ala Gly Ala Ile Lys Ala Lys Ile Asp
  740 745 750
Leu Tyr Lys Tyr Ser Gly Ser Asp Lys Glu Asn Ile Lys Ser
  755 760 765
Gln Val Glu Asn Leu Lys Asn Ser Leu Asp Val Lys Ile Ser Glu Ala
  770 775 780
Met Asn Asn Ile Asn Lys Phe Ile Arg Glu Cys Ser Val Thr Tyr Leu
  785 790 795 800
Phe Lys Asn Met Leu Pro Lys Val Ile Asp Glu Leu Asn Glu Phe Asp
  805 810 815
Arg Asn Thr Lys Ala Lys Leu Asn Leu Ile Asp Ser His Asn Ile
  820 825 830
Ile Leu Val Gly Glu Val Asp Lys Leu Lys Ala Lys Val Asn Ser
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Phe Gln Asn Thr Ile Pro Phe Asn Ile Phe Ser Tyr Thr Asn Asn Ser
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Leu Leu Lys Asp Ile Asn Glu Tyr Phe Asn Leu Glu Gly Gly Gly
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Gly Ser Gly Gly Gly Ser Gly Gly Gly Gly Ser Ala Leu Asp Asn
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Gly Val Cys Met Tyr Ile Glu Ala Leu Asp Lys Tyr Ala Cys Asn Cys
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<220> FEATURE:
<223> OTHER INFORMATION: Integrin binding peptide sequence

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<210> SEQ ID NO 22
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1. A method for inhibiting secretion from a non-neuronal inflammatory cell, said method comprising administering an agent comprising at least first and second domains, wherein the first domain cleaves one or more proteins essential to exocytosis and the second domain translocates the first domain into the inflammatory cell.

2. The method according to claim 1, for treatment of disease caused, exacerbated or maintained by secretion from said non-neuronal inflammatory cell.

3. The method according to claim 1 or 2, wherein the agent further comprises a third domain for targeting the agent to said non-neuronal inflammatory cell.

4. The method according to claim 3 wherein the third domain comprises or consists of a growth factor or an integrin-binding protein; or a ligand selected from (i) for mast cells, complement receptors in general, including C4 domain of the Fc IgE, and antibodies/ligands to the C3a/C4a-R complement receptor; (ii) for eosinophils, antibodies/ligands to the C3a/C4a-R complement receptor, anti VLA-4 monoclonal antibody, anti-IL-5 receptor, antigens or antibodies reactive toward CR4 complement receptor; (iii) for macrophages and monocytes, macrophage stimulating factor, (iv) for macrophages, monocytes and neutrophils, bacterial IPS and yeast B-glucans which bind to CR3, (v) for neutrophils, antibody to 0X42, an antigen associated with the IC3b complement receptor, or IL-8; (vi) for fibroblasts, mannose 6-phosphate/insulin-like growth factor-beta (M6P/IGFII) receptor and PA2.26, antibody to a cell-surface receptor for active fibroblasts in mice.

5. The method according to claim 1 for the treatment of a disease selected from the group consisting of allergies (seasonal allergic rhinitis (hay fever), allergic conjunctivitis, vasomotor rhinitis and food allergy), eosinophilia, asthma, rheumatoid arthritis, systemic lupus erythematosus, discoid
lupus erythematosus, ulcerative colitis, Crohn’s disease, hemorrhoids, pruritus, glomerulonephritis, hepatitis, pancreatitis, gastritis, vasculitis, myocarditis, psoriasis, eczema, chronic radiation-induced fibrosis, lung scarring and other fibrotic disorders.

6. The method according to claim 1, wherein the agent comprises a first domain that cleaves a protein selected from SNAP-25, synaptobrevin and syntaxin.

7. The method according to claim 1 wherein the first domain comprises a light chain of a clostridal neurotoxin, or a fragment, variant or derivative thereof which inhibits exocytosis.

8. The method according to claim 1, wherein the second domain comprises a HN region of a clostridial polypeptide, or a fragment, variant or derivative thereof that translocates the exocytosis inhibiting activity of the first domain into the cell.

9. The method according to claim 1 for inhibition of constitutive and regulated release from non-neuronal inflammatory cells.

10. The method according to claim 1, wherein the agent is in the form of a pharmaceutical composition comprising a pharmaceutically acceptable carrier.

11. The method according to claim 3, wherein the third domain is epidermal growth factor.

12. The method according to claim 3, wherein the third domain is an integrin-binding protein.

13. The method according to claim 12, wherein the third domain comprises the tri-peptide amino acid sequence Arg-Gly-Asp.

14. The method according to claim 12, wherein the third domain comprises a sequence selected from Arg-Gly-Asp-Phe-Val (SEQ ID NO: 23); Arg-Gly-Asp-[D-Phe]-[N-methyl-Val] (SEQ ID NO: 23); RGDFV (SEQ ID NO: 23); RGDINMeV (SEQ ID NO: 23); GGRGDMFGA (SEQ ID NO: 21); GGCRRGDMFGCA (SEQ ID NO: 22); GRGDSP (SEQ ID NO: 26); GRGESP (SEQ ID NO: 27); PLAEIDGIEI (SEQ ID NO: 24) and CPLAEIDGHEL (SEQ ID NO: 25), or a sequence having at least 80% identity therewith.

15. The method according to claim 2, wherein the agent further comprises a third domain for targeting the agent to said non-neuronal inflammatory cell.