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(54) Title: INFLUENZA VIRUS BINDING, SIALYLATED OLIGOSACCHARIDE SUBSTANCE AND USE THEREOF

(57) Abstract: The present invention is directed to human influenza virus binding substance containing at least one oligosaccharide chain, which comprises a terminal NeuNAc α 6 linked to: (a) a linear or branched poly lactosamine type structure consisting of at least three lactosamine residues, a linear sequence optionally containing one or two α 3-linked fucose residues in a non-sialylated lactosamine, a branched structure optionally carrying one or more additional NeuNAc α -residues at a terminal position in a branch, and/or (b) a linear or branched structure with two lactosamine and one lactose residue, a linear structure in addition containing one or two α 3-linked fucose residues in a non-sialylated lactoseamine or lactose, a branched structure optionally carrying one additional NeuNAc α -residue in a terminal position of the branch, or an analog or derivative of said oligosaccharide chain for use in binding of human influenza virus.

Influenza virusbinding, Sialylated oligosaccharide substance and use thereof

5 FIELD OF THE INVENTION

The present invention relates to a substance or a receptor binding to human influenza virus, as well as use thereof in pharmaceutical compositions and a method for treatment of a condition due to the presence of influenza virus in the human respiratory tract. The
10 invention is also directed to the use of the receptor for diagnostics of influenza viruses.

BACKGROUND OF THE INVENTION

Influenza virus attachment to host cells is mediated by specific interactions of the viral
15 envelope protein hemagglutinin (HA) with sialylated carbohydrate chains of cell surface glycoproteins and glycolipids (for reviews, Suzuki, 1994; Herrler *et al.*, 1995; Paulson, 1985; Wiley and Skehel, 1987). Natural sialylglycoproteins and gangliosides exhibit significant structural diversity, and different receptors are probably utilized by the viruses in different host tissues. It has been shown, that influenza A viruses isolated from avian
20 species preferentially bind to NeuAc α 3Gal-terminated sugar chains, while closely related human viruses reveal a higher binding affinity towards the NeuAc α 6Gal-terminated structures (Paulson, 1985; Suzuki, 1994; Connor *et al.*, 1994; Matrosovich *et al.*, 1997; Gambaryan *et al.*, 1997). Additional influential features for binding are inner parts of saccharide chains (Gambaryan *et al.*, 1995; Matrosovich *et al.*, 1997; Rogers and Paulson,
25 1983; Suzuki *et al.*, 1987; Suzuki *et al.*, 1992; Eisen *et al.*, 1997), polyvalency of receptor saccharides (Pritchett and Paulson, 1989; Matrosovich, 1989; Mammen *et al.*, 1995), spatial arrangement of sialyloligosaccharides in receptor glycoproteins (Pritchett and Paulson, 1989), or glycosylation of the viral hemagglutinins (Gambaryan *et al.*, 1998; Ohuchi *et al.*, 1997). Detailed molecular mechanisms of these effects and the importance
30 of a variation in fine structure of sialylated receptors for the virulence and pathogenicity of individual viral strains are not known. For example virulence of the 1918 influenza pandemic still remains unexplained (Laver *et al.*, 1999), and the actual strains have not been assayed for receptor specificity.

Studies on the structural characterization of biological receptors for human influenza viruses are hampered by the limited availability of the human respiratory tract tissues. However, characterization of the binding molecules from other human tissues may permit further specification of the receptor binding epitopes. Human leukocytes represent an attractive experimental model because they contain a series of gangliosides with high binding affinity for the virus. Binding species were detected in human leukocytes among common gangliosides (Müthing *et al.* 1993; Müthing, 1996). In contrast to the present invention these were described to contain $\alpha 3$ -sialylated sialyl-Lewis x and VIM-2 sequences. The receptor activity was also observed among highly complex glycolipid fractions, polyglycosylceramides (Matrosovich *et al.*, 1996). NeuAc $\alpha 6$ -containing glycolipids of human leukocytes with more than two lactosamine units in the core chain have not yet been characterized by other laboratories (Müthing *et al.*, 1993; Müthing *et al.* 1996; Müthing, 1996; Stroud *et al.*, 1995; Stroud *et al.*, 1996). They occur in human white cells in very small amounts and their existence has so far been neglected. However, these minor species may be of biological importance for *in vivo* events during influenza infections and may explain virulence variations between strains (Laver *et al.*, 1999).

Binding of influenza viruses to sialic acid-containing neutrophil receptor(s) depresses bactericidal activity of neutrophils (Abramson and Mills, 1988; Cassidy *et al.*, 1989; Daigneault *et al.*, 1992; Abramson and Hudnor, 1995) and stimulates apoptosis of these cells by a yet undefined mechanism (Colamussi *et al.*, 1999). This virus-mediated neutrophil dysfunction is a likely contributor to the development of secondary bacterial infections, which are the main cause of morbidity and mortality during influenza epidemics.

Several studies describe sialylated di- to heptasaccharide binding structures for human influenza viruses e.g. (Gambaryan *et al.*, 1995; Matrosovich *et al.*, 1997; Rogers and Paulson, 1983; Suzuki *et al.*, 1992; Eisen *et al.*, 1997). These have low affinities to the viruses and the studies do not describe larger saccharide receptors which have the high natural binding affinity. The divalent NeuNAc $\alpha 6$ Gal $\beta 4$ GlcNAc-saccharides constructed chemically on β -galactosides are described in Sabesan, S. *et al.* -92 and in US patents 5,254,676 and 5,220,008 and have only modestly better affinities than corresponding monosialocompounds even when linked on bovine serum albumin in multivalent form.

Several synthetic polymeric influenza inhibitors containing sialic acid (Mammen *et al.*, 1995) or sialylated lactose/N-acetyllactosamine have been described (Gambaryan *et al.*, 1997). The specific binding of human influenza virus to NeuNAc α 6Gal β 4GlcNAc-epitopes (Gambaryan *et al.*, 1997) and the presence of NeuNAc α 6Gal on the surface of human-ciliated tracheal epithelium (Baum and Paulson, 1990) were suggested to constitute an essential part of the biologically relevant cellular receptor for the viruses (Gambaryan *et al.*, 1997).

A polylactosamine containing and α 6-sialylated inhibitor of human influenza viruses has been described. This molecule is produced from a cryptically I-active (anti-I-antibodies are known to recognize polylactosamine and non-polylactosamine structures) glycoprotein 2 of bovine erythrocytes by removing sialic acid residues by sialidase treatment and by enzymatic resialylation by α 3- or α 6-sialyltransferases (Suzuki *et al.*, 1987). The semisynthetic bovine protein is not a natural receptor structure of human viruses. It is hardly useful as a therapeutic inhibitor of human influenza viruses, because it contains a protein structure and a substantial amount of Gal α 3Gal β 4GlcNAc-xeno-antigenic structures (Suzuki *et al.*, 1985). The Gal α 3Gal β 4GlcNAc-antigen is present in many mammalian species but not produced by human tissues, the structure is highly antigenic in human, and there are naturally large amounts of antibodies against the structure in humans. Foreign protein structures are known to be potential antigens and allergens for humans.

The authors (Suzuki *et al.*, 1987) discuss that inner I-active neolacto-series type II sugar chains may also be important as a common part of the receptor determinant toward the hemagglutinin of human influenza viruses A and B. Unfortunately their saccharide material is very heterogenous and the possible sialylated I-active components formed were not characterized chemically and no specific epitopes were assigned as virus binding structures. According to the data of Fig. 3 corresponding to oligosaccharides labelled at the reducing end, the large I-polylactosamines are actually very minor species in the glycoprotein II and smaller O-glycans are major species in molar amount, the smaller O-glycans without branched polylactosamine are also recognized by at least one anti-I-antibody used to determine the saccharides of the protein (Suzuki *et al.*, 1985). In another study the same authors show that α 3-sialylated and α 3-galactosylated branched

polylactosamine glycolipid (also an I-antigenic structure) was shown to be a binding compound for influenza virus (Suzuki *et al.*, 1986).

A BRIEF DESCRIPTION OF THE FIGURES

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Fig. 1. Binding of HRP conjugates of human (H1N1) and avian (H4N6) influenza A viruses to gangliosides isolated from different human tissues and separated on silica gel thin-layer plates. **Anis**, plate sprayed with anisaldehyde (4-methoxybenzaldehyde); **Human virus** and **Avian virus**, plates overlaid with respective conjugates. **Lane 1**, upper phase gangliosides (after Folch's partition) from human leukocytes; **Lane 2**, upper phase gangliosides from human erythrocytes; **Lane 3**, total gangliosides from human small intestine, sample 1; **Lane 4**, total gangliosides from human small intestine, sample 2; **Lane 5**, total gangliosides from human stomach; **Lane 6**, total gangliosides from human meconium; **Lane 7**, total gangliosides from human colon; **Lane 8**, total gangliosides from human pancreas; **Lane 9**, bovine brain gangliosides. The plates were developed in Chloroform/Methanol/0.25% KCl in H₂O, 50:40:10. **S-3-PG**, sialyl-3-paragloboside; **S-6-PG**, sialyl-6-paragloboside; **5s**, **7s** and **8s**, 5-, 7- and 8-sugar-containing monosialoganglioside fractions (NeuAc₁Hex₃HexNAc₁Cer, NeuAc₁Hex₄HexNAc₂Cer and Fuc₁NeuAc₁Hex₄HexNAc₂Cer, respectively). To visualize slowly migrating gangliosides some lanes were overloaded. Dotted lines were drawn to facilitate interpretation.

Fig. 2. Binding of human and avian influenza viruses HRP conjugates to leukocyte gangliosides on silica gel TLC plates before (**lane 1**) and after (**lane 2**) mild oxidation and reduction. **Lane 3**: reference brain gangliosides (from top: GM1, GD1a, GD1b, GT1b). **Anis**, gangliosides stained with anisaldehyde. Chromatographic conditions were as in Fig. 1. **x**, non-sugar spot acquired during dialysis.

Fig. 3A. Negative ion FAB mass spectra of gangliosides from human leukocytes before mild periodate oxidation and reduction.

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Fig. 3B. Negative ion FAB mass spectra of gangliosides from human leukocytes after mild periodate oxidation and reduction.

Fig. 4A. Binding of anti-sialyl-Lewis x (SLX) and Vim-2 (VIM) monoclonal antibodies to upper phase gangliosides (after Folch's partition) of human leukocytes separated on silica gel TLC plates. **Anis**, gangliosides stained with anisaldehyde; **Virus**, gangliosides overlaid with human influenza virus HRP conjugate. The plates were developed in
5 Chloroform/Methanol/0.25% KCl, 50:55:13.

Fig. 4B. Binding of anti-sialyl-Lewis x (SLX) and Vim-2 (VIM) monoclonal antibodies to upper phase gangliosides (after Folch's partition) of human leukocytes separated on silica gel TLC plates. **Anis**, gangliosides stained with anisaldehyde; **Virus**, gangliosides overlaid
10 with human influenza virus HRP conjugate. The plates were developed in Chloroform/Methanol/0.25% KCl, 50:40:10.

Fig. 5. Binding of NeuAc α 3- and NeuAc α 6-specific lectins from *Maackia amurensis* (MAA) and *Sambucus nigra* (SNA) to gangliosides separated by TLC and blotted to
15 PVDF membranes; **Human virus**, binding of human virus HRP conjugate on the corresponding TLC plate; **Anis**, TLC plate with separated gangliosides and visualized with anisaldehyde. The plates were developed in Chloroform/Methanol/0.25%KCl, 50:50:13. **Lane 1**, upper phase gangliosides (after Folch's partition) from human leukocytes; **Lane 2**, sialyl-3-paragloboside; **Lane 3**, bovine brain gangliosides (GM1, GD1a, GD1b and
20 GT1b).

Fig. 6. Binding of HRP conjugate of human (A, H1N1) influenza virus to ganglioside subfractions (**Fr. 1-6**) obtained from a mixture of human leukocyte gangliosides after preparative TLC; **TG**, Total ganglioside mixture. **Anis**, plate visualized with
25 anisaldehyde; **Human virus**, plate overlaid with virus conjugate.

Fig. 7. MALDI TOF spectrum of ganglioside fraction 6 of **Fig. 6**. Mass spectrometer was operated in a negative-ion reflectron mode.

30 DETAILED DESCRIPTION OF THE INVENTION

In the invention, the binding of human viruses to gangliosides from different human tissues and cells, including leukocytes, was studied. The human virus bound selectively to

only minor extended ganglioside species. So far other laboratories have not been able to detect and isolate the minor active species containing NeuNAc α 6 sequences even when starting with huge amounts of leukocytes. The combination of the older and newly developed special overlay techniques used by the inventors together with the most sensitive mass spectrometry experiments allowed analysis of the novel receptors for human influenza virus.

Sialyl-Lewis x and VIM-2-active saccharides were excluded as parts of the active receptors binding the human influenza virus. Human is known not to contain Gal α 3-structures (Larsen *et al*, 1990). It was also shown using SNA lectin that the binding was to some minor NeuNAc α 6-containing species. In addition, it was demonstrated, that the binding was dependent on the presence of the unchanged glycerol sialic acid tail. The monosaccharide and ceramide compositions of the smallest active molecules were indicated by mass spectrometric data of the active fraction. Mass spectrometry data together with the other evidence allowed us to describe further potential structural features present in the high affinity species. Assignments are based on the knowledge that the HexNAc $_x$ Hex $_{x+2}$ in the glycolipid fractions correspond to poly lactosamine sequences. The structural features described are in accordance with the previously known glycolipid structures of human leukocytes (Stroud *et al.*, 1995; Stroud *et al.*, 1996; Stroud *et al.*, 1996b; M \ddot{u} thing, 1996, Johansson and Miller-Podraza, 1998) except for the presence α 6-linked sialic acid on the structures.

The invention first describes the minimum structural elements present in the high affinity receptor of human influenza virus. The receptor active species contain at least one oligosaccharide chain, which comprises a terminal NeuNAc α 6 linked to

- (a) -a linear or branched poly lactosamine type structure consisting of at least three lactosamine residues, a linear sequence optionally containing one or two α 3-linked fucose residues in a non-sialylated lactosamine, a branched structure optionally carrying one or more additional NeuNAc α -residues at a terminal position in a branch, and/or
- (b) -a linear or branched structure with two lactosamine and one lactose residue, a linear structure in addition containing one or two α 3 -linked fucose residues in a non-sialylated lactoseamine or lactose, a branched structure optionally carrying one additional

NeuNAc α -residue in a terminal position of the branch. These active receptor epitopes are called virus binding structures.

The virus binding substances contain no Gal α 3Gal β 4GlcNAc-residues as active virus binding parts and is preferably not linked to a protein and preferably do not contain any Gal α 3Gal β 4GlcNAc-xenoantigenic structures. The virus binding substances are preferentially presented in clustered form such as by glycolipids on cell membranes, micelles, liposomes or on solid phases such as TLC-plates used in the assays. The clustered representation with correct spacing creates high affinity binding. According to a preferred embodiment the virus binding substance thus contains at least two oligosaccharides as defined, preferably at least three or at least four oligosaccharides as defined. It is noted from the TLC-assays that larger polylectosamine type compounds containing the NeuNAc α 6-terminal are also receptor for the viruses. These compounds present in even more minor amounts are very high affinity receptors for the virus.

Repeating virus binding epitopes containing branches with NeuNAc α 6Gal β 4GlcNAc on larger polylectosamine structures can effectively bind to several binding sites of virus surfaces, most probably on the hemagglutinin protein. The large glycolipid fraction is continuous with larger molecular weight polylectosamine glycolipid fraction called polyglycosylceramides. It has been recently shown that the polyglycosylceramide fraction has very high affinity for human influenza virus (Matrosovich *et. al*, 1996) and in a separate study these polylectosamines have been shown to carry NeuNAc α 6-terminal structures (Johansson and Miller-Podraza, 1998). In a preferred embodiment the virus binding substances are presented on polylectosamine-type oligo- or polysaccharide or glycosides thereof. In the middle and nonreducing terminal parts of natural virus binding polylectosamines the binding epitope does not contain lactose residues, but a lactose residue may be present at the reducing end.

According to the invention it is possible to use the influenza virus binding epitopes or naturally occurring or a synthetically produced analogue or derivative thereof having a similar or better binding activity with regards to human influenza virus. It is also possible to use a substance containing the virus binding substance, such as a receptor active polylectosamine ganglioside described in the invention or an analogue or derivative thereof having a similar or better binding activity with regards to human influenza virus.

The virus binding substance may be a glycosidically linked terminal epitope of an oligosaccharide chain. Alternatively the virus binding epitope may be a branch of a polylactosamine chain.

- 5 Further assignment of the most potential receptor structures is possible based on the following facts. The SNA-lectin recognizes the terminal NeuNAc α 6Gal-sequence. The presence of two NeuNAcs with α 6 or α 3-linkages indicates that the N-acetylactosamine structures are branched. The biosynthetic knowledge in the art indicates that terminal NeuNAc α 6Gal β 4GlcNAc sequences are not α 3-fucosylated (e.g. Paulson *et al.*, 1978; 10 DeVries *et al.*, 1995), indicating that fucose residues are located further away from terminal NeuNAc as in NeuNAc α 6Gal β 4GlcNAc β 3Gal β 4(Fuc α 3)GlcNAc β -. Branching and fucosylation are exclusive on the same N-acetylactosamine sequence of the known leucocyte type enzymes (Niemelä *et al.*, 1998; Mattila *et al.*, 1998). Furthermore, the Glc-residue is not fucosylated by the major fucosyltransferase of leukocytes (De Vries *et al.*, 15 1995; Clarke and Watkins, 1996), which is in accordance with the knowledge of the vast majority of the isolated human oligosaccharide structures. However, the analysis does not exclude the presence of other minor isomeric species which are synthesized by yet uncharacterized biosynthetic reactions. Under the assay conditions described, the linear chain glycolipids NeuNAc α 6Gal β 4GlcNAc β 3Gal β 4Glc β Cer or 20 NeuNAc α 6Gal β 4GlcNAc β 3Gal β 4GlcNAc β 3Gal β 4Glc β Cer did not show binding, indicating specific structural features on the larger binding glycolipids.

Mass spectrometry indicated the presence of disialylated and/or difucosylated molecules in the active fraction with an abundance of the the active species, disialylation and 25 difucosylation being structural features different from the non-active linear sequences. The disialylated species matches most correctly to the molecular masses of the saccharide sequences with the same lipid species previously known to be present in major sialylated glycolipids of leukocytes. These data indicate that the disialylated and/or difucosylated species belong to the high affinity receptor species.

30

The saccharide sequences of the receptor disialylated molecules are according to the experimental evidence and current biosynthetic and structural knowledge:

NeuNAc α 6Gal β 4GlcNAc β 3(NeuNAc α 6/or3Gal β 4GlcNAc β 6)Gal β 4GlcNAc β -

3Gal β 4Glc β Cer and NeuNA α 6Gal β 4GlcNAc β 3(NeuNA α 6/or3Gal β 4GlcNAc β 6)Gal β -
4Glc β Cer. The lectin binding assays indicate that most of the active species are devoid of
NeuNA α 3, therefore indicating species having both branches with NeuNA α 6:

NeuNA α 6Gal β 4GlcNAc β 3(NeuNA α 6Gal β 4GlcNAc β 6)Gal β 4GlcNAc β 3Gal β 4Glc β Ce
5 r and NeuNA α 6Gal β 4GlcNAc β 3(NeuNA α 6Gal β 4GlcNAc β 6)Gal β 4Glc β Cer. The
binding of the more elongated deca-saccharide sequences to the influenza virus may be
more effective. The similar terminal octa-saccharide sequences are defined as virus binding
substances. The preferred disialylated virus binding substances have the structures:

NeuNA α 6Gal β 4GlcNAc β 3(NeuNA α 6/or3Gal β 4GlcNAc β 6)Gal β 4GlcNAc,
10 NeuNA α 6Gal β 4GlcNAc β 3(NeuNA α 6/or3Gal β 4GlcNAc β 6)Gal β 4Glc,
NeuNA α 6Gal β 4GlcNAc β 3(NeuNA α 6Gal β 4GlcNAc β 6)Gal β 4GlcNAc, and
NeuNA α 6Gal β 4GlcNAc β 3(NeuNA α 6Gal β 4GlcNAc β 6)Gal β 4Glc.

The saccharide sequences of the receptor difucosylated molecules are according to the
15 experimental evidence and current biosynthetic and structural knowledge:

NeuNA α 6Gal β 4GlcNAc β 3Gal β 4(Fuca α 3)GlcNAc β 3Gal β 4(Fuca α 3)GlcNAc β 3Gal β 4Glc β -
-Cer and a less likely form of fucosylated lactose:

NeuNA α 6Gal β 4GlcNAc β 3Gal β 4(Fuca α 3)GlcNAc β 3Gal β 4(Fuca α 3)Glc β Cer, the
saccharide sequence of this is considered as an effective analog of the terminal
20 nona-saccharide sequence of the longer version. The specific structural feature of
difucosylation together with NeuNA α 6 is considered to be active part in the recognition
of the virus. Preferentially the difucosylated virus binding structures are
NeuNA α 6Gal β 4GlcNAc β 3Gal β 4(Fuca α 3)GlcNAc β 3Gal β 4(Fuca α 3)GlcNAc and
NeuNA α 6Gal β 4GlcNAc β 3Gal β 4(Fuca α 3)GlcNAc β 3Gal β 4(Fuca α 3)Glc.

25 A further extension of the glycolipid could be a structural feature increasing the receptor
activity in comparison to the non-active species. Mass spectrometry peaks indicating the
presence of the major non-receptor active α 3-sialylated species, may also contain signals
corresponding to isomeric α 6-sialylated molecules. The extended receptors have structures
30 NeuNA α 6Gal β 4GlcNAc β 3Gal β 4(Fuca α 3)₀₋₁GlcNAc β 3Gal β 4(Fuca α 3)₀₋₁
GlcNAc β 3Gal β 4Glc β Cer, where the molecule has 0 or 1 fucosyl residues. Furthermore a
branched monosialylated structure is possible with the same molecular weight as the

linear non-sialylated forms such as NeuNAc α 6Gal β 4GlcNAc β 3(Gal β 4GlcNAc β 6)-Gal β 4GlcNAc β 3Gal β 4Glc β Cer. The extended linear and branched structural features are very similar to the species indicated above and therefore these molecules are likely to also have receptor activity. The sequences NeuNAc α 6Gal β 4GlcNAc β 3Gal β 4(Fuc α 3)₀₋₁GlcNAc β 3Gal β 4(Fuc α 3)₀₋₁GlcNAc, where the molecule has 0 or 1 fucosyl residues and NeuNAc α 6Gal β 4GlcNAc β 3(Gal β 4GlcNAc β 6)Gal β 4GlcNAc are also virus binding structures.

When present as a branch of a longer poly lactosamine chain the virus binding substance has the structure R-3or6Gal β 4GlcNAc β 6/3(NeuNAc α 6Gal β 4GlcNAc β 3/6)Gal β -4GlcNAc β 4R₂, where the R is a glycosidically linked non-reducing end part of the poly lactosamine, and R₂ is glycosidically linked to the reducing end part of the poly lactosamine chain, preferably the R and/or R₂ contain more virus binding substances. The reducing end of the saccharide may be further derivatized.

The oligosaccharide chain of the invention can be a part of a poly lactosamine chain or a conjugate thereof. Preferably, the poly lactosamine chain contains at least 6 lactosamine residues or at least three sialic acid residues.

The virus binding substances may be conjugated to a carrier. The conjugation is performed by linking the virus binding substance preferably from the reducing end to a carrier molecule. When the conjugate is used in therapeutics, the carrier molecule is preferably not a protein.

The virus binding substances, preferably in oligovalent or clustered form, can be used to treat a disease or condition caused by the presence of the influenza virus in the respiratory tract of a patient. In such case the substances of the invention are used for anti-adhesion, i.e. to inhibit the binding of human influenza viruses to the receptor epitopes of the target cells or tissues. The target cells are neutrophils or epithelial cells of the respiratory tract.

When the substance or pharmaceutical composition according to the invention is administered it will compete with the receptor glycoconjugates on the target cells for the binding of the viruses. Some or all of the viruses will then be bound to the substance

according to the invention instead of the receptor on the target cells or tissues. The viruses bound to the substances according to the invention are then removed from the respiratory tract with secreted mucous material, resulting in a reduced effect of the viruses to the health of the patient. Preferably the pharmaceutical composition used is a soluble
5 composition comprising the substances of the invention. The substance according to the invention can be attached to a carrier substance which is preferably not a protein. When using a carrier molecule, several molecules of the substance according to the invention can be attached to one carrier and the inhibitory efficiency is improved.

10 According to the invention it is possible to incorporate the substance according to the invention, optionally with a carrier, in a pharmaceutical composition, which is suitable for the treatment of a condition due to the presence of influenza virus in the respiratory tract of a patient or to use the substance according to the invention in a method for treatment of such conditions. Examples of conditions treatable according to the invention are virus-
15 mediated neutrophil dysfunction which includes depression of bactericidal activity of neutrophils and stimulation apoptosis of these cells, prevention of secondary bacterial infections due the neutrophil dysfunction, and the primary infection by the influenza viruses in the respiratory tract.

20 The pharmaceutical composition according to the invention may also comprise other substances, such as an inert vehicle, or pharmaceutically acceptable carriers, preservatives etc, which are well known to persons skilled in the art.

The substance or pharmaceutical composition according to the invention may be
25 administered in any suitable way, although an oral or nasal administration especially in the form of a spray or inhalation are preferred.

The term "treatment" used herein relates both to treatment in order to cure or alleviate a disease or a condition, and to treatment in order to prevent the development of a disease or
30 a condition. The treatment may be either performed in a acute or in a chronic way.

The term "patient", as used herein, relates to any human or non-human mammal in need of treatment according to the invention.

5 Furthermore, it is possible to use substances specifically binding or inactivating the substances according to the invention when present on human tissues and thus to prevent the binding of influenza virus. Examples of such substances include the lectin *Sambucus nigra* agglutinin. When used in a human, such substances should be suitable for such use as a humanized antibody or an enzyme, such as recombinant sialidase of human origin which is non-immunogenic and capable of cleaving the terminal NeuNAc α 6-from the
10 substances of the invention.

It is possible to use the substance according to the invention with other pharmaceutical substance or substances effective against influenza viruses and achieve more effective pharmaceutical compositions and methods of treatment, provided that the substances used
15 do not inactivate each other, such as the substance inactivating the substances according to the invention as described above. It is especially beneficial to use substances according to the invention together with a pharmaceutical neuraminidase (sialidase) inhibitor (Influenza virus neuraminidase inhibitors have been reviewed in *The Lancet* (2000), 355:827-35), which could prevent partial degradation of the substance according to the invention and
20 prevent the viral activity with different mechanisms.

Furthermore, it is possible to use the substance according to the invention in the diagnosis of a condition caused by an influenza virus infection. Diagnostic uses also include the use of the substance according to the invention for typing of the influenza viruses. When the
25 substance according to the invention is used for diagnosis or typing, it may e.g. be included in a probe or a test stick, optionally constituting a part of a test kit. When this probe or test stick is brought into contact with a sample containing human influenza viruses, the viruses will bind the probe or test stick and can be thus removed from the sample and further analyzed.

30 Glycolipid and carbohydrate nomenclature is according to recommendations by the IUPAC-IUB Commission on Biochemical Nomenclature (*Carbohydr. Res.* 1998, 312, 167; *Carbohydr. Res.* 1997, 297, 1; *Eur. J. Biochem.* 1998, 257, 29).

It is assumed that Gal, Glc, GlcNAc, and NeuNAc are of the D-configuration, Fuc of the L-configuration, and all the monosaccharide units in the pyranose form. Glycosidic linkages are shown partly in shorter and partly in longer nomenclature, the linkages of the NeuNAc-residues $\alpha 3$ and $\alpha 6$ mean the same as $\alpha 2-3$ and $\alpha 2-6$, respectively, and $\beta 1-3$, $\beta 1-4$, and $\beta 1-6$ can be shortened as $\beta 3$, $\beta 4$, and $\beta 6$, respectively. Lactosamine refers to N-acetyllactosamine, Gal $\beta 1-4$ GlcNAc, and sialic acid is N-acetylneuraminic acid, NeuNAc. A lactose residue or a lactosamine residue can be derivatized or glycosidically conjugated from position 1 of Glc/GlcNAc or correspond to a free reducing end of a non-conjugated oligosaccharide chain. In the shorthand nomenclature for fatty acids and bases, the number before the colon refers to the carbon chain length and the number after the colon gives the total number of double bonds in the hydrocarbon chain. Glc/GlcNAc indicates an oligosaccharide containing either a Glc or a GlcNAc residue in the indicated position and 3/6, 6/3 indicates that the linkage can be either to the 3 or the 6 position.

The present invention is further illustrated in examples, which in no way are intended to limit the scope of the invention:

EXAMPLES

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Abbreviations used in the examples

TLC, thin-layer chromatography; C, chloroform; M, methanol; MAA, *Maackia amurensis* lectin; SNA, *Sambucus nigra* lectin; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; FAB MS, fast atom bombardment mass spectrometry; EI MS, electron ionization mass spectrometry. We use denotations **3s** to **8s** to indicate migration regions on TLC plates for three- to eight-sugar-containing monosialogangliosides. **S-3-PG**, sialyl-3-paragloboside (NeuAc $\alpha 3$ Gal $\beta 4$ GlcNAc $\beta 3$ Gal $\beta 4$ GlcCer); **S-6-PG**, sialyl-6-paragloboside (NeuAc $\alpha 6$ Gal $\beta 4$ GlcNAc $\beta 3$ Gal $\beta 4$ GlcCer). Glycolipid and carbohydrate nomenclature, see above.

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Materials and methods

Materials. Horseradish peroxidase (HRP) labeled egg-grown influenza A viruses (human X-113 reassortant vaccine strain bearing hemagglutinin and neuraminidase of A/Texas/36/91, H1N1, and avian virus A/duck/Czechoslovakia/56, H4N6) were prepared as described before (Matrosovich *et al.*, 1996). Total ganglioside fractions were obtained from the Institute of Medical Biochemistry, Göteborg University, Sweden, and prepared according to Karlsson, 1987. Some fractions were purified by phase partition (Folch *et al.*, 1957) before analysis, as indicated in legends to figures. The human virus-binding subfractions of human leukocyte gangliosides (Fig. 6) were prepared by preparative thin-layer chromatography (Miller-Podraza *et al.*, 1992) followed by further purification. After separation the fractions were suspended in C/M/H₂O, 60:30:4.5, by vol, applied to a small (0.25 ml) silica gel column packed in C/M, 2:1, by vol., and the sugar-positive fractions were eluted with C/M/H₂O, 60:35:8, by vol. Anti-sialyl-Lewis x and CDw65/clone VIM-2 monoclonal antibodies were from Seikagaku (Japan) and Dianova GmbH (Germany), respectively, and *Maackia amurensis* (MAA) and *Sambucus nigra* (SNA) lectins from Boehringer-Mannheim (Germany). Silica gel aluminum plates 60 were purchased from Merck (Germany).

Preparation of leukocytes. Mixtures of human white cells were prepared from venous blood of healthy donors. The buffy coats were lysed in 0.8% NH₄Cl (removal of erythrocytes, Fredlund *et al.*, 1988) and centrifuged at 400 x g. Fractions used contained from 70 % to 85 % of polymorphonuclear leukocytes.

Mild periodate oxidation of gangliosides (Veh et al., 1977)
Gangliosides (0.05-0.1 mM) were incubated in 1-2 mM NaIO₄ in 0.05 mM acetate buffer, pH 5.5, for 40 min on ice, after which an excess of Na₂SO₃ was added. The sample was concentrated by freeze-drying (about 5-fold) and reduced with an excess of NaBH₄ at room temperature overnight. Finally, the sample was dialyzed against distilled water and freeze dried.

TLC-overlay binding assays. The general overlay technique was previously described (Karlsson and Strömberg, 1987). Specific applications of this technique that we utilized in this study are given below.

Overlay with influenza viruses. Plates with separated glycolipids were treated with 0.3% polyisobutylmethacrylate (Aldrich Chemical Company, Inc., Milwaukee, USA) in diethyl ether: hexane, 5:1, by vol., for 1 min, dried and incubated in 2% BSA and 0.1%

Tween 20 in PBS for 2 h at room temperature. The plates were then overlaid with HRP-labeled virus suspension in 0.2% BSA, 0.01% Tween 20 in PBS and incubated as above for additional 2h. After washing four times with PBS, the plates were visualised by incubating at room temperature (in dark) in 0.02% DAB (3,3'-diaminobenzidine tetrahydrochloride, Pierce, Rockford, Illinois, USA) in PBS containing 0.03% H₂O₂.

Overlay with antibodies. Overlay with antibodies was performed as described earlier (Miller-Podraza *et al.* 1997).

Overlay with lectins on membrane blots. Detection of α 3- and α 6-linked sialic acids on membrane blots with lectins from *Maackia amurensis* (MAA) and *Sambucus nigra* (SNA), was performed as described (Johansson *et al.*, 1999).

Mass spectrometry. MALDI-TOF MS was performed on a TofSpec-E (Micromass, UK) mass spectrometer operated in a reflectron mode. The acceleration voltage was 20 kV and sampling frequency 500 MHz. The matrix was 6-aza-2-thiothymine dissolved in CH₃CN. FAB MS was performed on a SX102A mass spectrometer (JEOL) operated in a negative ion mode. The spectra were produced by Xe atoms (8 kV) using triethanolamine as matrix. EI MS of permethylated glycolipids was performed as described (Breimer *et al.*, 1980) using the same JEOL mass spectrometer.

EXAMPLE 1. Binding of human influenza virus to mixtures of human gangliosides

Fig. 1 shows binding of human and avian influenza viruses (avian viruses were used as a control experiment) to reference gangliosides (lane 9) and to mixtures of gangliosides isolated from different human tissues (lanes 1-8). The human influenza virus did not bind in the assay conditions to shorter gangliosides including abundant 5s and 7s species, but displayed a strong and selective binding to some extended glycolipids of human leukocytes (lane 1 in **Fig. 1**). There was also a weak binding to slow-moving species of other human tissues, in particular small intestine and pancreas (lanes 4 and 8). The avian virus bound to a variety of gangliosides in all lanes including two fractions of reference brain gangliosides, and displayed a preference for NeuAc α 3Gal-terminated species as compared with NeuAc α 6Gal-terminated species and species with sialic acid as internal branches. As shown in the figure, there was a binding to NeuAc α 3-paragloboside (S-3-PG) and gangliosides GD1a and GT1b (see **Table 1** for structures), but not to NeuAc α 6-paragloboside (S-6-PG) or gangliosides GM1 or GD1b. These results agree with earlier

reports on avian influenza virus binding specificities (see refs. in Introduction). The binding to GM3 was however not observed, although some lanes were overloaded with respect to less complex components. Binding to the fastest-moving band by both viruses in lane 6 of Fig. 1 was probably unspecific interaction with overloaded and charged sulfatide.

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EXAMPLE 2. Mild periodate oxidation of the gangliosides

To exclude binding to sulfated glycolipids in other lanes we used mild periodate oxidation and reduction which shortens specifically the sialic acid glycerol tail in gangliosides by one or two carbon atoms (Veh *et al.*, 1977). Binding of influenza viruses to sulfated galactosylceramide was previously reported by Suzuki *et al.*, 1996. The mild oxidation eliminated in our studies completely (human influenza) or almost completely (avian virus) binding to leukocyte glycolipids, as shown in Fig. 2. The result confirms the specific importance of the sialic acid for the virus attachment, and agrees with previous studies on binding of influenza viruses to chemically modified carbohydrates (Suttajit and Winzler, 1971; Matrosovich *et al.*, 1991).

The oxidized and reduced gangliosides were tested by FAB MS and by EI MS after permethylation. In FAB MS spectra the pseudomolecular ions $[M-H]^-$ seen clearly for 3s, 5s and 7s gangliosides were reduced by 30 or 60 (± 1) mass units, see Fig. 3. Thus, the main ions in Fig. 3A at m/z 1151.7 (GM3, d18:1-16:0), 1517.2 (SPG, d18:1-16:0) and 1882.7 (7s, d18:1-16:0) were replaced after oxidation and reduction in Fig. 3B by ions at 1091.1 and 1121.1, 1456.4 and 1486.4, and 1821.9 and 1851.8, respectively. In EI MS spectra the NeuAc fragment ions at m/z 376 and 344, were replaced after oxidation and reduction by m/z at 332 and 300, and 288 and 256 (not shown). Degradation of the core chains was not observed.

EXAMPLE 3. Analysis of the human influenza receptor glycolipids by antibodies

To test if the binding of the human virus was to the monofucosylated sialyl-Lewis x or VIM-2-active saccharides as reported earlier (Suzuki, 1994; Müthing, 1996), we used overlay of TLC plates with antibodies which react with these structures (Fig. 4). The polar solvent (A) allowed clear separation of VIM-2- and virus-positive fractions (Fig. 4A, lanes

VIM and Virus), at least within the less complex region. There was an overlapping of anti-sialyl-Lewis x and human virus bindings (**Fig. 4A and 4B**, lanes SLX and Virus), but the patterns were not identical, and there was no recognition by the virus of less complex sialyl-Lewis x-positive molecules.

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EXAMPLE 4. Characterization of the human influenza receptor glycolipids by lectins

Fig. 5 shows binding of MAA and SNA (lectins specific for α 3- and α 6-linked NeuAc, respectively) to leukocyte gangliosides in comparison with binding by human influenza virus. There was a co-migration of the more complex virus- and SNA-positive fractions, however, there was no binding of the virus to the less complex SNA-positive bands. The latter were earlier identified as 5s and 7s gangliosides with 6-linked NeuAc (Johansson and Miller-Podraza, 1998). MAA, which binds specifically to NeuAc α 3-containing carbohydrate chains of the neolacto series (Knibbs *et al.*, 1991; Johansson *et al.*, 1999), displayed a completely different pattern of binding than the virus. Binding of the human virus to minor NeuAc α 6-containing species were reproduced after ganglioside separation in a second TLC solvent (not shown). The weak binding of the virus seen in lane 3 of **Fig. 5** could represent cross reaction with brain gangliosides. However this binding was not reproducible (see **Fig. 1**).

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In an attempt to identify sequences that display efficient binding of human virus, gangliosides from human leukocytes were separated by preparative TLC and tested again for the binding (**Fig. 6**). The most complex fractions were not analysed in this way because of inadequate amounts and poor separation. Two fractions were shown to contain active components, see Fr. 5 and Fr. 6 in the figure. Tests in different chromatographic systems (TLC not shown) revealed however that the main components in these subfractions were inactive and that binding was to some minor overlapping fractions. This agrees with the fact that the binding was to slowly migrating NeuAc α 6-containing species (**Fig. 5**), which have earlier been detected by SNA lectin (Johansson and Miller-Podraza, 1998). They occur in human leukocytes in very small amounts and so far only NeuAc α 3-containing structures were detected by chemical methods among more complex gangliosides isolated from this source (Müthing *et al.*, 1996; Stroud *et al.*, 1995; Stroud *et al.*, 1996). The detection level on TLC plates by the human virus was 40-80 pmol in relation to the total

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ganglioside mixture, and apparently much lower for the active species, indicating that the binding was highly efficient (see binding to trace fractions in lane 6 of Fig. 6)

5 EXAMPLE 5. Mass spectrometry of glycolipid fractions containing receptors for human influenza viruses

The most active isolated fraction (**Fr. 6 of Fig. 6**) was shown by MALDI-TOF mass spectrometry to contain complex gangliosides with 8-11 monosaccharides per mol of ceramide (**Fig. 7 and Table 2**). Fragmentation pattern seen in FAB MS analysis confirmed
10 the presence of oligosaccharide chains with repeated HexHexNAc units (spectra not shown). The most abundant molecular ions corresponded to NeuAc₁Fuc₁Hex₅HexNAc₃Cer at *m/z* 2394.0 and NeuAc₁Hex₅HexNAc₃Cer at 2248.7. As mentioned, the main components were excluded as binding molecules by TLC in different solvent systems. The most probable candidates of the active species were
15 therefore minor disialylated or difucosylated gangliosides with 2 or 3 lactosamine units. Monofucosylated gangliosides NeuAc₁Fuc₁Hex₄HexNAc₂Cer, (8s), (*m/z* at 2030.4 in **Fig. 7**) with two N-acetyllactosamine units are less likely as binding molecules, as judged from chromatographic mobility and binding tests, see **Figs. 1, 4B and 6**, (8s region). In Fr. 5 of **Fig. 6** the main (non-active) component was NeuAc₁Hex₅HexNAc₃Cer.

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EXAMPLE 6. Analysis of data from mass spectrometry and TLC-experiments

The direct characterization of the exact binding structures was not possible because of the low amount of the material and presence of several active species. However, we have
25 excluded sialyl-Lewis x and VIM-2-active saccharides (**Table 1, Fig. 4**) as the binding sequences. We have also shown using SNA lectin that the binding was to some minor NeuAc_α6-containing species (**Fig. 5**). In addition, we have demonstrated, that the binding was dependent on the presence of the unchanged glycerol sialic acid tail (**Figs. 2 and 3**). Our results therefore do not support earlier suggestions on a preferential binding of human
30 influenza A viruses to sialyl-Lewis x (Suzuki, 1994) or VIM-2-active structures (Suzuki, 1994; Mütting, 1996). This discrepancy may be caused by differences in virus strains. The cited authors used influenza viruses A/PR/8/34 (H1N1) and the reassortant virus strain X-31, which bear HA and NA genes of A/Aichi/2/28 (H3N2), while we studied human

influenza virus X-113 (HA and NA genes of A/Texas/36/91, H1N1). PR/8/34 and X-31 are known to have a higher affinity to NeuAc α 3-containing receptors than more recently circulated influenza A viruses (Rogers and D'Souza, 1989; Matrosovich *et al.*, 1997), and this may explain strong interaction of PR/8/34 and X-31 with sialyl-Lewis x and VIM-2 structures and no binding to these structures in our tests. There was an overlapping binding by anti-sialyl-Lewis x antibody and the human virus in our studies (**Fig. 4**). However, the overall patterns were not identical and there was no interaction of the virus with less complex sialyl-Lewis x gangliosides reported to be present in human leukocytes (Müthing *et al.*, 1996). We have detected by FAB MS in fractions 1 through 3 (**Fig. 6**) increasing amounts of 8s gangliosides with a potential sialyl-Lewis x composition of Fuc₁NeuAc₁Hex₄HexNAc₂Cer with various ceramides (*m/z* at 2028.7 and 2138.4, not shown). These gangliosides migrated in the 7s region overlapping with more abundant non-fucosylated NeuAc₁Hex₄HexNAc₂Cer fractions. Of importance is a binding in this region of anti-sialyl-Lewis x antibody, but not of either VIM-2 antibody or virus (**Fig. 4**).

Structural microheterogeneity associated with ceramide parts and NeuAc α 3/ α 6 substitutions may explain the overlapping migration of different gangliosides and the complex multi-band patterns in lanes SLX of **Fig. 4**. The cross-binding to other fucosylated structures (Stroud *et al.*, 1995) of the lower TLC regions, may also contribute to these complex patterns.

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We have excluded that the main components of fractions 5 and 6 in **Fig. 6** are binding molecules by TLC analysis in different solvent systems and overlay tests. **Table 2** lists (in bold) candidates of active species which we could detect by MALDI-TOF MS. Of importance for the binding could be oligosialylation and/or repeated fucose branches, as judged from the presence of disialylated molecules and/or difucosylated molecules in the mixture (difference between masses of 2Fuc and 1NeuAc is only 1.03 amu). Like the oligosialylated species the monosialylated glycolipid may also be branched by β 6-linked lactosamine unit, molecular mass of such minor species would overlap the major non-active glycolipid with [M-H]⁻ molecular mass at 2248.5. Also length of the sugar should be considered as an important factor, since only complex gangliosides were binding. The extended carbohydrate chains may serve as spacers which reduce steric hindrance to recognition by viral hemagglutinins. It has been shown, that glycosylation of viral HAs in the vicinity of the receptor binding sites may decrease the virus binding to target cells and

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immobilized receptors (Matrosovich *et al.*, 1997; Gambaryan *et al.*, 1998; Ohuchi *et al.*, 1997). The impaired accessibility of the receptor binding pocket could explain why we did not see binding of the human influenza to less complex gangliosides like GM3 or SPG. In fact, the hemagglutinin of X-113 reassortant human virus, although not yet sequenced, is likely to contain glycans at Asn₁₂₉ and Asn₁₆₃ close to the tip of the HA globular head, similar to the HAs of other contemporary H1N1 human viruses, for which sequences are available. In contrast, the avian virus strain A/duck/Czechoslovakia/56 (H4N6) lacks carbohydrates in this portion of the HA (Matrosovich *et al.*, 1999). Also Mùthing (Mùthing, 1996) emphasized a stronger binding to longer fucosylated species (sialyl-Lewis x- and VIM-2-active species) compared to 5s and 7s gangliosides using X-31 (H3N2) influenza A strain.

In our studies there was however no binding at all to 5s NeuAc α 6-containing SPG nor to its 7s homologue, although the interaction of the human virus with some selected complex species was very strong (for characterization of 5s and 7s gangliosides of human leukocytes see Johansson and Miller-Podraza, 1998). Technical assay reasons for this unusual binding are unlikely, since the avian virus bound to S-3-PG and to other well defined common gangliosides under the same experimental conditions (**Fig. 1**). It is reasonable to assume, that both length and structural features of the receptor chain contributed to this result. Gangliosides with branched N-acetyl-lactosamine chains and NeuAc on more than one arm should be considered as highly efficient binding molecules. Polyvalency has earlier been shown as an important factor enhancing binding affinity of influenza virus to synthetic sialylated compounds (Mammen *et al.*, 1995), and branched polyglycosylceramides were very effective receptors for human influenza A and B viruses (Matrosovich *et al.*, 1996). The calculated molecular masses of the disialylated species having similar ceramide structures as previous reported from the human leukocyte gangliosides (Stroud *et al.*, 1996b; Mùthing, 1996) matched closely to the experimental data in Table 2. Branched α 3monosialylated polylactosamine ganglioside has been described from human gangliosides (Stroud *et al.*, 1996b). Human leukocytes are known to contain neolacto glycolipids with repeated fucose residues as Fuc α 3GlcNAc units (Stroud *et al.*, 1995; Stroud *et al.*, 1996; Mùthing, 1996), corresponding molecular weights are also seen in our analyses, see Table 2. Fucose may possibly interact with the hydrophobic methyl group with spots outside the NeuAc binding site of the viral HA. In fact, synthetic

NeuAc analogues with hydrophobic neighbouring groups have been shown to interact with hydrophobic patches adjoining the receptor binding site of influenza virus A hemagglutinin, considerably improving affinity (Watowich *et al.*, 1994).

Table 1. Carbohydrate and glycolipid structures discussed in this paper

NeuAc α 3Gal β 4Glc β Cer

GM3, 3s ganglioside

Gal β 3GalNAc β 4(NeuAc α 3)Gal β 4Glc β Cer

GM1

NeuAc α 3Gal β 3GalNAc β 4(NeuAc α 3)Gal β 4Glc β Cer

GD1a

Gal β 3GalNAc β 4(NeuAc α 8NeuAc α 3)Gal β 4Glc β Cer

GD1b

NeuAc α 3Gal β 3GalNAc β 4(NeuAc α 8NeuAc α 3)Gal β 4Glc β Cer

GT1b

NeuAc α 3Gal β 4GlcNAc β 3Gal β 4Glc β Cer

S-3-PG, NeuAc-3-paragloboside, sialyl-3-paragloboside,
5s ganglioside

NeuAc α 6Gal β 4GlcNAc β 3Gal β 4Glc β Cer

S-6-PG, NeuAc-6-paragloboside, sialyl-6-paragloboside,
5s ganglioside

NeuAc α 3Gal β 4GlcNAc β 3Gal β 4GlcNAc β 3Gal β 4Glc β Cer

7-sugar NeuAc-3-neolacto ganglioside, 7s ganglioside

NeuAc α 6Gal β 4GlcNAc β 3Gal β 4GlcNAc β 3Gal β 4Glc β Cer

7-sugar NeuAc-6-neolacto ganglioside, 7s ganglioside

NeuAc α 3Gal β 4(Fuc α 3)GlcNAc β -

Sialyl-Lewis x epitope

NeuAc α 3Gal β 4GlcNAc β 3Gal β 4(Fuc α 3)GlcNAc β -

"VIM-2" epitope (epitope reacting with CDw65/clone VIM-2
monoclonal antibody)

Table 2. Ganglioside composition of fraction 6 (Fr. 6 in Fig. 6) based on MALDI-TOF-MS mass spectrometry. The spectrum is shown in Fig. 7. The most probable binding components (as judged from combined results) are in bold.

M-H Observed	M-H Calculated	ΔM	Most probable compositions (listed according to decreasing abundance)
2394.0	2394.6	-0.6	NeuAc ₁ Fuc ₁ Hex ₅ HexNAc ₃ Cer (d18:1-16:0)
2248.7	2248.5	+0.2	NeuAc ₁ Hex ₅ HexNAc ₃ Cer (d18:1-16:0)
2504.4	2504.8	-0.4	NeuAc ₁ Fuc ₁ Hex ₅ HexNAc ₃ Cer (d18:1-24:1)
2173.7	2174.4	-0.7	NeuAc₂Hex₄HexNAc₂Cer (d18:1, 16:0) and/or
	2173.4	+0.3	NeuAc₁Fuc₂Hex₄HexNAc₂Cer (d18:1-16:1)
2649.3	2649.9	-0.6	NeuAc₂Hex₅HexNAc₃Cer (d18:1-24:1) and/or
	2651.0	-1.7	NeuAc₁Fuc₂Hex₅HexNAc₃Cer (d18:1-24:1) and/or
	2649.0	+0.3	NeuAc₁Fuc₂Hex₅HexNAc₃Cer (d18:1-24:2)
2030.4	2029.3	+1.1	NeuAc ₁ Fuc ₁ Hex ₄ HexNAc ₂ Cer (d18:1-16:0)

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CLAIMS

1. A substance comprising at least one oligosaccharide chain, which comprises a terminal NeuNAc α 6 linked to
- 5 (a) a linear or branched polylactosamine type structure consisting of at least three lactosamine residues, a linear sequence optionally containing one or two α 3-linked fucose residues in a non-sialylated lactosamine, a branched structure optionally carrying one or more additional NeuNAc α -residues at a terminal position in a branch, and/or
- (b) a linear or branched structure with two lactosamine and one lactose residue, a
- 10 linear structure in addition containing one or two α 3 -linked fucose residues in a non-sialylated lactosamine or lactose, a branched structure optionally carrying one additional NeuNAc α -residue in a terminal position of the branch,
- or an analog or derivative of said oligosaccharide chain for use in binding of
- human influenza virus.
- 15
2. The substance according to claim 1, containing two NeuNAc α 6 residues in a branched structure.
3. The substance according to claim 1, containing one fucose residue in a linear structure
- 20 (a), or two fucose residues in a linear structure (b).
4. The substance according to any one of claims 1 to 3, wherein the structure (a) contains three lactosamine residues.
- 25 5. The substance according to claim 1, which contains an oligosaccharide structure
- NeuNAc α 6Gal β 4GlcNAc β 3(NeuNAc α 3/6Gal β 4GlcNAc β 6)Gal β 4GlcNAc/Glc β 1-,
- NeuNAc α 6Gal β 4GlcNAc β 3(NeuNAc α 6Gal β 4GlcNAc β 6)Gal β 4GlcNAc/Glc β 1-,
- NeuNAc α 6Gal β 4GlcNAc β 3(Gal β 4GlcNAc β 6)Gal β 4GlcNAc/Glc β 1- ,
- 30 NeuNAc α 6Gal β 4GlcNAc β 3Gal β 4(Fuc α 3)₀₋₁GlcNAc β 3Gal β 4(Fuc α 3)₀₋₁GlcNAc β 1-, or
- NeuNAc α 6Gal β 4GlcNAc β 3Gal β 4(Fuc α 3)GlcNAc β 3Gal β 4(Fuc α 3)GlcNAc β 1.

6. The substance according to claim 1, which contains the structure
 $R6/3Gal\beta4GlcNAc\beta6/3(NeuNAc\alpha6Gal\beta4GlcNAc\beta3/6)Gal\beta4GlcNAc\beta4R2$,
where R is the glycosidically linked non-reducing end part of the polylactosamine chain,
and R₂ is the glycosidically linked reducing end part of the polylactosamine chain,
5 preferably R and/or R₂ containing further virus binding oligosaccharides, such as those
defined in the claim 1.
7. The substance according to any of claims 1-5, wherein the oligosaccharide is a part of a
glycolipid.
- 10 8. The substance according to claim 7, wherein the oligosaccharide (a) is linked over a
lactose residue to the lipid part of the glycolipid.
9. The structure according to claim 7 or 8, wherein the lipid part of the glycolipid is
15 ceramide.
10. A substance comprising at least one oligosaccharide chain, which comprises a terminal
NeuNAc α 6 linked to
- (a) -a linear or branched polylactosamine type structure consisting of at least three
20 lactosamine residues, a linear sequence optionally containing one or two α 3-linked fucose
residues in a non-sialylated lactosamine, a branched structure optionally carrying one or
more additional NeuNAc α -residues at a terminal position in a branch, and/or
- (b) -a linear or branched structure with two lactosamine and one lactose residue, a
linear structure in addition containing one or two α 3 -linked fucose residues in a non-
25 sialylated lactosamine or lactose, a branched structure optionally carrying one additional
NeuNAc α -residue in a terminal position of the branch,
- or an analog or derivative of said oligosaccharide chains, when said
oligosaccharide chain is a part of a polylactosamine chain or a conjugate thereof.

11. A substance comprising at least two oligosaccharide chains, which comprise a terminal NeuNAc α 6 linked to

5 (a) -a linear or branched polylactosamine type structure consisting of at least three lactosamine residues, a linear sequence optionally containing one or two α 3-linked fucose residues in a non-sialylated lactosamine, a branched structure optionally carrying one or more additional NeuNAc α -residues at a terminal position in a branch, and/or

10 (b) -a linear or branched structure with two lactosamine and one lactose residue, a linear structure in addition containing one or two α 3 -linked fucose residues in a non-sialylated lactosamine or lactose, a branched structure optionally carrying one additional NeuNAc α -residue in a terminal position of the branch, or an analog or derivative of said oligosaccharide chains.

12. A substance comprising at least one oligosaccharide chain, which comprises a terminal NeuNAc α 6 linked to

15 (a) -a linear or branched polylactosamine type structure consisting of at least three lactosamine residues, a linear sequence optionally containing one or two α 3-linked fucose residues in a non-sialylated lactosamine, a branched structure optionally carrying one or more additional NeuNAc α -residues at a terminal position in a branch, and/or

20 (b) -a linear or branched structure with two lactosamine and one lactose residue, a linear structure in addition containing one or two α 3 -linked fucose residues in a non-sialylated lactosamine or lactose, a branched structure optionally carrying one additional NeuNAc α -residue in a terminal position of the branch, or an analog or derivative of said oligosaccharide chains, in the form of a micelle or conjugated to a polyvalent carrier.

25

13. A composition comprising a substance according to any of the claims 1-12 for pharmaceutical use.

30 14. The composition according to claim 13, for the treatment of any condition due to the presence of human influenza virus.

15. The composition according to claim 13 or claim 14, for the treatment of virus-mediated neutrophil dysfunction and prevention of secondary bacterial infections due to the neutrophil dysfunction.
- 5 16. The composition according to claim 13 or claim 14, for the treatment of the primary infection by the influenza viruses in the respiratory tract.
17. Use of a substance according to any of claims 1 – 12, for the production of a pharmaceutical composition for treatment of any condition due to the presence of human
10 influenza virus.
18. A method for the treatment of a condition due to the presence of human influenza virus, wherein a pharmaceutically effective amount of a substance according to any of claims 1 – 12 is administered to the patient.
- 15 19. The method according to claim 18, for the treatment of any of the conditions indicated in claims 14, 15 or 16.
20. Use of a substance specifically binding or inactivating the substance according to any
20 of the claims 1-10 on human tissues, for the production of a pharmaceutical composition for the treatment of any of the conditions indicated in claims 14, 15 or 16, such substance being preferably lectin, an antibody or an enzyme.
21. Use of a substance specifically binding or inactivating the substance according to any
25 of claims 1 – 10 on human tissues, for the treatment of any of the conditions indicated in claims 14, 15 or 16.
22. The pharmaceutical composition according to any one of claims 13 to 16 containing one or more substances effective against influenza viruses, preferably a neuraminidase
30 inhibitor.
23. The method according to claim 18 or 19, wherein in addition a pharmaceutically effective amount of one or substances effective against influenza viruses, preferably a neuraminidase inhibitor, is administered.

24. Use of a substance according to any of the claims 1-12, for the diagnosis of a condition due to infection by human influenza virus, or for typing human influenza viruses.
25. An assay for determining the presence of human influenza virus in a sample using
5 substances according to any of claims 1 – 12.
26. An assay for determining the activity of a inhibitory substance for human influenza virus utilizing substances according to any one of claims 1 – 12.

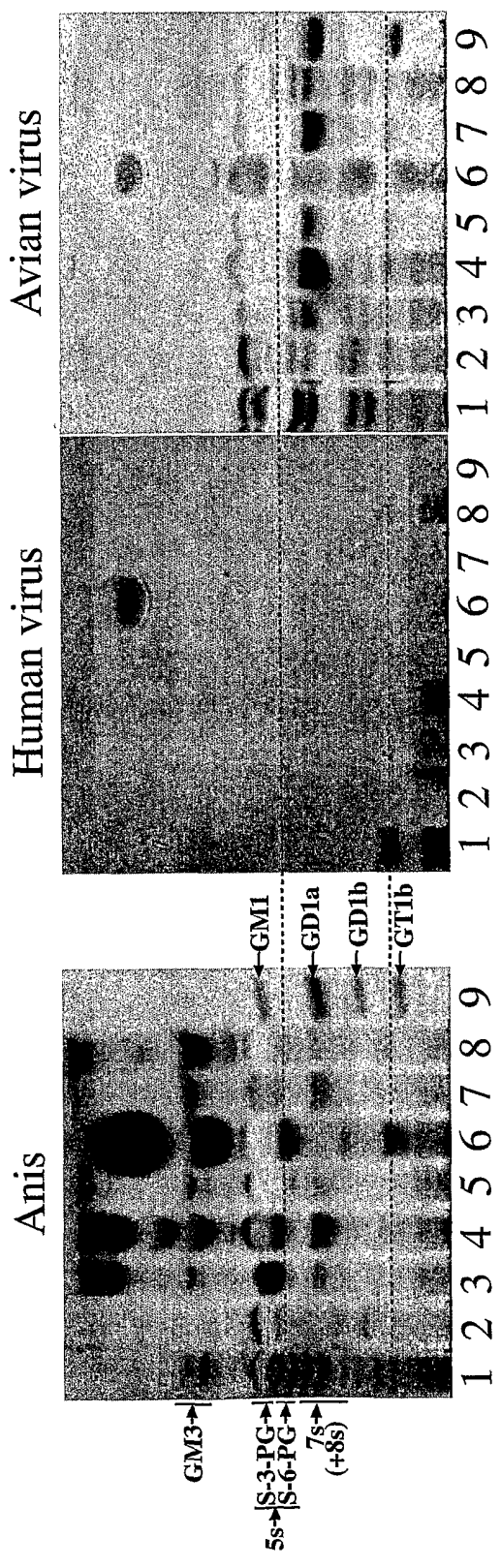


Fig. 1

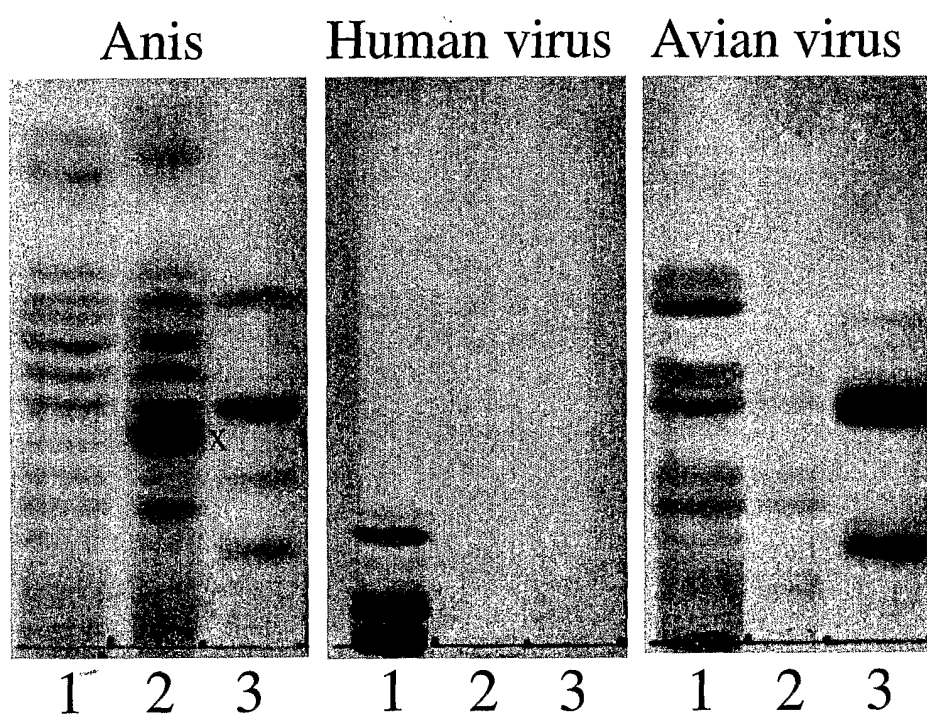


Fig. 2

Fig. 3A

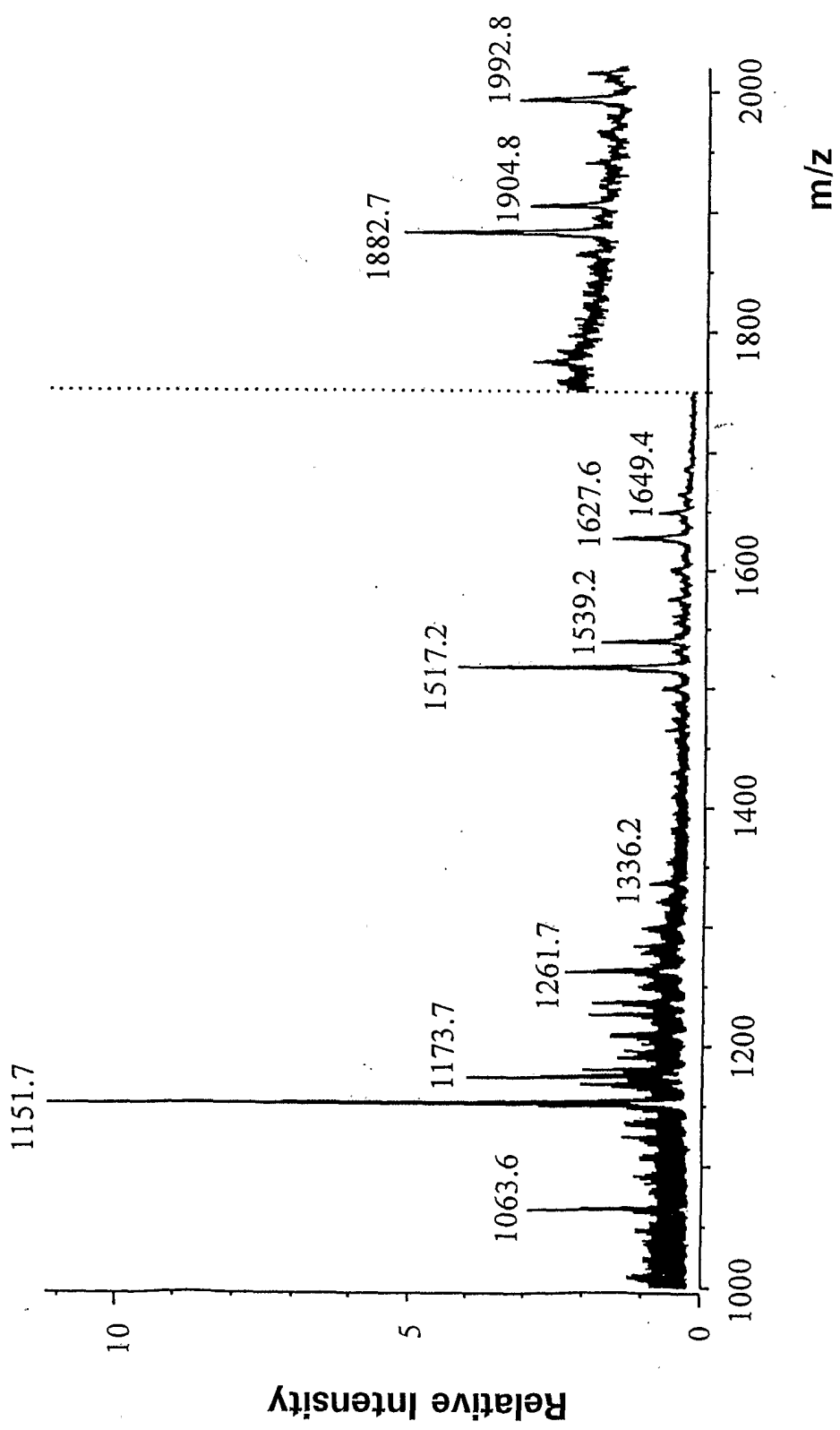


Fig. 3B

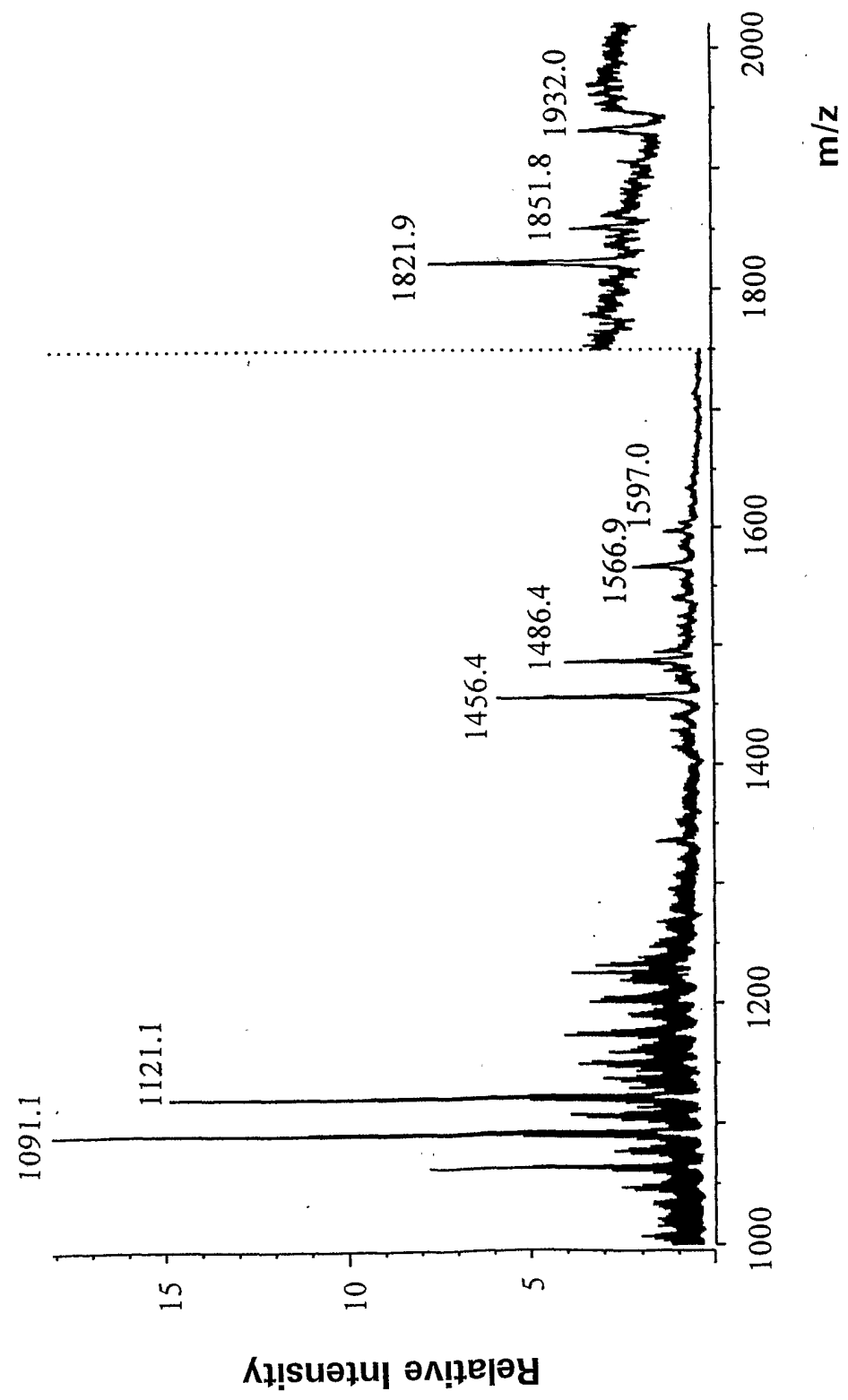


Fig. 4A

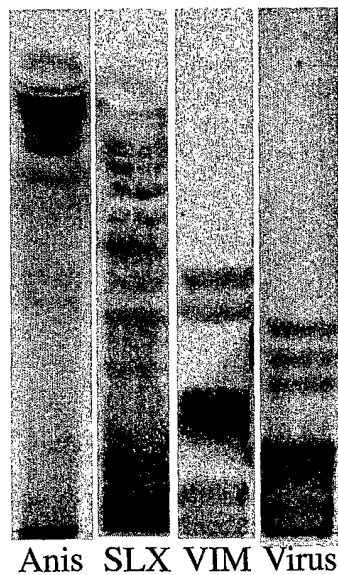
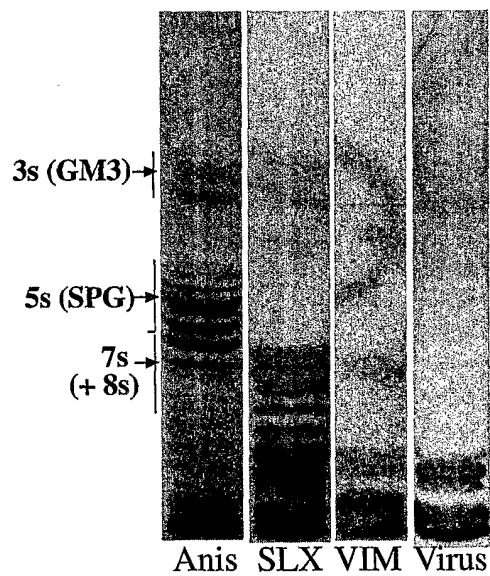


Fig. 4B



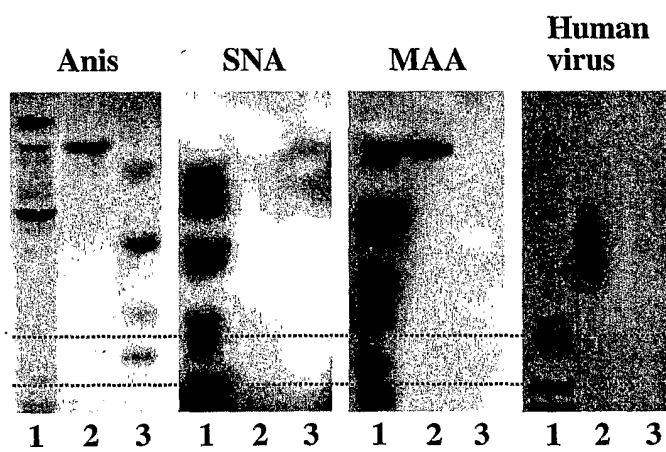


Fig. 5

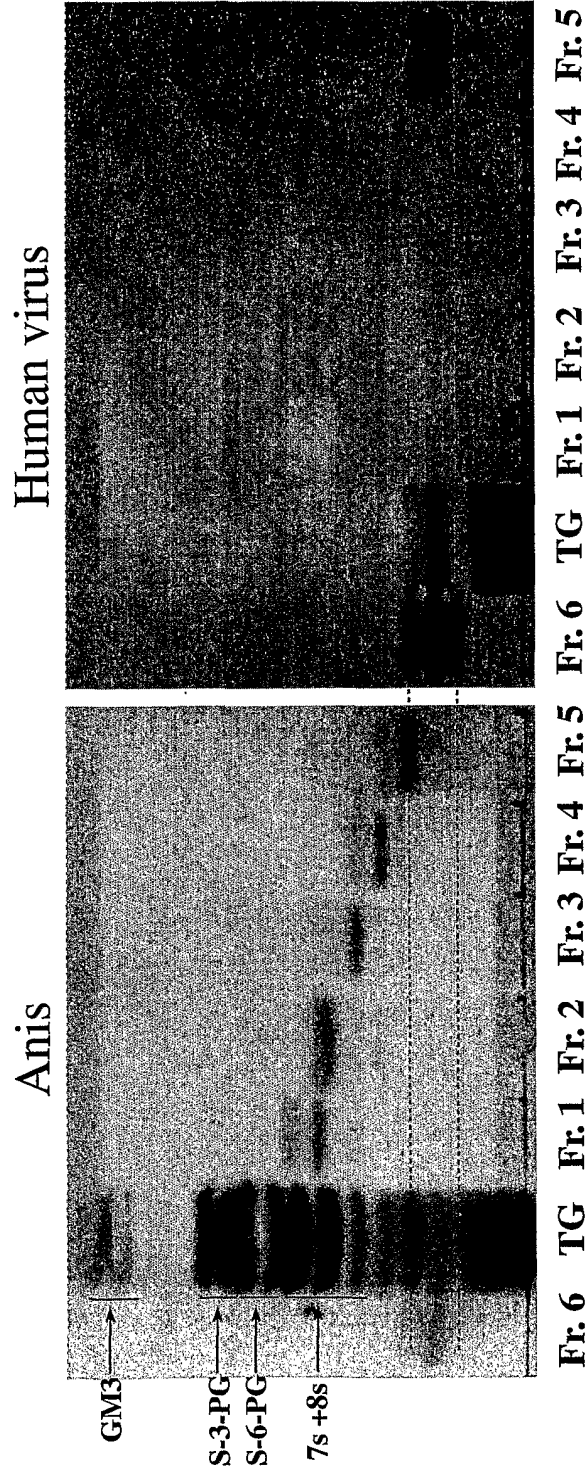
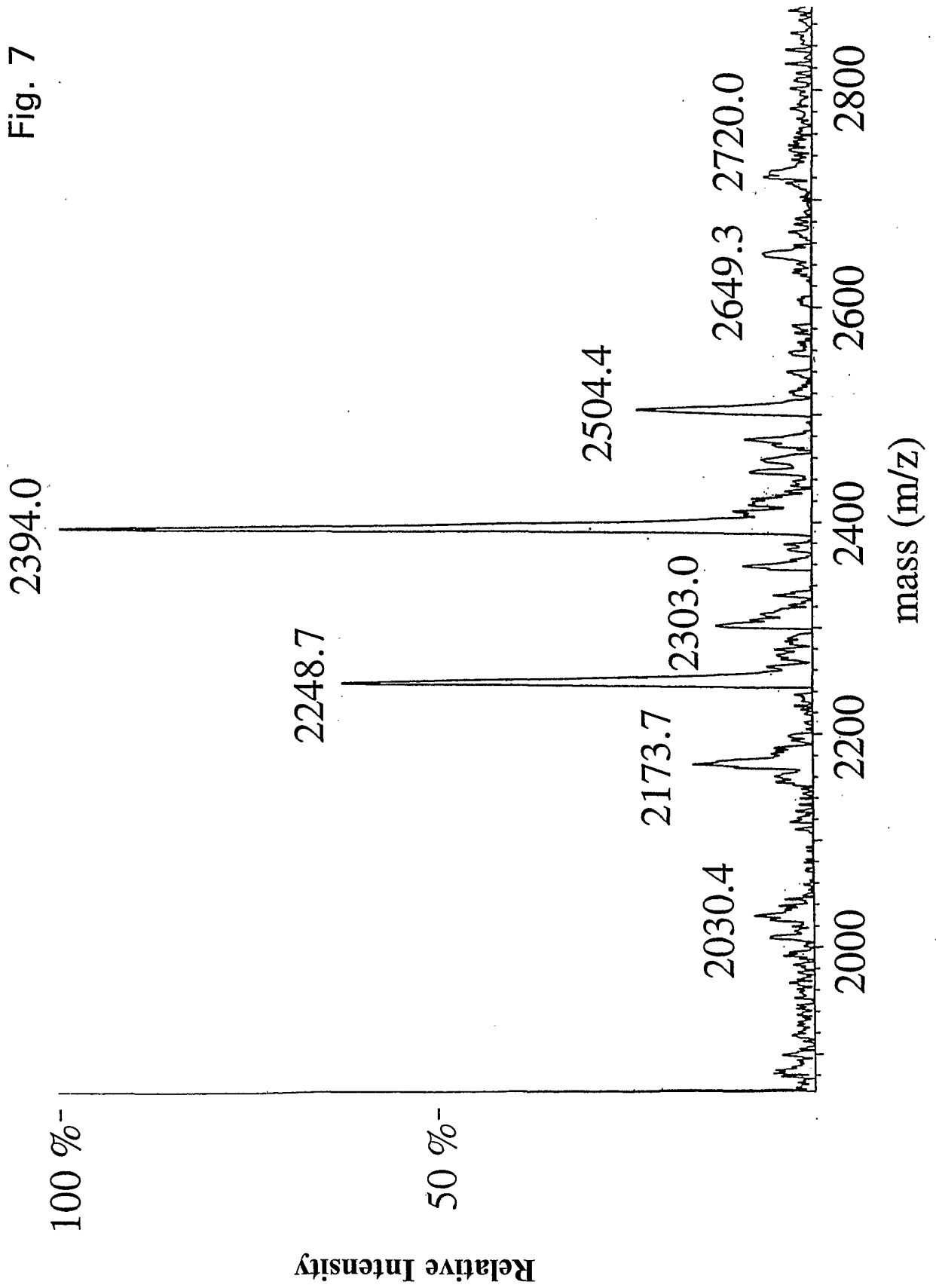


Fig. 6

Fig. 7



INTERNATIONAL SEARCH REPORT

International application No.

PCT/FI 01/00587

A. CLASSIFICATION OF SUBJECT MATTER

IPC7: A61K 31/715, A61K 31/739, C07H 5/06, C07H 3/06, A61P 31/12
 According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC7: A61K, C07H, A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CHEM ABS. DATA, EMBASE, EPO-INTERNAL, WPI DATA, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5254676 A (SUBRAMANIAM SABESAN), 19 October 1993 (19.10.93), column 8-9, heptasaccharides 27-31 --	1-26
X	US 5220008 A (SUBRAMANIAM SABESAN), 15 June 1993 (15.06.93), column 8-9, heptasaccharides --	1-26
A	Prog. Lipid Res., Volume 33, No. 4, 1994, Yasuo Suzuki, "Gangliosides as influenza virus receptors. Variation of influenza viruses and their recognition of the receptor sialo-sugar chains", page 429 - page 457 --	1-26

Further documents are listed in the continuation of Box C. See patent family annex.

* Special categories of cited documents:	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be of particular relevance	"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E" earlier application or patent but published on or after the international filing date	"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&" document member of the same patent family
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

22 November 2001

Date of mailing of the international search report

23 -11- 2001

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/FI 01/00587

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>Scientific American, Volume 280, No. 1, January 1999, W. Graeme Laver et al, "Disarming Flu Viruses. Coming soon: new medicines designed to treat the flu by halting viral replication in human tissues. The drugs may also serve as a novel kind of preventive", page 79 - page 87</p> <p style="text-align: center;">-- -----</p>	1-26

INTERNATIONAL SEARCH REPORT

Information on patent family members

06/11/01

International application No.

PCT/FI 01/00587

Patent document cited in search report			Publication date	Patent family member(s)			Publication date
US	5254676	A	19/10/93	US	5220008	A	15/06/93
US	5220008	A	15/06/93	US	5254676	A	19/10/93

INTERNATIONAL SEARCH REPORT

International application No.
PCT/FI01/00587

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. Claims Nos.: **18-19, 21, 23-24**
because they relate to subject matter not required to be searched by this Authority, namely:
see next sheet

2. Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- The additional search fees were accompanied by the applicant's protest.
 No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/FI01/00587

Claims 18-19, 21, 23-24 relate to methods of treatment of the human or animal body by surgery or by therapy/ diagnostic methods practised on the human or animal body/Rule 39.1.(iv). Nevertheless, a search has been executed for these claims. The search has been based on the alleged effects of the compounds/compositions.