Abstract: It is provided a multivalent ganglioside carbohydrate as a therapeutic cancer vaccine. The GD2 and GD3 carbohydrate conjugates disclosed are linked by a spacer to form a multimer which conserves the native structural feature of naturally occurring GD2 or GD3, the tetramer being immunogenic and elicits cytotoxic anti-gangliosides humoral and cellular responses in vivo.

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ANTI-GANGLIOSIDE COMPOUND FOR TARGETING CANCER AND GENERATING ANTIBODIES

TECHNICAL FIELD

[0001] The present description relates to ganglioside glycoconjugates and use thereof as anti-tumor vaccines.

BACKGROUND ART

[0002] Gangliosides are neuraminic acid-containing glycosphingolipids that accumulate in the outer leaflet of plasma membranes. Gangliosides such as GD2 and GD3 are prevalent tumor markers. They are expressed in neuroblastoma, melanoma, small cell lung cancer and gliomas (Hakomori, 1996, Cancer Research, 56: 5309) as well as breast cancer stem cells (Battula et al., 2012, The Journal of clinical investigations, 122: 2066), but they are absent in normal cells. Hence, GD2 and GD3 have been exploited as tumor targets, and they are validated clinical targets. Partial therapy can be achieved by passively administering purified anti-GD2 (Cheun et al., 1987, J Clin Oncol, 5: 1430) or anti-GD3 monoclonal antibodies (mAbs) (Houghton et al., 1985, Proc Natl Acad Sci USA, 82: 1242). However, passive immunity has high financial cost, significant side effects, limited frequency of intervention, and low therapeutic efficacy (Navid et al., 2010, Current Cancer Drug Targets, 10: 200).

[0003] As an alternative, many groups have pursued active immunotherapy gangliosides (Astronomo and Burton, 2010, Nat Rev Drug Discov, 9: 308). However, serious difficulties associated with gangliosides include poor immunogenicity, poor solubility and poor formulations, limited access and difficulty to prepare well-characterized and homogeneous immunogens, and the potential of poor selectivity with the risk of cross-reactivity to non-tumor gangliosides that are highly related in structure.

[0004] For example, a GD2 lactone chemically conjugated to keyhole limpet hemocyanin (KLH) is immunogenic, and can induce antibodies that delay tumor growth in mice (Chapman et al., 2000, Clinical Cancer Research, 6: 4658). The antibodies induced by this vaccine act through a complement-dependent cytotoxicity (CDC) mechanism (Kim et al., 2011, Cancer Immunology, Immunotherapy, 60: 621). However, the KLH-ganglioside conjugation yields chemically heterogeneous products (Danieshefsky and Allen, 2000, Angew Chem Int Ed, 39: 836), which is a serious drawback. Other ganglioside conjugates have shown poor immunogenicity and
generally elicited a low and transient anti-ganglioside antibody response (Ragupathi et al., 2000, International Journal of Cancer, 85: 659). Even the most immunogenic ganglioside, a GM2-KLH vaccine did not provide clinical benefits (Kirkwood et al., 2001, Journal of Clinical Oncology, 19: 2370) and was discontinued. Additional experimental approaches include GD2-peptide mimotopes (Wondimu et al., 2008, Cancer Immunology, Immunotherapy, 57: 1079), GD2-mimicking peptides (Bolesta et al., 2005, Cancer Research, 65: 3410), and GD2 mimotope DNA vaccines (Zeytin et al., 2000, Cancer Gene Therapy, 7: 1426) that can induce cross-reactive immunity to GD2. However, immune responses were not very effective at protecting the host in tumor-therapy paradigms (Bleeke et al., 2009, European Journal of Cancer, 45: 2915).

[0005] There is thus still a need to be provided with a new therapeutic approach using gangliosides as targets.

SUMMARY

[0006] In accordance with one aspect, there is provided a ganglioside carbohydrate of the formula: G- Aryl-NH2

wherein G is an oligosaccharide comprising one or more sialic acids (e.g. n-acetylneuraminic acid), wherein said G is covalently bonded to the aryl by the C1 anomic oxygen atom, and G is immunogenic, and Aryl is a C6 to C10 aryl, optionally substituted.

[0007] The ganglioside carbohydrate provided herein is immunogenic against tumors. More particularly, it is provided a ganglioside carbohydrate immunogenic against tumors for preventing or treating cancer.

[0008] In accordance with the present description there also provided a ganglioside carbohydrate multimer comprising at least one or at least two carbohydrate ganglioside analogues or ganglioside carbohydrate as described herein covalently bonded to a multimeric core molecule

[0009] There is provided an antibody specifically binding to the ganglioside carbohydrate or the ganglioside carbohydrate multimer as defined herein.

[0010] There is also provided a vaccine comprising the ganglioside carbohydrate or the ganglioside carbohydrate multimer as defined herein.
There is also provided the use of the ganglioside carbohydrate or the ganglioside carbohydrate multimer as defined herein for preventing or treating cancer.

There is also provided the use of the ganglioside carbohydrate or the ganglioside carbohydrate multimer as defined herein in the manufacture of a medicament for preventing or treating cancer.

There is also provided a method of preventing or treating cancer in a patient in need thereof comprising the step of administering to said patient an effective amount of the ganglioside carbohydrate or the ganglioside carbohydrate multimer as defined herein.

There is also provided a method of eliciting an immunogenic response in a patient in need thereof comprising the step of administering to said patient an effective amount of the ganglioside carbohydrate or the ganglioside carbohydrate multimer as defined herein.

There is also provided a pharmaceutical composition comprising an effective amount of the ganglioside carbohydrate multimer as defined herein and a pharmaceutically suitable carrier or excipient.

**BRIEF DESCRIPTION OF THE DRAWINGS**

Reference will now be made to the accompanying drawings, in which:

Fig. 1A illustrates the structure of gangliosides GM1, GD3 and GD2; Fig. 1B illustrates a scheme for the synthesis of GD2 and GD3 analogs as disclosed herein; Fig. 1C illustrates a scheme for the synthesis of an antigen as disclosed herein;

Fig. 2A illustrates a mass spectrum of amino phenyl ether-analog of GD2 (AP-GD2); Fig. 2B illustrates a H-NMR spectrum of amino phenyl ether-analog of GD2 (AP-GD2) in deuterated water; Fig. 2C illustrates a H-NMR spectrum of tetra-GD2 dendrimer in deuterated water;

Fig. 3A illustrates a representative FACScan data showing the presence of anti-GD2-reactive antibodies of the IgG class; Fig. 3B illustrates serial dilutions of test or control sera studied using anti-mouse IgG secondary reagents; Fig. 3C illustrates isotyping of the sera showing the increase of IgG and IgM isotypes after each round of immunization; Fig. 3D illustrates EL4-GD2^+ cells growing exponentially in complete
media cultured with the indicated antibodies and their survival/metabolism quantified by MTT after 24 hours;

[0020] Fig. 4A illustrates T cell proliferation evaluated by $^3$H-thymidine incorporation assays, wherein tumor cells were cultured at a 1:10 ratio with T cells purified from vaccinated or from naive mice; Fig. 4B illustrates GD2-dependent T cell proliferation measured by Trypan Blue exclusion;

[0021] Fig. 5A illustrates measured tumor volume of mice vaccinated intraperitoneally; Fig. 5B illustrates average tumor volumes measured for immunized versus control mice; Fig. 5C illustrates tumor volume measured in mice vaccinated intraperitoneally with tetra-GD2; Fig. 5D illustrates average tumor volumes for immunized versus control mice; Fig. 5E illustrates adoptive transfer therapy effect; Fig. 5F illustrates the quantification of metastasis to the lymph nodes;

[0022] Fig. 6 illustrates purified T cells from 2 groups of tumor-bearing mice naive (control or vaccinated), and CD4/CD8 profiles of the cells quantified by FACScan;

[0023] Fig. 7 is a representative ELISA data for sera showing anti-GD3-reactive antibodies of the IgG sub-class;

[0024] Fig. 8 and 9 represent the results of vaccination with tetra-GD3; and

[0025] Fig. 10 illustrates the count of metastatic nodules after vaccination using the GD3 vaccine.

DETAILED DESCRIPTION

[0026] The present disclosure relates to a ganglioside carbohydrate consisting of

![Chemical Structure Image]
The present disclosure specifically provides a ganglioside carbohydrate consisting of

or

As used herein, G refers to part of the respective ganglioside (such as GD2, GD3, GM2 and GT1b) comprising only the oligosaccharide and sialic acids (e.g. n-acetyleneuraminic acid, NANA) corresponding to the respective ganglioside. Stated differently, G refers to the oligosaccharide and sialic acids of the corresponding ganglioside, excluding the ceramide portion of the ganglioside which is herein replaced by an amino-aryl residue (such as an amino-phenyl). Examples of G as used herein include the residues of GD3 and GD2 such as
The term "aryl", as used herein, is understood as referring to 6 to 10 membered aromatic groups, for example phenyl or naphthyl, preferably a phenyl. The aromatic ring can be substituted at one or more ring positions, preferably at no substituent is present, and the amino group is preferably in a para position of a phenyl relative to the sugar moiety. Preferably, the "-aryl-NH₂" is therefore a 4-aminophenyl.

The term "optionally substituted" with regard to the aryl means optionally substituted with one or more of an alkyl, aryl, or halogen, at any available position or positions. Preferably, there is no substituent.

The present disclosure provides a ganglioside carbohydrate multimer comprising at least one or at least two carbohydrate ganglioside analogues covalently bonded to a multimeric core molecule.

In one embodiment, the carbohydrate ganglioside analogue is an analogue of at least one of GD2, GD3, GM2 and GT1 b.

In one embodiment, the carbohydrate ganglioside analogue or the ganglioside carbohydrate multimer comprises at least one GD2 carbohydrate ganglioside analogue, or at least one GD3 carbohydrate ganglioside analogue.
In one embodiment, the carbohydrate ganglioside analogue or the ganglioside carbohydrate multimer is a tetramer of the carbohydrate ganglioside analogues. In one embodiment, the carbohydrate ganglioside analogue or the ganglioside carbohydrate multimer is useful for preventing or treating cancer. In one embodiment, the carbohydrate ganglioside analogue or the ganglioside carbohydrate multimer is useful for treating cancer. In one embodiment, the cancer is a ganglioside-positive cancer. In another embodiment, the cancer is a neuroblastoma, a melanoma, or a glioma. In one embodiment, the cancer is breast cancer or small cell lung cancer.

In one embodiment, there is provided an antibody specifically binding to the carbohydrate ganglioside analogue or the ganglioside carbohydrate multimer as defined herein. In one embodiment, the antibody is a monoclonal antibody, a polyclonal antibody or a humanized antibody. In one embodiment, the antibody is for preventing or treating cancer. In one embodiment, the cancer is a ganglioside-positive cancer. In one embodiment, the cancer is neuroblastoma, a melanoma, or a glioma. In one embodiment, the cancer is breast cancer or small cell lung cancer. In one embodiment, the cancer is breast cancer or small cell lung cancer.

In one embodiment, there is provided a vaccine comprising the carbohydrate ganglioside analogue or the ganglioside carbohydrate multimer as defined herein and a carrier. In one embodiment, the vaccine is for preventing or treating cancer. In one embodiment, the cancer is a ganglioside-positive cancer. In one embodiment, the cancer is neuroblastoma, a melanoma, or a glioma. In one embodiment, the cancer is breast cancer or small cell lung cancer.

In one embodiment, the carbohydrate ganglioside analogue or the ganglioside carbohydrate multimer for the methods or use defined herein is formulated for injection. In one embodiment, the vaccine is formulated for a transdermal administration or a parental administration. In one embodiment, the the parental administration is an intramuscular administration, a subs-cutaneous administration or an intravenous administration.

In a particular embodiment, GD2 glyconjugates that are immunogenic are disclosed herewith which are applied as anti-tumor vaccines.
[0039] It is disclosed herein the design and characterization of a water-soluble analog of GD2 and GD3 carbohydrate conjugated to form a dendrimeric (e.g. tetrameric, hereafter "tetra-GD2" or "tetra-GD3").

[0040] Tetrameric gangliosides carbohydrate conserves the native structural features of naturally occurring GD2 or GD3 for example, but are immunogenic and elicits cytotoxic anti-gangliosides humoral and cellular responses in vivo. Tetra-GD2 for example is effective as a GD2-cancer vaccine in prophylactic and in therapeutic paradigms. It is provided an effective anti-tumor vaccine, targeting cell surface carbohydrates, that rapidly elicits humoral and cellular immune responses that are protective in therapeutic paradigms.

[0041] Gangliosides accumulate on the outer leaflet of cell membranes, with the ceramide and lipids embedded and the carbohydrate head exposed. This should enable recognition by the immune system because complex gangliosides are neo-antigens and are defined as tumor markers. In addition, even antigenic gangliosides are poor immunogens. The immune system may recognize a carbohydrate as "self without mounting a response or it may generate cross-reactive pathological responses (e.g. Guillain-Barre syndrome is due to an anti-GM1 antibody).

[0042] A synthetic carbohydrate analog of GD2 and GD3 that is immunogenic, and which can be used to generate selective immunity against tumors was generated.

[0043] It is believed that the analog of G (such as an analogue of at least one of GD2, GD3, GM2 and GT1b) can be multimeric, i.e. a dimeric, trimeric, tetrameric or any other form suitable for allowing a proper spatial positioning.

[0044] It is believed, in particular that the analog of GD2 and GD3 can be multimeric, i.e. a dimeric, trimeric, tetrameric or any other form suitable for allowing a proper spatial positioning of GD2 or GD3. Encompassed herein is a multimer of 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 or 16 of the carbohydrate ganglioside analogue as described herein.

[0045] Scheme 1 below illustrates an immunogenic synthetic carbohydrate analog of GD2 and GD3 in a tetrameric form.
[0046] The analog essentially comprises a central mutimeric (or as shown above tetrameric) core allowing to covalently bond a spacer which in turn is also covalently bound to the desired GD2 or GD3. A typical example of such spacer is -(C=X)-, wherein X is N or X (such as -(C=S)- or -(C=0)-). The spacer can be introduced by the use of the isocyanate or isothiocyanate chemistry. Fig. 1c demonstrate a specific example, however the chemistry would be applicable to GD2/GD3-aminoaryl compounds disclosed above.

[0047] Preferably, the central core comprises an ethylene diamine residue on which is attached a multiplicity of terminal (i.e. primary) amino groups as generally described in scheme 2

[0048] An example of such core can be a PAMAM compound, a number of which are commercially available (see Aldrich Catalog at www.sigmaaldrich.com). Preferably, the central mutimeric core has a multiplicity of terminal (i.e. primary) amino groups. Examples of PAMAM compounds include generation 0.0 PAMAM
Higher generations of PAMAM compounds include PAMAM on which the terminal -NH$_2$ are further (partially or completely) functionalized with residues:

![Higher generation PAMAM structure](image)

A partial representation of such higher generation PAMAM is as follows:

![Partial representation of higher generation PAMAM](image)

wherein the remaining "arms" (showing a wavy line) can also optionally have amidoamine residues to provide multimer of up to 8 that can further be expanded.

An example of immunogenic synthetic carbohydrate analog of G (such as GD2 and GD3) in a tetrameric when the central core is a generation 0.0 as displayed above can be illustrated by the following:

![Example of immunogenic synthetic carbohydrate](image)

wherein G is as defined above, or preferably
X is O or S, preferably X is S.

[0052] GD2 or GD3 derivatives can be synthesized as described in Gilbert et al., 2002, J Biol Chem, 277: 327, while modifying the process of Tong et al., 2010, Chem Biol, 17: 183 to use a suitably functionalized β-D-lactopyranoside bearing an amino group that can be used to covalently bond GD2 or GD3 to the central multimeric core. For example, the functionalized β-D-lactopyranoside can be a C1-p-D-lactopyranoside amino aryl derivative, or preferably an aminophenyl-p-D-lactopyranoside or more preferably a p-aminophenyl-p-D-lactopyranoside.

[0053] The functionalized β-D-lactopyranoside can be further derivatized so that the central mutimeric (or as shown above tetrameric) core can be covalently bound. The functionalized β-D-lactopyranoside can be reacted with a suitable reagent to provide an activated carbonyl residue. For example, the C1-amino aryl derivative, or preferably an aminophenyl-p-D-lactopyranoside or more preferably a p-aminophenyl-β-D-lactopyranoside can be reacted with phosgene, diphosgene, triphosgene, thiophosgene, carbonyl dimidazole, disuccinimidyl carbonate, or other suitable reagent to provide the corresponding isocyanato, isothiocyanato, carbonylimidazolyl or succinimidyl carbonyl derivative.
The central mutimeric core can then be reacted with the functionalized β-D-lactopyranoside having the activated carbonyl residue described above to provide the desired immunogenic molecule.

As an alternative synthetic approach, the central mutimeric core (such as PAMAM) can be first reacted with a suitable reagent to provide an activated carbonyl residue as described above. Then the functionalized β-D-lactopyranoside comprising an amino group can be added to provide the desired immunogenic molecule.

It will be clear to a person of ordinary skill that if a further additional therapeutic agent is required or desired, ratios will be readily adjusted. It will be understood that the scope of combinations described herein is not particularly limited, but includes in principles any therapeutic agent useful for the prevention and treatment of cancer.

It will be appreciated that the amount of a compound of the invention required for use in treatment will vary not only with the particular compound selected but also with the route of administration, the nature of the condition for which treatment is required and the age and condition of the patient and will be ultimately at the discretion of the attendant physician.

The desired dose may conveniently be presented in a single dose or as divided dose administered at appropriate intervals, for example as two, three, four or more doses per day.

Pharmaceutical compositions include, without limitation, those suitable for transdermal, or parenteral (including intramuscular, sub-cutaneous and intravenous) administration.

The methods for preparing a pharmaceutical composition can include the steps of bringing into association the compound as defined herein and pharmaceutically acceptable excipients.

The compounds and combinations as defined herein may also be formulated for parenteral administration (e.g. by injection, for example bolus injection or continuous infusion) and may be presented in unit dose form in ampoules, pre-filled syringes, small volume infusion or in multi-dose containers with an added preservative. The compositions may take such forms as suspensions, solutions, or emulsions in oily
or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents. Alternatively, the active ingredient may be in powder form, obtained by aseptic isolation of sterile solid or by lyophilisation from solution, for constitution with a suitable vehicle, e.g. sterile water or saline, before use.

[0062] A specific embodiment is described below, followed by examples.

[0063] An amino phenyl ether-GD2 analog (herein AP-GD2), which has the correct GD2 carbohydrate structure but with an amino phenyl group replacing the ceramide and lipids is disclosed (see Fig. 1B). This analog is water-soluble. In AP-GD2, the bond between the phenyl group and the first sugar is preferably in the β-configuration, which is the configuration between ceramide and the first sugar in native gangliosides. This bond is critical for displaying a proper and homogeneous structure throughout the whole carbohydrate. The expected mass and configuration was verified by mass spectrometry (Fig. 2A), and NMR spectroscopy respectively (Fig. 2B).

[0064] AP-GD2 was converted to its corresponding isothiocyanatophenyl analog, and this intermediate was coupled to the terminal free amines of a tetravalent PAMAM G0 linker dendritic core (Fig. 1C). The formation of the thiourea-bridge between AP-GD2 and the PAMAM G0 linker was verified by a carbon chemical shift at 178ppm that appears in the spectrum of HMBC (NHC(S) NHPh). The formation of the tetrameric product was verified by 1D-1H-NMR. The 1D-1H-NMR spectra showed a single signal pattern for all sugar units and indicated a 40:36:8 ratio of the amide NHs, NHC(S) NHPh, H-ortho, H-meta, NHAc/CH3/H ax or H eq, demonstrating that the GD2 dendrimer is indeed tetravalent (Fig. 2C).

[0065] The tetra-GD2 antigen was designed to be used as an immunogen in tumor therapeutic studies. The rationale for the design of tetra-GD2 as a potential immunogen was based on the following concepts. First, presentation of tetra-GD2 may more closely mimic the oligomeric display of GD2 normally clustered in membrane rafts. Second, the sugar AP-GD2 analog can be easily conjugated to PAMAM linker at room temperature under mild conditions. Third, chemo-enzymatic synthesis guarantees the appropriate configuration of the glycosidic linkages, thus maintaining the conformation of the whole carbohydrate. Fourth, the antigens would be homogeneous and well-characterized chemically. Fifth, the resulting product would be water soluble and stable. All of these features are desirable for a vaccine and can be translated to AP-GD3 disclosed herewith.
Mice were immunized up to four times with tetra-GD2. Sera were collected four days after each immunization, and samples were tested for the presence of anti-ganglioside antibodies. Immunization with tetra-GD2 generated high titer and selective antisera against tumor-marker GD2 and GD3 gangliosides in 22 out of 25 mice (88%).

Data from FACScan assays demonstrated that sera from immunized mice had antibodies that reacted selectively with cell surface GD2 and GD3 in tumor cells (see Fig. 3A and Table 1). Their sera contained antibodies of the IgG and IgM isotypes (based on the isotype-specific secondary reagents used). Sera bound to EL4-GD2\(^+\) and/or EL4-GD3\(^+\) cells. Negative control normal mouse serum pre-immune (NMS) had no reactivity. Positive control mAbs show that EL4-GD2\(^+\) cells express GD2 but not GD3, and that EL4-GD3\(^+\) cells express GD3 but not GD2. In cellular controls, the sera did not bind to Jurkat cells that do not express GD2 or GD3, but do express many other gangliosides such as GM1.

<table>
<thead>
<tr>
<th>Mouse</th>
<th>FACScan on cells</th>
<th>ELISA</th>
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<tbody>
<tr>
<td></td>
<td>EL4 GD2</td>
<td>EL4 GD3</td>
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<tr>
<td>M1</td>
<td>++++</td>
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<td>M2</td>
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Sera were also tested by ELISA for direct binding to immobilized gangliosides. Gangliosides GD2, GD3, or controls were immobilized on plates and the indicated dilutions of test and control pre-immune sera were assayed. Antibodies generated through vaccination bound selectively to GD2 (see Fig. 3B). One immunization was sufficient to elicit an increase in circulating anti-GD2 IgG, ~2-fold above background, and after a boost there was a ~3-fold increase above background (Fig. 3C). The antibodies also bound to GD3, but did not bind to GM1. Both anti-GD2 IgG and anti-GD2 IgM isotypes were detected. The IgG isotypes increased after each
boost, suggesting B cell maturation and class switching (Fig. 3C). These ELISA data are consistent with the results obtained by FACScan.

[0069] Antibodies from mice vaccinated with tetra-GD2 killed EL4-GD2\(^+\) cells in culture (Fig. 3D). EL4-GD2\(^+\) cells were killed by immune serum in the absence of complement, an effect that has been reported for some anti-GD2 mAbs (Yoshida et al., 2001, Cancer Research, 61: 4244) (e.g. mAb 3F8 kills cell but mAb ME361 does not). In addition, killing of EL4-GD2\(^+\) cells was confirmed by counting cells stained with the vital dye trypan blue, and by assessing their morphology.

[0070] Together, these data validate tetra-GD2 as an immunogen to generate and to mature a cytotoxic humoral immunity.

[0071] It has been demonstrated that glycopeptide vaccines could activate the adaptive immune system by binding to histocompatibility class II (MHC II) (Avci et al., 2011, Nature Medicine, 17: 1602). This suggested that T cell mediated immunity might also be activated by tetra-GD2. Consequently, primary T cells in a mixed lymphocyte reaction (MLR) measuring \(^3\)H-thymidine incorporation were tested. As target cells stimulators of T cell activation, mytomicin-treated EL4-GD2\(^+\) were used, and Jurkat cells (GD2\(^-\)) were used as negative control (Fig. 4A). The EL4-GD2\(^+\) or Jurkat cells treated with Mytomicin do not incorporate \(^3\)H-thymidine (188 ± 4 cpm and 107 ± 5 cpm respectively), hence they act only as stimulators.

[0072] T cells from mice vaccinated twice with tetra-GD2 proliferated robustly when challenged with EL4-GD2\(^+\) cells, but they did not proliferate when challenged with Jurkat cells. In cellular controls, T cells from non-vaccinated mice did not proliferate when challenged with either EL4-GD2\(^+\) or with Jurkat cells. In positive controls, treatment with ConA stimulated proliferation of T cells from control or vaccinated mice to a similar degree (21,457 ± 504 cpm, and 19,834 ± 309 cpm respectively). Similar data were obtained in related assays counting, by trypan blue exclusion, the number of proliferating T cells in each group (Fig. 4B). In these assays, 40,000 responding T cells from tetra-GD2 vaccinated mice were seeded with live cells Jurkat or EL4 as stimulators. After 7 days \textit{in vitro}, all the EL4 target cells were dead, whereas Jurkat cells were alive and multiplying.

[0073] Together, these data validate tetra-GD2 as an immunogen to generate cellular immunity.
In a tumor-preventive paradigm, immunocompetent C57/Bl6 mice were immunized intraperitoneal\(^{a}\) twice at one-week intervals, followed by subcutaneous implantation of syngeneic EL4-GD2\(^{+}\) cells, which are very aggressive and highly metastatic. EL4 cells are syngeneic and grow and metastasize very rapidly in these mice. Immunized mice (5<n<9) had primary tumors of significantly smaller size than control mice at all days measured (11, 14, 16 post tumor implantation) (Fig. 5A). The tumor-preventative vaccine experiments were reproduced independently three times (total n=22 immunized versus n=22 control mice). To compare all three independent experiments, the average tumor volumes of control mice for each experiment at day 16 were standardized to 100%. Using this criterion, overall the tumor volumes in the immunized mice were reduced by ~57% (Fig. 5B).

In a tumor-therapeutic paradigm, mice were first implanted with EL4-GD2\(^{+}\) cells, and when tumors were visible/palpable, the mice were immunized two times with tetra-GD2 or control vehicle. In this more clinically relevant paradigm, immunized mice (4<n<6) had significantly delayed primary tumor growth compared to control mice, at all days measured (Fig. 5C). After 18 days all the control mice developed primary tumors averaging ~6,000 mm\(^3\), and extensive lymph node metastasis. In contrast, the immunized mice had primary tumors ~2,300 mm\(^3\).

The tumor-therapeutic vaccine data were reproduced in two independent experiments (N=13 immunized versus n=18 control mice). To compare all experiments, the average tumor volumes of control mice for each experiment at day 18 were standardized to 100%. Using this criteria, overall the tumor volume in the immunized mice were reduced by ~51% (Fig. 5D).

Mice bearing subcutaneous tumors received, by intraperitoneal injection, 4 x 10\(^{6}\) T cells purified form tetra-GD2-immunized donor mice. The adoptively-transferred mice (n= 6) had significantly delayed primary tumor growth compared to control untreated mice (n= 6), at all days measured (Fig. 5E). After 14 days of tumor growth, the control groups developed primary tumors averaging ~1200 mm\(^3\). In contrast, the adoptively transferred group had primary tumors ~500 mm\(^3\).

Notably, the adoptively transferred group had no evidence of metastasis to the lymph nodes or the thymus, organs which are the major sites of metastasis observed for EL4 tumors. EL4 tumor metastasis causes enlargement of the tissue, and a corresponding increase in weight that can be quantified. Mice bearing tumors (n=6)
have bigger lymph nodes than naive mice not bearing tumors, the size is increased by ~10-fold. Mice bearing tumors (n=6) but receiving adoptive transfer of T cells had smaller lymph nodes, comparable to normal lymph nodes (Fig. 5F).

The T cells activated after vaccination were characterized further as to their CD4 and CD8 phenotype. T cells were isolated from tumor-bearing vaccinated mice or control mice, and the cells were cultured in dishes that had GD2 immobilized on the plastic. T cells from vaccinated mice proliferated robustly in GD2-coated dishes. In contrast, T cells from control mice did not proliferate on GD2-coated dishes. Although all mice are bearing tumors and are exposed to GD2, only the vaccinated mice can respond to this antigen. FACScan assays characterizing the cultures showed that the phenotype of the proliferating cells was predominantly CD8+. The CD4/CD8 ratio in these cells changed due to the expansion of the CD8 subset, whereas the T cells from control mice retained the normal CD4/CD8 ratio (Fig. 6).

These data indicate that after vaccination activated T cells can respond to immobilized (multivalent) antigen in the absence of antigen presenting cells.

The presence of tumor-infiltrating lymphocytes was verified by immunostaining cryosections prepared from primary tumors. The EL4 tumors are "double negative" T cells, and do not stain with anti-CD4 or anti-CD8 antibodies. In mice that received adoptive transfer of T cells from immunized mice there was a significantly higher number of infiltrating CD8+ cells in the primary tumors, compared to tumor-bearing mice that did not receive adoptive transfer. Anti-CD4 antibodies detected infiltrating CD4+ cells in both groups.

Together, the data show that vaccination is sufficient to manage primary tumor growth and tumor metastasis in a realistic therapeutic paradigm, in which the vaccine is given after the tumor is established.

A novel tetravalent GD2 carbohydrate dendrimer has been developed as an effective carbohydrate immunogen, and as a therapeutic cancer vaccine. Biophysical characterization of the synthetic immunogen showed that it is a homogenous tetramer that maintains the desired β-linkages. Presentation of oligomeric carbohydrate structures to the immune system may stimulate more genuine and cytotoxic anti-tumor responses by mimicking the rafted GD2 in tumor membranes. In vivo and in vitro biological studies showed that the vaccine induces anti-GD2 cytotoxic antibodies and
cytotoxic cellular immunity. Immunity elicited by the vaccine can delay the growth and the metastasis of an established tumor. *In vivo* studies disclosed herein confirm that the ganglioside vaccine can not only prevent tumor growth, but also suppress established tumor growth.

[0084] From the data generated for the tetravalent GD2 carbohydrate dendrimer described herein, the development of other cancer vaccines targeting carbohydrates, and that are adapted to other tumor-associated gangliosides such as GD3 are encompassed herein. A tetrameric GD3 carbohydrate, prepared from the amino phenyl ether-GD3 analog is disclosed herein (see Fig. 1B), similarly to AP-GD2, which conserves the native structural features of naturally occurring GD3, predictably is immunogenic and will elicits cytotoxic anti-gangliosides humoral and cellular responses *in vivo*.

[0085] Using the same synthetic strategy as described for AP-GD2, a GD3 dendrimer (tetra-GD3) analog was generated and after two rounds of immunization, high titers of anti-GD3 antibodies was detected. As seen in Fig. 7, the humoral immunity elicited by vaccination with tetra-GD3 was detected.

[0086] The novel GD3 vaccine was evaluated as an anti-cancer agent *in vivo* and it was determined that immunized mice had primary tumors of significantly smaller size than control mice at all days measured (Fig. 8) and metastasis was virtually absent to the lymph nodes (Fig. 9) and to the lungs (Fig. 10). GD3 vaccine reduces melanoma lung metastasis (Fig. 10). Accordingly, a novel tetravalent GD3 carbohydrate dendrimer has been developed as an effective carbohydrate immunogen, and as a therapeutic cancer vaccine.

[0087] The present disclosure will be more readily understood by referring to the following examples which are given to illustrate embodiments rather than to limit its scope.

**EXAMPLE I**

**Synthesis AP-GD2 and tetra-GD2 dendrimer**

[0088] The carbohydrates were synthesized as described (Gilbert et al., 2002, J Biol Chem, 277: 327), by modification of the process for phenylthio-GD2 (Tong et al., 2010, Cehm Biol, 17: 183) in which the thio-phenyl analog originally reported was substituted with an p-aminophenyl-p-D-lactopyranoside (AP-Lac), from Toronto
Research Chemicals), for subsequent conjugation to the dendrimer (see below). AP-GD2 was water soluble (>20 mg/ml), and was purified to >99% purity by size-exclusion (Superdex 30 16 mm X 85 cm column, GE Health Care). The measured molecular weight of AP-GD2 was 1218 g/mol and corresponded to expected values. Structures were verified by 1D and 2D NMR spectroscopy and mass spectrometry (EI-MS) (see Figs. 2A and B). The chemoenzymatic synthesis of AP-GD2 had a final yield of ~90% pure material.

[0089] Thiophosgene (2 µl) was added to a stirred solution of AP-GD2 (2mg) in 80% ethanol (300 µl), and the mixture was allowed to stand at room temperature for 3h, when thin layer chromatography (ethyl acetate-methanol, 4:1) showed that all starting material had reacted and a single product had formed. Concentration almost to dryness gave a solid to which water was added. Filtration with washing of the product with water gave the isothiocyanatophenyl GD2 solution, which was freeze-dried to white powder (1.8 mg, 90% yield). The volatiles from a methanol solution of PAMAM GD (Dendritech, Inc.) were evaporated under reduced pressure, and the resulting residue was dissolved in dimethylformamide (DMF). A solution of isothiocyanatophenyl GD2 (1.8 mg) in DMF (110 µl) was added drop-wise to a stirred DMF solution (100 µl) of N,N-diisopropylethylamine (0.5 µl) and PAMAM GD (2 µl of 0.854 µg/µl). The reaction was stirred at room temperature for 20h, until no starting material was detected by TLC. The reaction mixture was diluted with 3 ml of water and dialyzed against water (MW cutoff 2 kDa, Spectrum Laboratories Inc.). The resulting solution was freeze-dried to give tetravalent PAMAM based GD2 as white powder in 80% yield (1.34 mg). The tetravalent PAMAM based GD2 was verified by 1D and 2D NMR spectroscopy. Mouse lymphoma EL4-GD2+ (wild type EL4 cells) and Jurkat leukemia cells were obtained from ATCC. EL4-GD3+ cells were developed by negative selection of EL4-GD2+ with anti-GD2 mAbs, followed by limiting dilution sub-cloning. EL4-GD3+ cells are stable and have the same in vitro growth properties and kinetics as EL4-GD2+ cells. All cells were grown in RPMI 1640 medium (Wisent INC) supplemented with 5% fetal bovine serum, 2 mM glutamine, 10 mM Heps and penicillin/streptomycin at 37°C in 5% C0₂ humidified atmosphere. Flow cytometry showed that all cell lines express equal levels of cell surface GM1. Flow cytometry, and thin layer chromatography of ganglioside extracts, confirmed that EL4-GD2+ cells express GD2 but not GD3, that EL4-GD3+ cells express GD3 but not GD2, and that Jurkat cells do not express either GD2 or GD3.
EXPOSED II
In vivo characterization of Tetra-GD2

Immunization for FACS and ELISA characterization of sera

[0090] Tetra-GD2 (50 µg) in PBS was administrated intraperitoneally to C57/B16 mice. After 10 days, the mice were re-immunized intraperitoneally (25 µg) + subcutaneously (25 µg) in PBS. Four days later, blood samples were collected for analyses.

[0091] FACScan: 2 x 10⁵ cells of EL4-GD2⁺, EL4-GD3⁺, and Jurkat cells were washed with FACS buffer (PBS, 0.5% BSA, 0.05% NaN₃), and incubated for 20 minutes on ice with 2 µl mouse antisera (1:50 dilution) or positive control anti-GD2 mAb (13 nM) or anti-GD3 mAb (13 nM). Cells were washed 2x with ice-cold FACS buffer, and incubated for 20 minutes on ice with FITC-conjugated anti-mouse IgG or FITC-conjugated anti-mouse IgM (Sigma). Cells were washed with FACS buffer, and freshly studied in a flowcytometer (Becton-Dickinson), and data were analyzed using CellQuest software. Mouse IgG or IgM and normal mouse sera were used as negative control antibodies. Jurkat cells were used as negative control cells.

[0092] Direct binding ELISA: Gangliosides (Advanced Immunochemical Inc.) were immobilized onto polystyrene Corning Strip Well 96-well plates (10 ng/well) (Fisher Scientific). The wells were then "blocked" with phosphate buffered saline containing 0.5% bovine serum albumin (PBS-0.5% BSA) for one hour. Wells were incubated for two hours with primary antibodies, including test sera, control pre-bleed mouse sera, mouse IgG (Sigma), or specific anti-ganglioside monoclonal antibodies. The plates were washed three times with PBS-0.5% BSA, followed by horseradish peroxidase (HRP)-conjugated anti-mouse antibody (Sigma) specific for mouse IgG isotypes, or mouse IgM isotype. After three washes with PBS-0.5% BSA and two with PBS, the colorimetric substrate TMB One Solution (Promega) was added, and the reaction was stopped with 0.5 N H₂SO₄. Plates were read at 450 nm (Benchmark Plus, Bio-Rad) (31).

[0093] Sera isotyping: Blood was collected after each round of immunization using a capillary blood collection system (Microvette, Sardstedt) and was centrifuged at 10000 x g for 5 minutes at room temperature for serum separation. Isotyping of Ig present in serum was then performed using a mouse subisotyping kit (Calbiochem,
cat# 386445) following the manufacturer's specifications. Experiments were performed 4 times in triplicate for each serum.

**Cytotoxicity evaluated by MTT assays**

[0094] EL4-GD2+ cells (5,000/well, in 96-well plates, Corning) were cultured in regular media supplemented with the indicated reagents. The survival/metabolic profile of the cells were quantified after 24h using the tetrazolium salt reagent (MTT, Sigma) and UV absorption. Assays were done 5 times each in quadruplicate. Test reagents include positive control anti-GD2 mAbs (7 nM final concentration), negative control normal mouse IgG (7 nM final concentration). Sera collected from naive mice (negative control), or from test mice vaccinated twice intraperitoneally. In these experiments vaccination was at days 3 and 10 and sera was collected at day 13. The times for serum collection followed the timelines of the tumor therapeutic paradigm (see below). The sera were semi-purified and ~50 µg/well of serum antibodies were applied. A small fraction of the serum antibodies would be anti-ganglioside antibodies, whereas the mAbs controls contain 100% anti-ganglioside IgG.

**Mixed lymphocyte reaction (MLR) evaluated by ³H-thymidine incorporation assay**

[0095] EL4-GD2+ cells and control Jurkat cells were treated with 25 µg/ml Mitomycin (Sigma) for 1 hr to arrest their proliferation. After the cells were washed three times with media to remove Mitomycin, they were plated in 96-well plates at 2 x 10^5 cells/well. Single cell suspension of splenocytes were obtained from twice immunized (as above) and from vehicle injected control mice. Cells were separated following the protocol of the EASYSEPTM magnet (Stem Cell Tech). Similar amounts of T cells were obtained from each mouse spleen (>95% purity, data not shown). The T cells were plated at a ratio of 10:1 with Mitomycin-treated EL4 or Jurkat cells. As controls, cultures of T cells alone (no stimulating tumor cells) or T cells treated with ConcanavilinA were used. After 5 days of culture, 0.1 µCi/µl³H-thymidine (Sigma-Aldrich) was added. DNA-incorporated ³H-thymidine was counted by liquid scintillation, and data are reported as average cpm ± sd.

**EXAMPLE III**

**Tumor-preventative studies**

*Immunization for tumor-preventative studies*
C57BL/6 mice were vaccinated intraperitoneal^ four-times, each one week apart (50 µg each time). Control mice received only vehicle injections. One week after the fourth vaccination, 5 x 10^5 EL4-GD2^+ were injected subcutaneously. Ten days after tumor challenge, tumors were measured at the indicated times post-tumor implantation.

**Immunization for tumor-therapeutic studies**

5 x 10^5 EL4-GD2^+ cells were injected subcutaneously in C57BL/6 mice, on the left flank. After three days, when the tumor was visible/palpable, mice were randomized and were vaccinated twice intraperitoneally on the right side, with either vehicle control or with tetra-GD2-dendimer (50 µg in PBS). Tumors were measured at the indicated times post-tumor implantation.

**Adoptive T cell transfer therapeutic studies**

C57BL/6 mice were vaccinated intraperitoneally twice (one week apart) with 50 µg of tetra-GD2. Seven days after the second immunization, T cells from spleen and lymph nodes were isolated using the EasySep Negative Selection Mouse T Cell Enrichment Kit (Stemcell Technologies). Approximately 4 x 10^6 T cells were injected intraperitoneally to C57BL/6 mice that had been injected subcutaneously with 2.5 x 10^5 EL4-GD2^+ cells 3 days prior to the adoptive transfer. Tumors were measured at the indicated times and mice were euthanized 14 days post-tumor implantation in order to dissect the ipsilateral inguinal and axillary lymph nodes and assess metastasis.

**Evaluation of tumor growth**

The primary tumor was measured with a digital caliper, and data were analyzed by the following equation: \( V (\text{mm}^3) = 0.5 \times \text{width} \times (\text{length})^2 \). After euthanasia, mice were dissected and examined microscopically for evidence of metastasis to lymph nodes and thymus (organs to which EL4 cells are known to home).

Differences in tumor growth for the two groups were analyzed by two-tailed student t-tests; with significance at \( p<0.05 \) (*) and \( p<0.01 \) (**). Elsewhere, one-way ANOVA with Tukey-Kramer Multiple Comparisons Test compared the five different groups. A difference between results was considered significant at \( p<0.05 \) (*) and \( p<0.01 \) (**).
EXAMPLE IV

In vivo characterization of Tetra-GD3

[00101] Safety. Vaccinated mice did not exhibit any signs of adverse effects that could be predicted from known side effects. All endpoints measured were negative, including those reported as problematic in clinical trials using their forms of GD2 or GD3 antigens (hyperalgesia, changes in behavior, mobility, and learning/memory). Hyper-immunized mice did not develop cross-reactive immunity to normal gangliosides, and there were no alterations to hematological profiles, liver or kidney enzyme profiles.

[00102] Using the same synthetic strategy as described in Example i, the GD3 dendrimer (tetra-GD3) analog was generated. After two rounds of immunization, high titers of anti-GD3 antibodies were detected, but not anti-GM1 Abs. Safety evaluation, as above, revealed no problems.

[00103] Fig. 7. Shows the humoral immunity elicited by vaccination with tetra-GD3. Representative ELISA data for sera showing anti-GD3-reactive antibodies of the IgG sub-class. Negative controls are normal mouse pre-vaccination serum and mouse Ig (Sigma). Binding is selective for GD3, whereas GM1 ELISA plates are negative (not shown). Data shown is n=8 individual samples averaged ± sem.

[00104] The novel GD3 vaccine was evaluated as an anti-cancer agent in vivo. EL4-GD3+, expressing high levels of GD3 was used. In a preventative paradigm, mice were immunized twice prior to implantation of a very aggressive syngeneic EL4-GD3+. In a clinically relevant tumor-therapeutic model, adoptive transfer of T cells from vaccinated donor mice into mice bearing established subcutaneous tumors was performed. In both paradigms, immunized mice had primary tumors of significantly smaller size than control mice at all days measured (Fig. 8) and metastasis was virtually absent to the lymph nodes (Fig. 9) and to the lungs (Fig. 10).

[00105] Fig. 8 illustrates the results of vaccination with tetra-GD3 protects against GD3+ tumor challenge. Mice were vaccinated intraperitoneally with tetra-GD3 (2x, 1 week apart, 10 ug/mouse each time, no adjuvant) before subcutaneous tumor implantation of EL4-GD3+ cells. Average primary tumor volumes ± sd.

[00106] Fig. 9 represents the results of vaccination with tetra-GD3 protects against GD3+ tumor challenge. Mice were vaccinated intraperitoneally with tetra-GD3 (2x, 1 week apart, 10 ug/mouse each time, no adjuvant) before subcutaneous tumor
implantation of EL4-GD3+ cells. Average lymph node volume± sd (indicative of metastasis, measured by detecting EL4 cells in the lymph node), n=8 each group. Lymph nodes are shown as example.

[00107] Fig. 10 illustrates the observation that the GD3 vaccine reduces melanoma lung metastasis. 5x10^5 B16-GD3 melanoma cells were injected in the tail vein of C57BL/6 mice. After three days, mice were randomized and were vaccinated twice IP. Mice were sacrificed after 14 days. Lungs with dark spots of metastatic nodules were quantified (tumors contain melanin and can be easily seen).

[00108] While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention, as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth, and as follows in the scope of the appended claims.
WHAT IS CLAIMED IS:

1. A ganglioside carbohydrate of formula
   
   $$ G - \text{Aryl-NH}_2 $$

   wherein $G$ is an oligosaccharide comprising one or more sialic acids (e.g. $n$-acetylneuraminic acid), wherein said $G$ is covalently bonded to the aryl by the C1 anomeric oxygen atom, and Aryl is a C6 to C10 aryl, optionally substituted.

2. The ganglioside carbohydrate of claim 1 having the formula

   or

3. The ganglioside carbohydrate of claim 1 having the formula

   or
4. A ganglioside carbohydrate multimer comprising at least one or at least two carbohydrate ganglioside analogues covalently bonded to a multimeric core molecule.

5. The ganglioside carbohydrate multimer of claim 4, comprising at least one or at least two carbohydrate ganglioside analogues consisting of the ganglioside carbohydrate of claim 1.

6. The ganglioside carbohydrate multimer of claim 4 or 5, wherein said at least two carbohydrate ganglioside analogues comprises at least one GD2 carbohydrate ganglioside analogue, or at least one GD3 carbohydrate ganglioside analogue.

7. The ganglioside carbohydrate multimer of any one of claims 4-6, wherein said ganglioside carbohydrate multimer is a tetramer of the carbohydrate ganglioside analogues.

8. The ganglioside carbohydrate multimer of claim 4, wherein said at least one or at least two carbohydrate ganglioside analogues are analogues of at least one of GD2, GD3, GM2 and GT1b.

9. The ganglioside carbohydrate multimer of claim 4, having the formula
wherein Aryl is a C6 to C10 aryl, optionally substituted; X is O or S, and G is 10. The gangloside carbohydrate of any one of claims 1-9, wherein said gangloside carbohydrate is immunogenic against tumours.

11. The gangloside carbohydrate of any one of claims 1-10, for preventing or treating cancer.

12. The gangloside carbohydrate of claim 11, wherein the cancer is a ganglioside-positive cancer.
13. An antibody specifically binding to the ganglioside carbohydrate of any one of claims 1-12.

14. The antibody of claim 13, wherein said antibody is a monoclonal antibody, a polyclonal antibody or a humanized antibody.

15. The antibody of claim 13 or 14, for preventing or treating cancer.

16. The antibody of claim 15, wherein said cancer is a ganglioside-positive cancer.

17. A vaccine comprising the ganglioside carbohydrate of any one of claims 1-12 and a carrier.

18. The vaccine of claim 17, for preventing or treating cancer.

19. A method of preventing or treating cancer in a patient in need thereof comprising the step of administering to said patient an effective amount of the ganglioside carbohydrate of any one of claims 1-12, the antibody of any one of claims 13-16 or the vaccine of any one of claims 17-18.

20. The method of claim 19, wherein said cancer is a ganglioside-positive cancer.

21. The method of claim 19 or 20, wherein said cancer is neuroblastoma, a melanoma, or a glioma.

22. The method of any one of claims 19-21, wherein said cancer is breast cancer or small cell lung cancer.

23. A method of eliciting an immunogenic response in a patient in need thereof comprising the step of administering to said patient an effective amount of the ganglioside carbohydrate of any one of claims 1-12, the antibody of any one of claims 13-16 or the vaccine of any one of claims 17-18.
Fig. 1B

\[
\begin{align*}
\alpha-(2-3)\text{-sialyltransferase} & \quad \text{CMP-Neu5Ac} \\
\alpha-(2-8)\text{-sialyltransferase} & \quad \text{CMP} \\
\beta-(1-4)\text{-GalNAc-transferase} & \quad \text{UDP-GalNAc} \\
\end{align*}
\]

Fig. 1
Fig. 1C

\[
\text{S} \quad \text{Cl–C–Cl} + R-O-\text{NH}_2 \xrightarrow{70\% \text{ EtOH/H}_2\text{O}} 3\text{hr, RT} \quad R-O-\text{N=C=S}
\]
90% yield

PAMAM Dendrimer G0
DMF+DIPEA
24hr, RT

80% yield
Fig. 4
Fig. 5
**Fig. 7**

Fold Change in ELISA

- pre-immune
- 1st immunization
- 2nd immunization

**Fig. 8**

Tumor Volume [mm³]

- Control
- GD3 vaccination

Day 7 | Day 9 | Day 11
INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA2014/051165

A. CLASSIFICATION OF SUBJECT MATTER
IPC: C07H 15/203 (2006.01), A61K 39/385 (2006.01), A61K 39/395 (2006.01), A61P 35/00 (2006.01), A61P37/04 (2006.01), C07H 15/10 (2006.01), C07K 16/44 (2006.01), C12P 19/44 (2006.01)

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC: C07H 15/203 (2006.01), A61K 39/385 (2006.01), A61K 39/395 (2006.01), A61P 35/00 (2006.01), A61P37/04 (2006.01), C07H 15/10 (2006.01), C07K 16/44 (2006.01), C12P 19/44 (2006.01)

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

Electronic database(s) consulted during the international search (name of database(s) and, where practicable, search terms used)
Questel-Orbit, Canadian Patent Database, Scopus, CAplus

ganglioside, GD+, GM+, conjugate, dendrimer, multimer, multivalent, antigen, antibody, vaccine, immunogen, cancer

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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<th>Category*</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
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<td>MUROZUKA et al, &quot;Lyso-GM3, its dimer, and multimer: their synthesis, and their effect on epidermal growth factor-induced receptor tyrosine kinase&quot;, Glycoconj J., Dec 2007, 24(9), 551-63. Epub 19 Jul 2007. Fig. 1, p.552; Fig. 3, compounds 17-19, p.554.</td>
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<td>WO03003985A2, (SLOAN-KETTERING INSTITUTE FOR CANCER RESEARCH) 16 January 2003 (16-01-2003), claims 2, 4, 13, 16, 26, 30, 47-49; p.13, 1.27 to p.14, 1.6</td>
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* Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier application or patent but published on or after the international filing date
- "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)
- "O" document relating 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

- "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
- "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
- "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
- "&" document member of the same patent family

Date of the actual completion of the international search:
16 February 2015 (16-02-2015)

Date of mailing of the international search report:
03 March 2015 (03-03-2015)

Name and mailing address of the ISA/CA
Canadian Intellectual Property Office
Place du Portage 1, C14 - 1st Floor, Box PCT
50 Victoria Street
Gatineau, Quebec K1A 0C9
Facsimile No.: 001-819-953-2476

Authorized officer:
Yong-Huang Chen (819) 956-41 13

Form PCT/ISA/210 (second sheet) (July 2009)
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**INTERNATIONAL SEARCH REPORT**

**Box No. II  Observations where certain claims were found unsearchable (Continuation of item 2 of the 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. Claim Nos.: 19-23  
   because they relate to subject matter not required to be searched by this Authority, namely:
   Claims 19-23 are directed to a method for treatment of the human or animal body by surgery or therapy, which the International Searching Authority is not required to search under Rule 39.1(iv) of the PCT. However, this Authority has carried out a search based on the alleged effect or purpose/ use of the product defined in claims 19-23.

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

**Box No. III  Observations where unity of invention is lacking (Continuation of item 3 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 additional fees, this Authority did not invite payment of additional fees.

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 claim 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 claim Nos.:

**Remark on Protest**

- The additional search fees were accompanied by the applicant=s protest and, where applicable, the payment of a protest fee.
- The additional search fees were accompanied by the applicant=s protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- No protest accompanied the payment of additional search fees.
<table>
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