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(54) Title: METHODS AND COMPOSITIONS FOR TREATMENT OF LIPID STORAGE DISORDERS
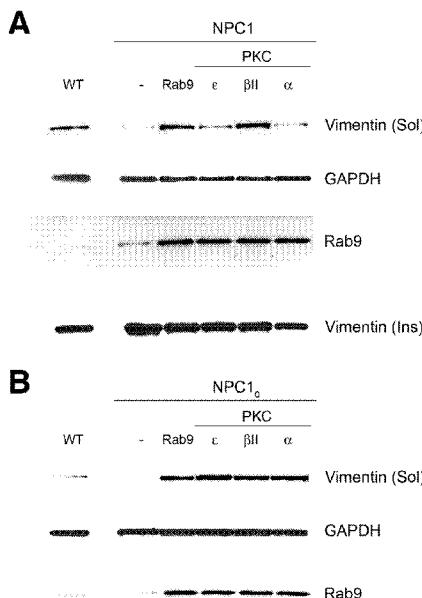
(57) **Abstract**: Treating subjects having a lipid storage disorder with a composition comprising a PKC activator, such as bryostatins, bryologs, and polyunsaturated fatty acids. Accordingly, the present disclosure provides methods for treating human subjects suffering from lipid storage disorders, such as Niemann-Pick disease, by administering PKC activators. The present disclosure provides, according to certain embodiments, methods comprising administering to a subject with Niemann-Pick Type C disease a pharmaceutically effective amount of bryostatin 1.

Figure 1

Methods and Compositions for Treatment of Lipid Storage Disorders

Priority Claim

[001] This application claims priority to United States provisional patent application number 61/981,473 filed April 18, 2014 and United States provisional patent application number 61/987,360 filed May 1, 2014, the contents of which are expressly incorporated by reference.

BACKGROUND

[002] All references cited herein are expressly incorporated by reference.

[003] Lipid storage disorders (or lipidoses) are a group of inherited metabolic disorders in which harmful amounts of lipids accumulate in some of the body's cells and tissues. People with these disorders generally either do not produce enough of one of the enzymes needed to metabolize lipids or they produce enzymes that do not work properly. Over time, this excessive storage of fats can cause permanent cellular and tissue damage.

[004] Many lipid storage disorders lack adequate therapeutics for treatment. These disorders include, for example, Niemann-Pick disease types A, B and C, Gaucher disease Type II, Fabry disease (note that an enzyme replacement is available), gangliosidoses including Tay-Sachs disease, Sandhoff disease, Krabbe disease, Metachromatic leukodystrophy, and cholesteryl ester storage disease (Wolman's disease).

[005] Niemann-Pick disease is an inherited autosomal recessive lipid storage disorder characterized by excessive accumulation of sphingomyelin in the lysosomes of cells such as macrophages and neurons, which impairs normal cellular function. Niemann-Pick Type A results from a deficiency of acid sphingomyelinase and is a rapidly progressive neurodegenerative disease. It typically results in death within two to three years of age. Niemann-Pick Type B is a milder form that results in the enlargement of the liver and spleen, and respiratory distress with death generally ensuing by early adulthood. These two forms of Niemann-Pick disease which are both associated with acid sphingomyelinase (ASM) deficiencies are referred to collectively herein as Niemann-

Pick disease, or ASM deficiency (ASMD). Other types of Niemann-Pick disease, e.g., Type C, do not involve mutations in the ASM gene and are not directly attributable to the function of ASM. The nature of the biochemical and molecular defects that underlie the remarkable clinical heterogeneity of the A and B subtypes remains unknown. Although patients with both subtypes have residual ASM activity (about 1 to 10% of normal), biochemical analysis cannot reliably distinguish the two phenotypes. Moreover, the clinical course of Type B NPD is highly variable, and it is not presently possible to correlate disease severity with the level of residual ASM activity.

[006] Niemann-Pick Type C is results from mutations in NPC1 and NPC2 genes. In Niemann-Pick Type C, the protein product of the major mutated gene NPC1 is not an enzyme but appears to function as a transporter in the endosomal-lysosomal system, which moves large water-insoluble molecules through the cell. The protein coded by the NPC2 gene has been shown to be a small cholesterol-binding protein that resides in the lysosome lumen. The disruption of this transport system results in the accumulation of cholesterol and glycolipids in lysosomes.

[007] Niemann Pick disease, as well as other lipid storage disorders, is a disorder for which there remains an overwhelming need for therapeutics for treatment. Presently there is no FDA approved therapy for Neimann Pick disease in the United States; and treatments for this disease are limited with most people afflicted with Type A dying by age 18 months, while those with Type B or Type C, frequently live into their teenage years.

SUMMARY

[008] The protein kinase C (PKC) family of enzymes is responsible for a multitude of cellular processes through the enzymes' ability to regulate proteins via signal transduction cascades. The members of this kinase family are structurally and functionally similar and are categorized into conventional (α, βI, βII and γ), novel (δ, ϵ, η, and θ), and atypical isoforms (ζ and λ). These isoforms have been implicated in a variety of diseases and pathological conditions.

[009]   The present disclosure is based in part on the previously unappreciated role for PKCs in lipid storage disorders such as Niemann-Pick Type C (NPC1) disease. We observed that the intermediate filament, vimentin, is hypophosphorylated in NPC1 cells compared to wild-type (WT) cells and that this hypophosphorylation results from reduced activity [5]. Vimentin is involved in a variety of cellular processes, including vesicular membrane transport [6,7], signal transduction [8,9] and cell motility [10]. Similar to NPC1 cells, cells lacking vimentin are unable to transport LDL-derived cholesterol from their lysosomes to the endoplasmic reticulum for esterification [11]. The decreased vimentin phosphorylation in NPC1 cells reduces the pool of soluble vimentin, likely disrupting the vimentin cycle, which is necessary for transport to take place [12,13]. Vimentin has been shown to be phosphorylated by several proteins, including the PKCs [14] and in particular the α [15], ε [10] and βII [16,17] isoforms.

[0010] Accordingly, the present disclosure provides methods for treating human subjects suffering from lipid storage disorders, such as Niemann-Pick disease, by administering PKC activators.

[0011] The present disclosure provides, according to certain embodiments, methods comprising administering to a subject with a lipid storage disorder a pharmaceutically effective amount of a PKC activator.

[0012] The present disclosure provides, according to certain embodiments, methods comprising administering to a subject with Niemann-Pick Type C disease a pharmaceutically effective amount of bryostatin 1.

[0013] The features and advantages of the present disclosure will be readily apparent to those skilled in the art upon a reading of the description of the embodiments that follows.

DRAWINGS

[0014] The patent or application file contains at least one drawing executed in color. Copies of this patent or patent application publication with color drawing(s) will be provided by the Office upon request and payment of the necessary fee.

[0015] Some specific embodiments of the disclosure may be understood by referring, in part, to the following description and the accompanying drawings.

[0016] **Figure 1A and 1B** is a Western blot showing the effects of transient PKC expression on vimentin solubilization in human NPC1 cells. Representative Western blot analyses of soluble and insoluble vimentin levels in human NPC1 3123 (A) and human nullNPC1o (B) cells transfected with PKC ε, β, or α show that the three isoforms increase levels of soluble vimentin and Rab9 with a concurrent decrease of insoluble vimentin relative to untransfected cells (-). The levels of vimentin solubilized are similar to that seen in cells expressing Rab9 (Rab9). The blots shown are representative of at least 3 independent experiments.

[0017] **Figure 2** is a Western blot showing Rab9 release from insoluble vimentin fraction of NPC1 cell lysates. The insoluble vimentin fraction from NPC1 cell lysates was incubated with various PKC isoforms. All isoforms tested can affect Rab9 release to some degree from the insoluble vimentin fraction, with PKCα being the most effective and PKCγ being the least effective. The blots shown are representative of at least 3 independent experiments.

[0018] **Figure 3** is a graph showing the effects of PKCs and fatty acids on cholesterol esterification in M12 NPC1 CHO cells. M12 cells were treated with 50 μg/ml fatty acids for 2 days and then transfected with the indicated PKC isoforms. Following transfection, cholesterol transport was assessed by esterification assay. Both free fatty acids and PKCs alleviate the cholesterol transport defect of NPC1 cells and their effects appear to be additive.

[0019] **Figure 4** are images showing the effects of transient PKC expression on the NPC1 phenotype. M12 cells were transfected with PKC isoforms or Rab9 for 48 hrs and then analyzed by filipin staining for cholesterol storage. Cells positive for transfection stain positive for GFP (left panel) and show decreased filipin staining (outlined cells, right panel) compared to surrounding untransfected cells, confirming the role of PKCs in mobilizing stored cholesterol from the NPC1 endosomes. Bar, 20 μm.

[0020] **Figure 5** shows the effects of fatty acids on vimentin solubilization and the NPC1 phenotype. (A) Human NPC1 3123 cells were treated with 50 μg/ml linoleic or oleic acid for 24 hrs, after which the levels of soluble vimentin were analyzed by Western blotting. Cholesterol storage in NPC1 CHO (B) or human 3123 (C) cells was analyzed by filipin staining. Fluorescence intensity was quantitated in at least 150 cells for each sample. The bar graph represents average values ±SEM from 3 independent experiments. * and *** denote statistically significant differences between treated and untreated cells with $P < 0.05$ and $P < 0.0001$, respectively, as determined by Student's t-test.

[0021] **Figure 6** shows the effects of PKC activation on the NPC1 phenotype. NPC1 CHO cells (B through F) were treated with 100 μM DCP-LA (C), 10 μM DHA (D), or 100 μM diazoxide (E) and cholesterol storage was quantified by filipin fluorescence. WT CHO cells are shown in (A). Filipin intensity was quantitated in at least 150 cells for each sample. The bar graph represents average values ±SEM from 3 independent experiments. *, **, and *** denote statistically significant differences between treated and untreated cells with $P < 0.05$, $P < 0.01$ and $P < 0.0001$, respectively, as determined by Student's t-test.

[0022] **Figure 7** are images showing the effects of PKC activation on sphingolipid transport. Human NPC1 3123 cells were treated with (B) 20 μM DCP-LA, (C) 2 μg/ml oleic acid, (D) 2 μg/ml linoleic acid, or (F) 100nM PMA for 48 hours before BODIPY-LacCer staining was performed. In untreated cells (A), transport of the lipid to the trans-Golgi network (TGN) is inhibited and staining is visible only in punctate endocytic vesicles. In contrast, in treated cells the lipid can be seen in the TGN (arrows) in treated cells, indicating release of the transport block that characterizes NPC1.

[0023] **Figure 8** is a graph showing the ability of DCPLA, diazoxide and bryostatin 1 to decrease stored cholesterol levels in NPC3-SV cells at 48h.

[0024] **Figure 9** is a graph showing the ability of DCPLA, and bryostatin 1 to decrease stored cholesterol levels in NPC3-SV cells at 72h.

[0025] **Figure 10** is a graph showing the ability of DCPLA, and bryostatin 1 to decrease stored glycosphingolipid levels in NPC3-SV cells at 72h using verotoxin B (VTB) as a

probe stain.

[0026] **Figure 11** is a graph showing the ability of DCPLA and bryostatin 1 to decrease filipin accumulation using an NPC24-SV cell line.

[0027] **Figure 12** are photomicrographs showing the ability of DCPLA, and bryostatin 1 to release the ganglioside transport block in NPC3SV cells.

[0028] **Figure 13** is a graph showing the ability of bryostatin 1 (0.1-100 nM) to decrease cholesterol accumulation in human NPC24-SV cells.

[0029] **Figure 14** is a graph showing the ability of bryostatin 1 (0.1-100 nM) to decrease glycosphingolipid accumulation in human NPC24-SV cells.

[0030] **Figure 15** are images showing representative fields of treated cells used in the analysis shown Figures 13-14.

[0031] **Figure 16** is a graphic representation of an NPC1 cell showing the itineraries of various lipids stored.

[0032] **Figure 17** is a graph showing untreated C57 NPC1 mice. Weight gain is observed up to around day 70-72 of life after which a rapid weight loss is observed.

[0033] While the present disclosure is susceptible to various modifications and alternative forms, specific example embodiments have been shown in the figures and are herein described in more detail. It should be understood, however, that the description of specific example embodiments is not intended to limit the invention to the particular forms disclosed, but on the contrary, this disclosure is to cover all modifications and equivalents as illustrated, in part, by the appended claims.

DESCRIPTION

[0034] In general, the present disclosure provides methods for treating lipid storage disorders using PKC activators. As used herein, "protein kinase C activator" or "PKC activator" refers to a substance that increases the rate of the reaction catalyzed by protein kinase C, upregulates the expression of PKC (e.g., upregulates the expression of PKCα,

PKC βII , PKC γ and/or PKC ε), or otherwise facilitates the activation of PKC.

[0035] In certain embodiments, the present disclosure provides methods comprising administering to a human subject with a lipid storage disorder a pharmaceutically effective amount of a PKC activator. The PKC activator may be administered as part of a composition suitable for administration to a human subject.

[0036] In certain embodiments, the PKC activator may be any of bryostatin 1-20, a bryolog, neristatin, a polyunsaturated fatty acid, or a combinations thereof.

[0037] Bryostatins may be used in the methods of the present disclosure. The bryostatins are a family of naturally occurring macrocyclic compounds originally isolated from marine bryozoa. Currently, there are about 20 known natural bryostatins which share three six-membered rings designated A, B and C, and which differ mainly in the nature of their substituents at C7 (OR^A) and C20 (R^B).

Formula A
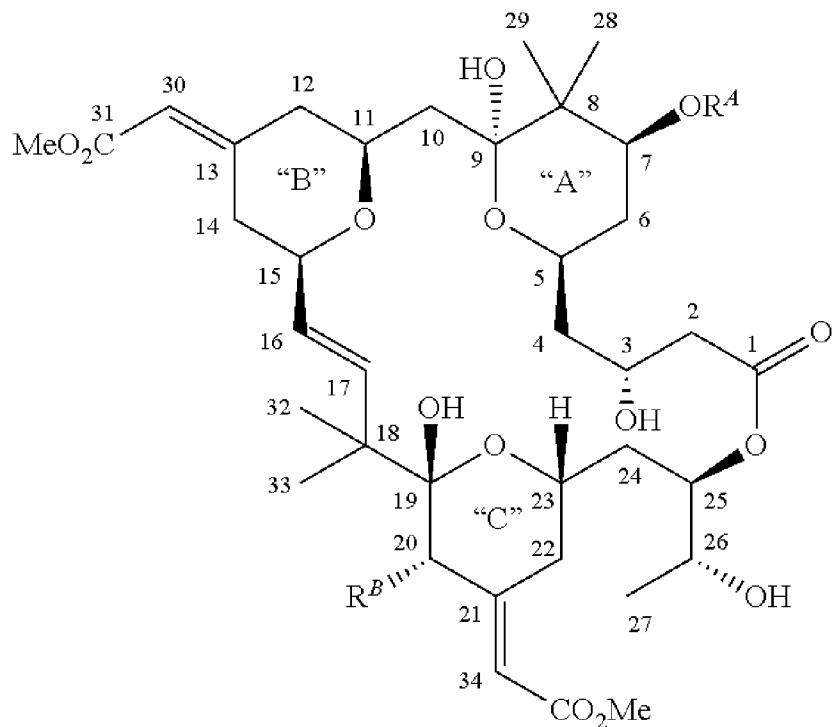
Bryostatin 1 and derivatives of bryostatin 1 are described in U.S Pat. No. 4,560,774 (incorporated herein by reference). Examples of suitable bryostatins that may be used with the methods of the present disclosure include, bryostatin 1, bryostatin 2, bryostatin 3, bryostatin 4, bryostatin 5, bryostatin 6, bryostatin 7, bryostatin 8, bryostatin 9, bryostatin 10, bryostatin 11, bryostatin 12, bryostatin 13, bryostatin 14, bryostatin 15, bryostatin 16, bryostatin 17 bryostatin 18, bryostatin 19, and bryostatin 20.

[0038] Analogs of bryostatins, commonly referred to as bryologs, also may be used in the methods of the present disclosure. Bryologs are structural analogues of bryostatin. While bryostatin has two pyran rings and one 6-membered cyclic acetal, in most bryologs one of the pyrans of bryostatin is replaced with a second 6-membered acetal ring. This modification reduces the stability of bryologs, relative to bryostatin, for example, in both strong acid or base, but has little significance at physiological pH. Bryologs also have a lower molecular weight (ranging from about 600 to 755), as compared to bryostatin (988), a property which may facilitate transport across the blood-brain barrier. Examples of suitable bryologs include, but are not limited to analogs and derivatives of bryostatins such as those disclosed in U.S. Pat. Nos. 6,624,189, 7,256,286 and 8,497,385 (the disclosures of which are incorporated herein by reference).

[0039] In certain embodiments, polyunsaturated fatty acid esters (PUFAs or polyenoic fatty acids)) may be used in the methods of the present disclosure for treating lipid storage disorders. A PUFA is a fatty acid containing more than one double bond. There are three classes of PUFAs, omega-3 PUFAs, omega-6 PUFAs, and omega-9 PUFAS. In omega-3 PUFAs, the first double bond is found 3 carbons away from the last carbon in the chain (the omega carbon). In omega-6 PUFAs the first double bond is found 6 carbons away from the chain and in omega-9 PUFAs the first double bond is 9 carbons from the omega carbon. As used herein, the term PUFA includes both naturally-occurring and synthetic fatty acids. A major source for PUFAs is from marine fish and vegetable oils derived from oil seed crops. Examples of PUFA's suitable for use in the methods of the present disclosure include, but are not limited to, esters of 8-[2-(2-pentyl-cyclopropylmethyl)cyclopropyl]-octanoic acid (DCPLA), as well as those described in United States patent 8,163,800 and in PCT publication WO 2010014585 A1.

[0040] Another example of suitable PKC activators include potassium channel activators such as, for example, diazoxide.

[0041] In certain embodiments, neristatins, such as neristatin 1, may be used in the methods of the present disclosure for treating lipid storage disorders.

[0042] Other suitable PKC activators include, but are not limited to, phorbol-12-myristate-13-acetate (PMA), okadaic acid, 1α,25-dihydroxyvitamin D3, 12-deoxyphorbol-13-acetate (prostratin), 1,2-dioctanoyl-sn-glycerol (DOG), 1-oleoyl-2-acetyl-sn-glycerol (OAG), (2S,5S)-(E,E)-8-(5-(4-(trifluoromethyl)phenyl)-2,4-pentadienoylamino)benzolactam (α-amyloid precursor protein modulator), cis-9-octadecenoic acid (oleic acid), ingenol 3-angelate, resiniferatoxin, L-α-Phosphatidyl-D-myo-inositol-4,5-bisphosphate, triammonium salt (PIP2), phorbol-12,13-dibutyrate, 8(S-hydroxy-(5Z, 9E, 11Z, 14Z)-eicosatetraenoic acid (8(S)-HETE), 12β-[(E,E)-5-Phenyl-2,4-pentadienoyloxy]daphnetoxin (merzerein), clomiphene citrate, sodium oleate, phorbol 12,13-diacetate, phorbol-12,13-didecanoate, 1,2-dipalmitoyl-sn-glycerol, 1-Stearoyl-2-linoleoyl-sn-glycerol, phorbol-12,13-didecanoate, 1,2-dipalmitoyl-sn-glycerol, 1-stearoyl-2-linoleoyl-sn-glycerol, phorbol 12, 13-dihexanoate, prostratin and its analogs, resiniferonol 9,13,14-ortho-phenylacetate, C-8 ceramide, 1,6-bis(Cyclohexyloximinocarbonylamino)hexane; 1,6-Di(O-(carbamoyl)cyclohexanone oxime)hexane (RHC-80267), (+/-)-1-oleoyl-2-acetylglycerol, 5(S),6(R),15(S)-TriHETE (Lipoxin A4), (-)-Indolactam V, SC-9, SC-10, zoledronic acid monohydrate, 12-deoxyphorbo 13-angelate 20-acetate, 6-(N-decylamino)-4-hydroxymethylindole, 4α-phorbol 12,13-dibutyrate, 1,2-dihexanoyl-sn-glycerol, zoledronic acid disodium salt tetrahydrate, arachidonic acid methyl ester, arachidonic acid-d8.

[0043] As used herein, "a pharmaceutically effective amount" is an amount of a pharmaceutical compound or composition having a therapeutically relevant effect on a lipid storage disorder. A therapeutically relevant effect relates to some improvement in a biomechanical process (e.g., gait, use of limbs, and the like) or a change in the cellular, physiological or biochemical parameters associated with any of the causes of a particular lipid transport disorder (e.g., vimentin solubility, cholesterol esterification, cholesterol

accumulation and transport, glycosphingolipid accumulation and transport).

[0044] In certain embodiments, a pharmaceutically effective amount for bryostatins and bryologs may be from about 0.0000001 to about 500 mg per kg host body weight per day, which can be administered in single or multiple doses. In some embodiments, the dosage level may be: from about 0.0000001 mg/kg to about 250 mg/kg per day; from about 0.0000005 mg/kg to about 100 mg/kg per day; from at least about 0.0000001 mg/kg to about 250 mg/kg per day; from at least about 0.00000005 mg/kg to about 100 mg/kg per day; from at least about 0.000001 mg/kg to about 50 mg/kg per day; or from about 0.00001 mg/kg to about 5.0 mg/kg per dose. In other embodiments, the dosage may be about 0.00000001 mg/kg to about 0.00005 mg/kg; 0.00005 mg/kg to about 0.05 mg/kg; ; about 0.0005 mg/kg to about 5.0 mg/kg per day; about 0.0001 mg/kg to about 0.5 mg/kg per dose; or 0.001 to 0.25 mg/kg per dose.

[0045] In certain specific embodiments, in which the lipid storage disorder is Niemann-Pick disease, a pharmaceutically effective amount of a PKC activator may be an amount sufficient to solubilize vimentin and/or release trapped Rab9.

[0046] In certain embodiments, the dosing is from about 1 μg/kg (3-25 μg/m^2) to 120 μg/kg (360-3000 μg/m^2). In other embodiments, the dosing is from about 0.04-0.3 μg/kg (1 μg/m^2) to about 1-10 μg/kg (25 μg/m^2). In other embodiments, the dosing is from about 0.01 μg/m^2 to about 25 μg/m^2. In other embodiments, the dosing is from about 0.0002-0.0004 μg/kg to about 0.05-1 μg/kg.

[0047] In certain embodiments, the PKC activator is a PUFA administered at a dosage of about 0.001 to 100 mg/kg; 0.01 to about 50 mg/kg; about 0.1 to about 10 mg/kg.

[0048] In certain embodiments, the PKC activator present in the compositions used in the methods of the present disclosure is a bryostatin or bryolog, and the bryostatin or bryolog is used in an amount from about 0.0001 to about 1000 milligrams. In some embodiments, the bryostatin or bryolog is used in an amount from at least about 0.0001, 0.0005, 0.001, 0.002, 0.003, 0.004, 0.005, 0.006, 0.007, 0.008, 0.009, 0.01, 0.02, 0.03, 0.04, 0.05, 0.06, 0.07, 0.08, 0.09, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 5.0, 10.0, 15.0, 20.0, 25.0,

50.0, 75.0, 100.0, 150.0, 200.0, 250.0, 300.0, 400.0, 500.0, 600.0, 750.0, 800.0, 900.0, or about 1000.0 milligrams.

[0049] The compositions used in the methods of the present disclosure may be administered via any suitable route; for example, orally, intraperitoneally, subcutaneously, intranasally, buccally, trans-dermally intramuscularly, intrarectally, intravenously, and by oral inhalation.

[0050] The compositions used in the methods of the present disclosure may be administered on a regimen of 1 to 4 times per day, and in some embodiments, the compositions are administered twice a week, once a week, once every two weeks, once every three weeks, once every four weeks, once every six weeks, once every eight weeks or even less frequently depending on the needs of the patient.

[0051] The compositions used in the methods of the present disclosure may be administered as part of a course of treatment lasting for about 1 to about 30 days; about 1 to about 90 days; about 1 to about 120 days; about 1 to about 180 days; about 1 to 365 days; one year; two years; three years; or for the patient's lifetime.

[0052] It will be understood, however, that the specific dose level and frequency of dosage for any particular host may be varied and will depend upon a variety of factors including the activity of the specific compound employed, the metabolic stability and length of action of that compound, the age, body weight, general health, sex, diet, mode and time of administration, rate of excretion, drug combination, the nature of the disorder, the severity of the particular disorder, and the host undergoing therapy.

[0053] To facilitate a better understanding of the present invention, the following examples of specific embodiments are given. In no way should the following examples be read to limit or define the entire scope of the invention

EXAMPLES

[0054] **Example 1**

[0055] **Materials and Methods**

[0056] Dulbecco's Modified Eagle Medium (DMEM), trypsin, L-glutamine, gentamicin, and NuPage gels and buffers were obtained from Invitrogen (Carlsbad, CA) while FBS was from Hyclone, Thermo Scientific (Rockford, IL). The monoclonal anti-vimentin (V9), conjugated anti-mouse-IgG and anti-rabbit- IgG antibodies were from Santa Cruz Biotechnologies, Inc. (Santa Cruz, CA). The anti-GAPDH antibody was from Millipore (Billerica, MA) and the anti-Rab9 polyclonal antibody has been described elsewhere [27]. Filipin was from Polysciences, Inc. (Warrington, PA). Lumilight Plus substrate and FuGENETM 6 transfection reagent were both from Roche Diagnostics (Indianapolis, IN). [9,10-3H(N)] oleic acid (15 Ci/mmol) was obtained from NEN Life Science Products (Boston, MA) and LDL was from EMD Biosciences Inc. (La Jolla, CA). All other chemicals were acquired from Sigma-Aldrich (St. Louis, MO).

[0057] **Cell culture and transfection**

[0058] The human wild-type fibroblast (GM05387), NPC1o fibroblast (GM09341), and NPC1 fibroblast (GM03123) cell lines were obtained from Coriell Cell Repositories (Camden, NJ). The M12 Chinese hamster ovary (CHO) cell line and its wild-type parental line were obtained and cultured. Fibroblast cell lines were cultured in DMEM, and CHO cells were cultured in DMEM/F12 (50:50) medium, supplemented with 10% FBS, 2 mM L-glutamine, and 50 μg/ml gentamicin in a humidified incubator at 37°C with 5% CO_2.

[0059] The cDNA for PKC α was cloned into the bicistronic vector pIRES (Stratagene), which contains GFP for monitoring successful transfection. The cDNAs for PKC βII, and PKC ε (ATCC) were cloned into vector pYDual, which expresses a nuclear-targeted RFP (Ioannou, unpublished). A Rab9-YFP fusion construct (described in [5]) was used for Rab9 expression. Transient overexpression was achieved by transfecting cells at 70% confluency using the FuGENETM 6 reagent (Roche Diagnostics) according to the manufacturer's suggestions.

[0060] **Protein analyses**

[0061] Transfected cells were harvested with PBS containing 2 mM EDTA at 2 days

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post-transfection. Soluble and insoluble cell fractions were prepared as described previously [5]. Briefly, to obtain the soluble/cytoplasmic fraction, cells were incubated on ice for 30 min in cold "phospho" buffer [150 mM NaCl, 20 mM NaF, 100 μM Na$_3$VO$_4$, 20 mM Hepes, pH 7.5), 1% (v/v) Igepal, 10% (v/v) glycerol, and 1 μL/20 mg tissue of protease inhibitor cocktail] and then centrifuged for 20 min at 14,000 rpm at 4°C; the clear supernatant was frozen in aliquots at a concentration of 1 μg/μl. The pellet (insoluble fraction) was washed 3 times in ice-cold PBS containing 2 mM EDTA and then resuspended in a volume of "Triton" buffer [PBS, 1% (w/v) SDS, and 0.1% (v/v) Triton X-100] equal to the "phospho" buffer. This solution was boiled for 10 min and sonicated until the solution became clear. The protein concentration of this fraction was adjusted to 1 μg/μl according to the protein concentrations determined for the soluble/cytoplasmic fraction. Protein concentrations were determined using the fluorescamine method as we have described [36]. 4-12% Bis-Tris precast gel (Invitrogen, Carlsbad, CA) and then transferred onto a Protran membrane using an XCell II apparatus (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Blots were processed as described previously [27].

[0062] For Rab9 dissociation studies, 1.0 x 107 NPC1 3123 cells were collected in ice-cold PBS and lysed by sonication 4 times for 10s each. The lysate was centrifuged at 14,000 rpm for 10 minutes to separate the soluble from the insoluble fractions and the total protein concentration was determined using a modified Bradford assay (Bio-Rad, Hercules, CA). An equal amount of each insoluble fraction was mixed with each purified PKC isoform from a PKC isozyme panel (Sigma, St. Louis, MO) and incubated for 60 min at 37°C. An equal volume of each sample was resolved through a 4-12% Bis-Tris precast gel, transferred to a membrane, and processed as described above.

[0063] **Cholesterol esterification**

[0064] The preparation of [^{3}H] oleate substrate and esterification assays were performed as previously described [5]. Cells were treated with 50 μg/ml fatty acid for 2 days and then transfected with PKE α or PKC ε for 24 hrs before esterification assays. All values were generated in triplicate and normalized for total cell protein.

[0065] **Immunofluorescence microscopy**

[0066] For filipin staining in transfected cells, cells were transfected with PKC or Rab9 using Fugene 6 according to the manufacturer's recommendations. After 48 hrs, cells were stained with filipin as we have previously described [37]. Cells were mounted onto slides using Fluoromount-G (SouthernBiotech, Birmingham, AL) and analyzed on a Nikon Eclipse microscope fitted with a charge-coupled-device camera (Nikon, Melville, NY). Images were acquired with MetaVue software and then deconvoluted using AutoDeblur software from AutoQuant Imaging, Inc (Troy, NY). For quantitation of filipin fluorescence, cells were seeded at 3×10^5 cells/well in 6-well dishes and allowed to settle overnight, after which the medium was replaced with medium containing 10% lipoprotein deficient serum (LPDS) for 4 days. Cells were incubated with oleic/linoleic acids for 48 hours, DCPLA/DHA for 24 hrs, or diazoxide for 72 hrs before fixing and staining with filipin as we have previously described. Images were acquired using the same exposure time for all samples. Fluorescence intensity was determined using the integrated intensity function of MetaVue software; at least 150 cells were quantitated for each sample and each experiment was repeated 3 times. For analysis of sphingolipid transport, cells were incubated with oleic/linoleic acids, DCP-LA, or PMA for 48 hours before BODIPY-LacCer staining was performed as previously described [30].

[0067] **PKC Expression Increases the Levels of Soluble Vimentin in NPC1 Cells**

[0068] NPC1 cells with missense or null (NPC1o) mutations contain decreased or virtually undetectable levels of soluble phosphorylated vimentin relative to WT cells, respectively [5]. Furthermore, the vimentin present in NPC1 cells exists as large disorganized filaments (dephosphorylated state) near the plasma membrane. Thus, NPC1 cells behave essentially as vimentin-null cells, which, similar to NPC1 cells, are unable to esterify LDL-derived cholesterol [11]. In extending those studies, it was hypothesized that decreased vimentin phosphorylation was the result of protein kinase C (PKC) inhibition in NPC1 cells. In support of this, it was observed in that study that treatment of NPC cells with the PKC activator phorbol-12-myristate-13-acetate (PMA) increased levels of soluble vimentin and ameliorated the NPC lipid storage phenotype, whereas

conversely, treatment of WT cells with PKC inhibitors resulted in the disappearance of soluble vimentin in those cells. These results strongly implicate PKC in the maintenance of the soluble vimentin pool in cells and by extension normal lysosomal cholesterol efflux. Extending those studies by evaluating different PKC isoforms and their effects on soluble vimentin levels in NPC cells, it can be shown that the PKC isoforms α, βII, and ϵ have been implicated in vimentin phosphorylation [10, 17, 18]; therefore, the focus on these isoforms. They were transiently expressed in human NPC1 cells and their effects on soluble vimentin levels were characterized. Expression of PKC βII caused a significant increase in soluble vimentin levels (~38-fold higher than untransfected NPC1 cells), which was higher than the levels seen in WT cells (~20-fold higher than NPC1 cells), whereas expression of PKCs α or ϵ caused smaller but still significant increases (~3-fold and ~7-fold, respectively) in soluble vimentin levels (Figure 1). As a control, expression of Rab9 in these cells also led to a significant increase (~30-fold) in soluble vimentin, consistent with what was previously reported [5]. As noted all three isoforms resulted in an increase of soluble Rab9 levels to a similar degree (~2500-fold higher than untransfected NPC1 cells). Furthermore, insoluble vimentin levels decreased as soluble vimentin levels increased in PKCexpressing cells, suggesting that the increase in soluble vimentin was due to solubilization (phosphorylation) of insoluble vimentin (Figure 1A).

[0069] Similarly, in the severely affected NPC1o cells, which normally have almost no detectable soluble vimentin, expression of any of the three PKC isoforms resulted in increased levels of soluble vimentin (Figure 1B). With respect to vimentin solubilization, all three isoforms work equally well in the NPC1o cells, in contrast to the NPC1 cells, in which the βII isoform seemed to be the most effective in solubilizing vimentin. Furthermore, soluble Rab9 levels also increased to similar levels as a result of PKC expression (Figure 1B), a result also seen in PKC-expressing NPC1 cells (Figure 1A).

[0070] **PKC Expression Induces Rab9 Dissociation from Vimentin**

[0071] The observations that the small GTPase Rab9 is trapped in vimentin filaments in NPC1 cells [5] and that Rab9 overexpression corrects the NPC1 phenotype [19] strongly suggest that Rab9 availability is reduced in NPC1 cells. The disease cells may attempt to

compensate for this deficit by upregulating Rab9 protein expression. This idea is supported by the fact that NPC1 cells do contain more Rab9 protein than WT cells (Figure 1A and [19]). As described above, PKC expression increased not only soluble vimentin levels but also soluble Rab9 levels significantly (Figure 1A and 1B). To determine whether the increased Rab9 levels in PKC-expressing cells was a result of Rab9 release from insoluble vimentin after it was phosphorylated, PKC assays were performed in vitro with nine purified PKC isoforms using the insoluble vimentin fraction of NPC1 cell lysates as the PKC substrate. All isoforms were able to effect Rab9 release from insoluble vimentin to varying degrees (Figure 2), suggesting that, at least in vitro, most PKC isoforms can catalyze vimentin phosphorylation and Rab9 release. Interestingly, PKC α caused the greatest increase in Rab9 release, while PKC βII and ε were less effective and PKC γ was almost ineffective. These results differ from the results of transiently transfected PKC-expressing cells, which suggest that PKC βII is more effective in increasing soluble vimentin levels than PKC α and ε (Figure 1A). The discrepancies in isoform effectiveness may be due to the nature of the assays, the inherent activity of each isoform, or the in vivo subcellular location of the different isoforms and their access to vimentin [10,16,17,20]; however, it is clear that PKC is able to solubilize vimentin and in doing so release entrapped Rab9.

[0072] **Overexpression of PKCs Induces a Partial Correction of the NPC1 Phenotype**

[0073] Based on the data above and observations that Rab9 overexpression results in increased soluble vimentin and correction of the NPC1 phenotype [5], it was determined whether increased vimentin solubility caused by PKC overexpression would also result in correction of the NPC1 phenotype. NPC1 CHO (M12) cells containing a deletion of the NPC1 locus [21] were transfected with PKC α or PKC ε and the amount of LDL-derived free cholesterol transported from the E/L system to the ER for esterification by acyl-CoA: cholesterol acyltransferase (ACAT) [22] was measured. Esterification levels for M12 cells were less than 10% of the esterification activity of the parental WT CHO cells (Figure 3), which is consistent with a block in cholesterol transport out of the E/L system. Expression of PKC α or PKC ε ameliorated the cholesterol transport block, increasing the

level of M12 cell esterification by approximately 4- and 6.5-fold, respectively, over that of untransfected M12 cells (Figure 3, +PKCα, +PKCε). These results indicate that solubilization of vimentin mediated by expression of PKCs can partially release the NPC1 lipid transport block.

[0074] Cholesterol storage in PKC-transfected cells was also determined qualitatively by staining with filipin, a fluorescent probe that binds to free cholesterol [23]. This analysis yielded similar results to the esterification studies shown in Figure 3. Cells that were positive (as determined by GFP co-expression) for PKCα, PKCε, or PKCβII showed significantly less filipin staining than surrounding untransfected cells (Figure 4). These results were similar to those seen in cells over-expressing Rab9 (Figure 4, Rab9), which has previously been shown to correct the NPC1 cholesterol storage phenotype [19].

[0075] **Exposure to Fatty Acids Increases Soluble Vimentin Levels in NPC1 Cells**

[0076] Fatty acids and in particular oleic acid have been shown to induce PKC activity [24], whereas a downstream metabolite of linoleic acid, DCP-LA (8-[2-(2-pentyl-cyclopropylmethyl)- cyclopropyl]-octanoic acid), has been shown to potently activate PKC ε [25,26]. Furthermore, it was shown previously that NPC1 endosomes store large amounts of fatty acids [27], which could potentially limit the amount of free fatty acids available to the cell for PKC activation and other processes.

[0077] To determine if exogenously added fatty acids can increase vimentin solubilization in NPC1 cells, human NPC1 fibroblasts were treated with oleic or linoleic acid for 48 hours and the levels of soluble vimentin in cell lysates were analyzed. NPC1 fibroblasts contain very little soluble vimentin (Figure 5A and [5]). Treatment with either oleic or linoleic acid significantly increases the amount of soluble vimentin, with oleic acid being slightly more effective. These results suggest that exogenously added fatty acids can effect vimentin solubilization in NPC1 cells, presumably by activating PKCs.

[0078] **Exposure to Fatty Acids Induces Correction of the NPC1 Phenotype**

[0079] Since fatty acids increase solubilization of vimentin in NPC1 cells, they might also improve the NPC1 phenotype. M12 CHO cells (Figure 5B) and human NPC1

fibroblasts (Figure 5C) were treated with each fatty acid and then stained with filipin. WT cells (Figures 5B/C, 1) stain very weakly with filipin, whereas NPC1 (Figures 5B/C, 2) cells contain bright, punctate staining that is indicative of free cholesterol in endocytic vesicles. Filipin fluorescence in NPC1 cells from both species was significantly decreased after exposure to either fatty acid (Figures 5B/C, 3 and 4). Following quantitation of filipin fluorescence levels by integrated morphometry, both fatty acids were found to dramatically reduce the levels of cholesterol accumulation in both NPC1 cell lines, reducing filipin fluorescence to ~75% of levels in untreated cells (Figure 5B/C, graphs). Human fibroblasts exhibit more heterogeneous filipin staining patterns than either CHO or mouse NPC1 cell lines, which is reflected in the higher standard deviation in untreated 3123 cells (Figure 5C, graph). The effect of fatty acids on cholesterol esterification in M12 cells was also evaluated. Linoleic acid increased cholesterol esterification by greater than 2-fold compared to untreated M12 cells (Figure 3, +Lin). In M12 cells treated with oleic or linoleic acids followed by expression of PKC ε, the correction of the NPC1 phenotype was more pronounced, with esterification levels that were ~3.5-fold over untreated cells (Figure 3, +PKCε/oleic and +PKCε/linoleic). These results suggest that fatty acids and PKC expression have an additive effect on correction of the NPC1 cholesterol transport block.

[0080] To further characterize the effects of fatty acids on the NPC phenotype, the ability of docosahexanoic acid (DHA) and a metabolite of linoleic acid, DCP-LA, were tested for their ability to decrease cholesterol storage in M12 CHO cells. Both fatty acids have been shown to potently activate PKC ε [24,28]. Treatment of M12 CHO cells with these compounds overnight resulted in decreased cholesterol storage (Figure 6C/D) relative to untreated cells (Figure 6B). DCP-LA (Figure 6C) was slightly more effective than DHA (Figure 6D). Quantitation of filipin intensity in these cells revealed that both compounds reduced cholesterol storage in M12 CHO cells by ~50% (Figure 6G), lending further support to the positive effect of free fatty acids on the NPC disease phenotype, possibly through activation of PKC ε.

[0081] To provide further support for the role of PKCs in NPC rescue, M12 cells were treated with diazoxide, which has been shown to activate PKC ε [29]. This treatment

resulted in reduced cholesterol accumulation in M12 CHO cells (Figure 6F) relative to untreated cells (Figure 6E). Quantitation of the filipin intensity in these cells indicated that diazoxide reduced cholesterol storage by ~50%, similar to the results seen with the free fatty acids (Figure 6G).

[0082] To further confirm the positive effects of DCP-LA and fatty acids on the NPC1 phenotype, human NPC1 cells were treated with DCP-LA, fatty acids, or PMA to activate PKCs. Cells were then labeled with BODIPY-LacCer, which has previously been shown to provide a dynamic view of the endocytic pathway [30]. Following absorption to the plasma membrane, the LacCer sphingolipid enters normal cells via endocytosis and eventually reaches the trans-Golgi network (TGN; [30]). Due to the lipid transport block in NPC1 cells however, this sphingolipid is trapped in endosomes and its targeting to the TGN is dramatically inhibited [30]. Human NPC1 cells treated as indicated in Figure 7 show a dramatic improvement in lipid transport with the BODIPY-LacCer effectively reaching the TGN (Figure 7; arrows) compared to untreated cells that show only punctate, endosomal fluorescence (Figure 7A). These results provide further support that these agents are able to release the NPC1 lipid block.

[0083] Taken together, these results indicate that exposure to free fatty acids, which may act by activating PKCε, has a positive effect on the NPC cholesterol storage phenotype.

[0084] **Discussion**

[0085] Rab9 expression in NPC1 cells restored lipid transport from the E/L system and normalized cholesterol esterification [19] and subsequently showed that Rab9 was entrapped in insoluble vimentin filaments in NPC1 cells [5]. Consequently, accumulated lipids, such as sphingosine [31, 32] in NPC1 cells, might exert an inhibitory effect on various PKC isoforms, resulting in a disruption of the vimentin phosphorylation/dephosphorylation cycle [19].

[0086] To characterize the nature of PKC inhibition and vimentin hyposphorylation in NPC1 cells, a number of PKC isoforms (α, βII and ε) in NPC1 cells were expressed and their effect characterized on vimentin solubilization and correction of the NPC1

phenotype. All three isoforms had a positive effect on vimentin solubilization to varying degrees (Figure 1).

[0087] Furthermore, PKC-induced vimentin solubilization was accompanied by the release of the entrapped Rab9 (Figure 1). To further determine which PKC isoforms might be more effective in vimentin phosphorylation and release of Rab9, eight different PKC isoforms were tested in an in vitro assay. Most isoforms were able to release Rab9 from vimentin to varying degrees (Figure 2), which may not be in case in vivo. This discrepancy is likely due to the different in vivo subcellular locations of PKC isoforms and their access to vimentin filaments [16, 20]. However, it is also possible that vimentin may not be a direct substrate for certain PKCs, as has been shown with regards to PKC ε-controlled phosphorylation of vimentin [10]. In that study, PKC ε mediated vimentin phosphorylation, which was shown to be critical for proper integrin recycling through the cell. These studies indicate that expression of PKC isoforms in NPC1 cells results in the partial correction of the NPC1 disease phenotype, i.e., cholesterol accumulation in the endosomal/lysosomal system.

[0088] Also, many studies have shown that long chain fatty acids such asoleic and linoleic, along with downstream metabolites such as DCP-LA, are able to activate PKC ε [24, 25], an isoform shown to phosphorylate vimentin filaments [10]. Since it has been previously reported that the availability of free fatty acids may be limited in NPC1 cells [27,33], exogenously added fatty acids were considered to have a positive effect on the NPC1 phenotype, presumably by activating PKC ε and leading to phosphorylation of vimentin and release of Rab9. As predicted, addition of oleic acid, linoleic acid, or DCP-LA resulted in an increase of soluble vimentin in NPC1 cells (Figure 5). Furthermore, fatty acid addition resulted in a significant improvement in cholesterol esterification by NPC1 cells (Figure 3), indicating that lipid transport from the E/L system was restored. The ability of diazoxide, a known activator of PKC ε [29], was tested to correct the NPC1 phenotype, providing further support for the involvement of insufficient PKC phosphorylation of vimentin in contributing to NPC1 pathogenesis. In agreement with the results presented here, diazoxide was able to reduce cholesterol accumulation in NPC1 cells by 50% (Figure 6). Although results with multiple PKC ε activators strongly suggest

that PKC ε is mediating these changes within the NPC cells, the possibility that these agents may be acting through some other pathway or protein besides PKC cannot be excluded.

[0089] These data are consistent with previous observations of aberrant PKC expression in NPC mouse liver [34]. In those studies the expression of PKC α, δ, ε, and ζ were evaluated by immunoblot. Whereas PKC α and δ were about 3-fold higher in NPC1 livers compared to WT livers, PKC ε was not significantly increased and PKC ζ was higher only in heterozygous livers. It is interesting to postulate that PKC ε does not render itself amenable to upregulation but can be activated, via fatty acids for example, and such activation can yield beneficial results in NPC1 cells. There is strong evidence that PKC ε is responsible for phosphorylating vimentin, which in turn controls the vesicular transport of various ligands such as integrins [10]. Considering the difficulties of delivering proteins as therapeutics, which are significantly amplified in diseases with neuropathology such as NPC1, a small lipid activator of a key regulator such as PKC ε would be greatly advantageous. These results suggest that identification of the PKC isoform(s) responsible for vimentin phosphorylation may provide new therapeutic targets for the treatment of Niemann-Pick type C disease, as well as other lysosomal storage disorders that lead to E/L lipid accumulation [35].

[0090] **Example 2**

[0091] We also studied the effect of the PKC activators bryostatin 1 and DCPLA on the phenotype of Niemann-Pick C disease using probes for free cholesterol (Filipin), glycosphingolipid levels (VTB) and gangliosides movement (CTB), following treatment of human NPC1 cells for 48-72 hrs.

[0092] **Materials and Methods**

Table 1: Human NPC1 Cell Lines

NPC3---SV:	SV40 largeT immortalized
Species:	Human
Source:	Mount Sinai-Proprietary
NPC genotype:	V1165M and 3741-44delACTC

NPC24---SV:	SV40 largeT immortalized
Species:	Human
Source:	Mount Sinai-Proprietary
NPC genotype:	p.I1061T/p.I1061T

Table 2: Reagents

PKC Activators:	Bryostatin 1, DCPLA, Diazoxide
Source:	Santa Cruz Biotech, Sigma-Aldrich

Staining Reagents:	Filipin, Cholera toxin B, Verotoxin B
Source:	Polysciences, Sigma-Aldrich, MS Proprietary

[0093] **General Methodology**

[0094] Cells are plated in 6-well dishes and treated with the appropriate compound (dissolved in DMSO) at the indicated dose daily for 48 hrs. Control cells receive DMSO. After 48 hrs cells are transferred to cover slips with fresh compound and grown for another 24 hrs. Cover slips are collected and processed for microscopy.

[0095] **Assay Protocol**

[0096] *Filipin*: For detection of unesterified cholesterol in lysosomes, cells were fixed in formalin for 30 min at 4°C, washed 2X, 5 min in 0.9% NaCl, incubated for 45 min with 0.01% filipin in PBS at room temperature, and then washed 2X, 5 min. Fluorescence was observed using a Nikon Eclipse fluorescence microscope (Nikon, Melville, NY) equipped with a CCD camera. Fluorescence signals were quantified using Nikon's imaging and quantitation software package *NIS Elements,* v.3.22.

[0097] *Verotoxin B (VTB)*: For VTB staining (glycosphingolipid detection) cells are washed with PBS and fixed in formalin, 30 min, 4°C. Following a wash with 0.9% sodium chloride, 2X, 5 min at room temperature cells are permeabilized with digitonin 50μg/well in 1.5ml PBS. Cells are washed with PBS, and alexa-labeled VTB is added at 0.5μg/well in 1ml PBS. Cells are incubate 45min at RT on a shaker in the dark, washed with 0.9% sodium chloride, 2X, 5 min at RT, and mounted for viewing.

[0098] *Cholera toxin B (CTB)*: For CTB labeling (ganglioside movement) cells are washed with PBS, and 0.5 μg/well CTB is added in 1.5 ml Opti-MEM per well. Cells are incubated in a 37°C incubator for 1 hr. Complete DMEM with FBS media is added and incubation is continued for 4 hr at 37°C. Cells are washed with PBS and fixed with formalin for 15 min at 4°C. Cells are washed with 0.9% sodium chloride, 2X, 5 min at RT and mounted for viewing.

[0099] **Analysis, Results and Conclusions**

[00100]   Niemann--Pick C1 (NPC1) cells have a defective NPC1 protein and are characterized by the extreme accumulation of a number of lipids such as cholesterol, sphingolipids and gangliosides in various endosomal vesicles (Figure 2). In the characterization of NPC1 cells from various patients and evaluation of potential compounds that may be beneficial in treating this disease a number of different assays that measure the clearance and/or movement of various NPC1-specific lipids were performed. For example filipin was used to detect the level of stored cholesterol (Assay protocol) whereas verotoxin B (VTB) allows the assessment of the levels of glycosphingolipids which are sometimes stored in endosomes distinct from those that contain cholesterol (Assay protocol). In addition, movement of gangliosides from the plasma membrane by using a different probe, cholera toxin can be monitored (Assay protocol).

[00101]   These studies indicate that all three PKC activators, bryostatin 1, DCPLA and diazoxide are able to induce clearance of cholesterol from NPC1 cells to varying degrees and at different drug concentrations (Figure 8). However, bryostatin 1 appears to be the most active compound showing activity in the nanomolar concentration range (Figure 9). At 10 nM bryostatin 1 also shows statistically significant effectiveness in decreasing the glycosphingolipid accumulation of NPC1 cells (Figure 10).

[00102]   To determine whether the effects of bryostatin 1 is dependent on the genotype of the NPC1 cells used in the above experiments (i.e. the specific NPC1 gene mutations carried by a particular patient) a different patient cell line with a completely distinct NPC1 genotype was used. Bryostatin 1 was still able to effect cholesterol

clearance from these cells at 10 nM concentration indicating that the activity of bryostatin 1 is independent of the NPC1 genotype (Figure 11).

[00103]   Bryostatin 1 also was effective in a kinetic assay that monitors the movement of lipids from the plasma membrane to the Golgi network through endosomes. At 10 mM bryostatin 1 was able to clear the endosome--trapped cholera toxin (CTB) from NPC1 cells (Figure 12).

[00104]   To confirm these results bryostatin 1 was used at a range of 0.1 nM to 100 nM and evaluated both in cholesterol and sphingolipid assays (Figure 13). At 0.1 nM bryostatin has no effect in both assays. The effect becomes statistically significant at 1 nM and increases with increasing bryostatin 1 concentration.

[00105]   Accordingly, bryostatin 1 showed a positive therapeutic effect for human NPC1 disease cells at a concentration of 10-100 nM.

[00106]   **Example 3**

[00107]   Niemann-Pick C disease is a severe inherited lipidosis that leads to neurodegeneration and early childhood death. The biochemical and cellular events that lead to neurodegeneration are currently poorly understood. However, it has been shown that PKCε activation can restore the blocked lipid transport pathway and lead to a reduction of stored lipid material in the NPC endosomes/lysosomes. Thus, treatment of NPC1 mice with bryostatin 1, a natural product activator of PKCε, should lead to an improvement of disease progression in this animal model.

[00108]   A total of 30 C57Bl6 NPC1 mice, mixed sex, were used. These mice were separated into 5 groups of 5 mice each, Groups 1-5.

[00109]   **Study Drug**

[00110]   Bryostatin 1 (purity ≥95%) from Aphios (Woburn, MA), 1 vial 2 mg, was solubilized in a 5% DMSO, 20% Solutol and 75% Saline solution and used as the study drug. The negative (vehicle) control is a 5% DMSO, 20% Solutol and 75% Saline solution. DCP-LA from Sigma-Aldrich: 5 mg oil/vial was used as an additional in vitro

active compound. Bryostatin 1 (API) was stored at or below -20°C and formulated as needed. The formulated bryostatin 1 was stored at 2° to 8°C for less than 24 hour(s). The DCP-LA and the vehicle controls were stored at 2°-8°C.

[00111]   The stock solution of bryostatin 1 is 10mg/ml in DMSO, which is kept at -80°C in aliquots. The dosing formulation is made by diluting the stock first in DMSO, then adding solutol, and lastly saline—with more insoluble compounds, adding the drug stock to complete vehicle will cause the compound to come out of solution, so we make formulations stepwise. The dosing volume is always 100 µl. Mice weigh approximately 20 g.

[00112]   **Dosing and Frequency**

[00113]   The study agents are dosed intraperitoneally (IP) as shown in Table 3. The mice are dosed twice weekly (Mon and Thu) starting at 30 days for up to 150 days.

Table 3: Dosing regimen

Group	Agent	Dosing
Group 1:	Vehicle control (mL)	
Group 2:	Bryostatin-1	[BHD-1] 40 µg/kg (120 µg/m^2)
Group 3:	Bryostatin-1	[BHD-2] 30 µg/kg (90 µg/m^2)
Group 4:	Bryostatin-1	[BHD-3] 20 µg/kg (60 µg/m^2)
Group 5:	Bryostatin-1	[BHD-4] 10 µg/kg (30 µg/m^2)
Group 6:	Bryostatin-1	[BHD-5] 5 µg/kg (15 µg/m^2)
Group 7	Active	(DCP-LA 3 mg oil/kg)

[00114]   **Study Duration**

[00115]   C57 NPC1 mice have an average life span of ~110 days. By Day 70 mice reach an average weight of 18-22 grams. Untreated NPC1 mice develop ataxia and will begin to lose weight at ~70 days (see Figure 17). Ataxia (or lack of voluntary muscle control) is a clinical feature of NPC1 mice, which may be described as shaking. Treatment will be initiated at ~30 days of age, average NPC mouse weight of 10 g. If the drug is efficacious injections will continue past day 70-80. An extension of life of about 20-30% will mean that animals will need to be treated up to day 130-150.

[00116]   **Data Collection**

[00117]   Initially mice will be weighed and weights will be recorded prior to each injection. Mice may periodically be tested in a rotarod and time on rotarod will be recorded.

[00118]   Upon euthanasia blood will be collected and the brains will be frozen and stored for evaluation of cholesterol storage and Purkinje cell survival. In addition, the livers and spleens from these mice will be extracted, prepared for histology and stored frozen for the option of determining the effects of bryostatin 1 treatment on peripheral organ lipid accumulation.

[00119]   **Data Analysis**

[00120]   Primary outcome will be weights collected on dosing days (2x/week) over the course of the study. Differences will be examined between vehicle control and different doses of bryostatin 1, particularly after age 70 days, when animals have historically experienced a weight loss.

[00121]   Secondary outcomes include: (1) lipid accumulation in liver and spleen, and (2) cerebellar Purkinje cell survival and cholesterol storage (which are to be conducted only if the primary outcome is positive).

[00122]   **Results**

[00123]   Mice in dose groups 30, 20 and 10 μg/kg lived past the age of 100 days.

[00124]   One of skill in the art will appreciate that the examples herein are not intended to be limiting and that one of skill in the art will readily be able to apply the teachings herein to treating lipid storage disorders. Therefore, the present invention is well adapted to attain the ends and advantages mentioned as well as those that are inherent therein. While numerous changes may be made by those skilled in the art, such changes are encompassed within the spirit of this invention as illustrated, in part, by the appended claims.

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We claim:

1.   A pharmaceutically effective amount of bryostatin 1 for use in treatment of
        Niemann-Pick Type C disease.

2.   A pharmaceutically effective amount of broystatin 1-20, a bryolog, a
        polyunsaturated fatty acid, or combinations thereof, for use in treatment of
        Niemann-Pick Type C disease or a lipid storage disorder.

3.   The method of claim 1 or 2 wherein the pharmaceutically effective amount is
        from about 0.0000001 mg/kg to about 250 mg/kg per dose.

4.   The method of claim 1 or 2 wherein the pharmaceutically effective amount is
        from about 0.00001 mg/kg to about 5.0 mg/kg per dose.

5.   The method of claim 1 wherein the pharmaceutically effective amount of
        bryostatin 1 is provided in a dose from 0.01-25 $\mu g/m^2$ IV.

Drawings

Figure 1
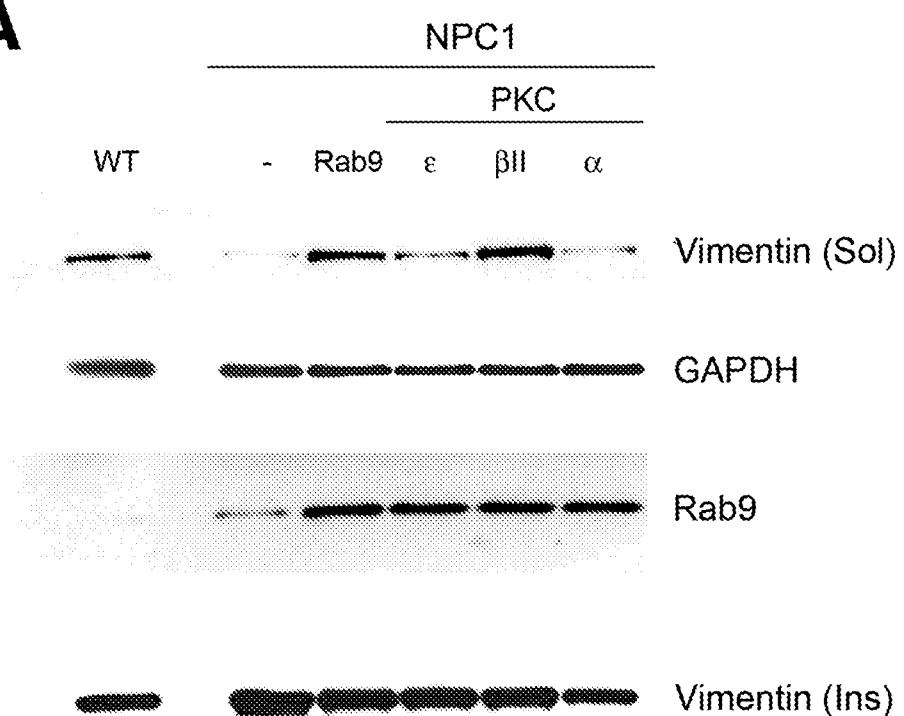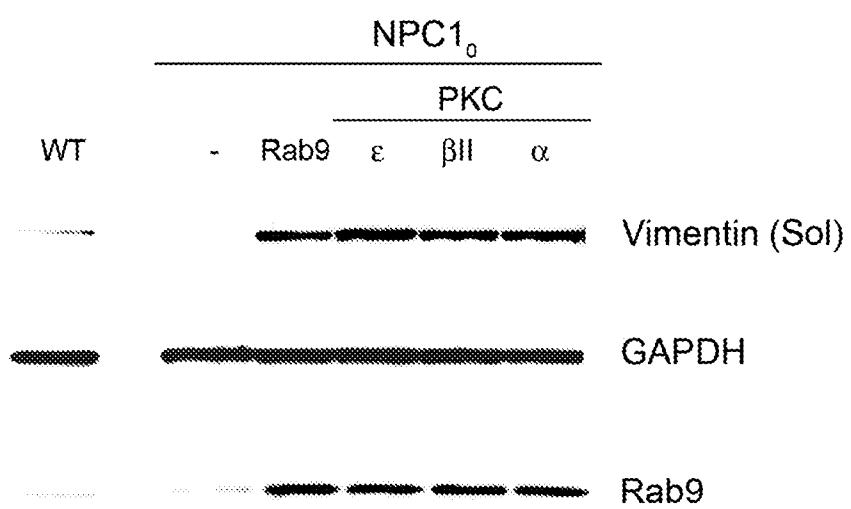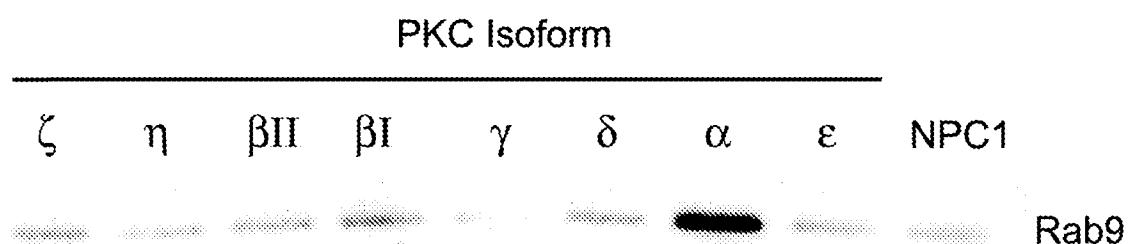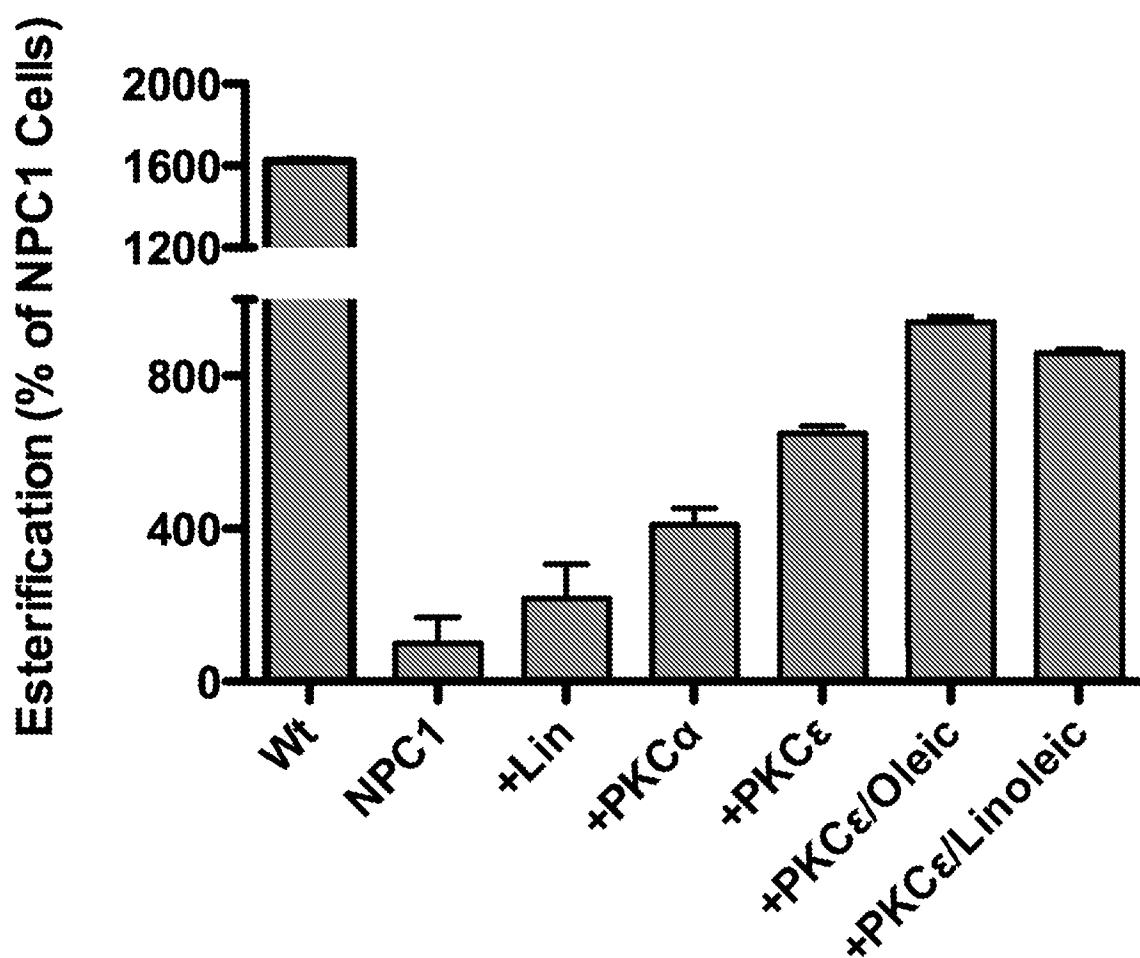
Figure 2

Figure 3

Figure 4
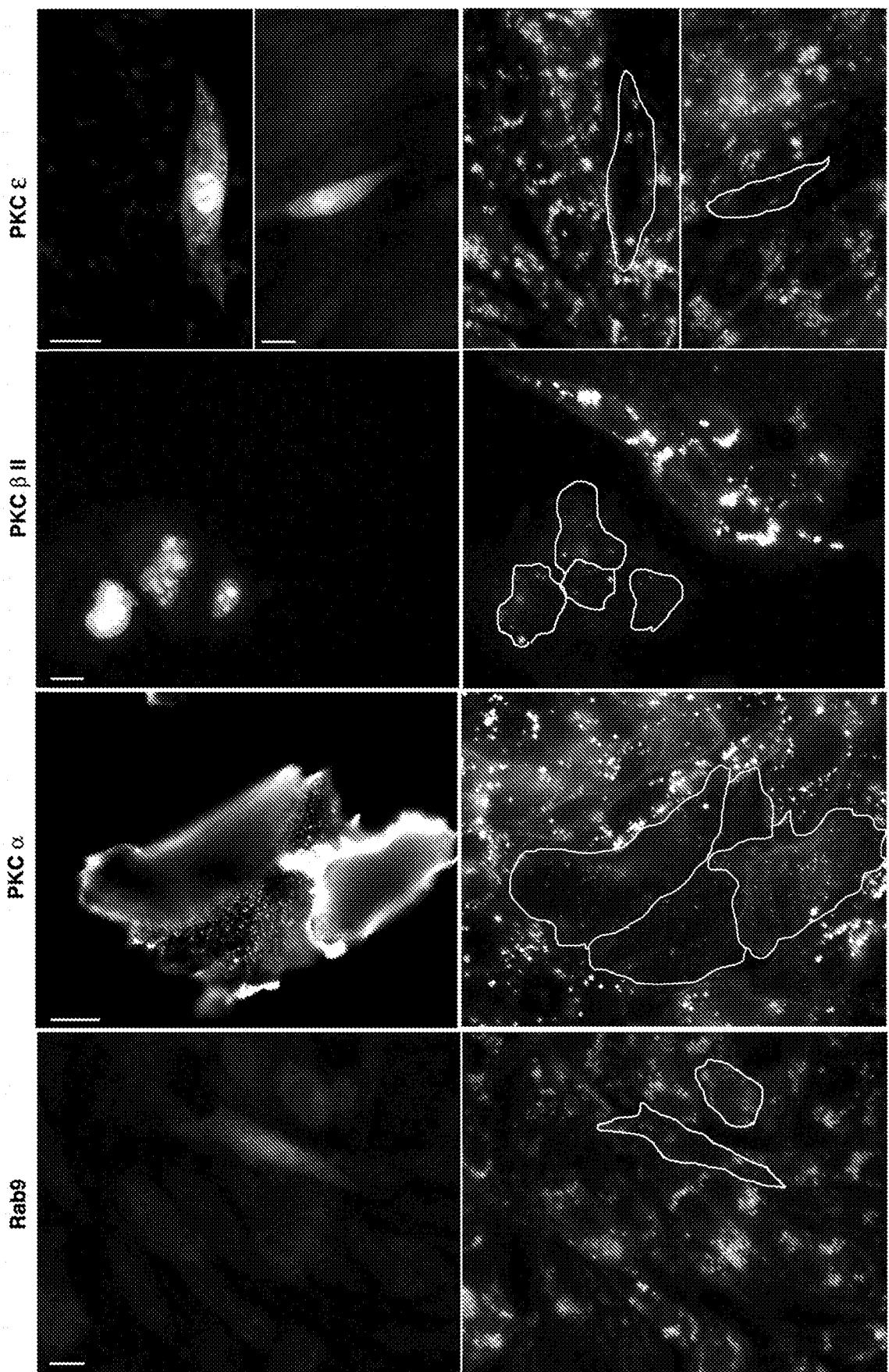
Figure 5

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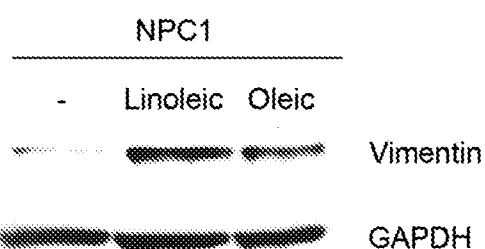
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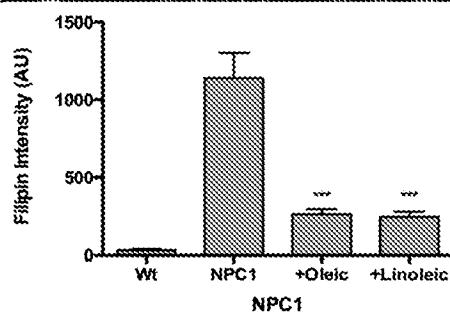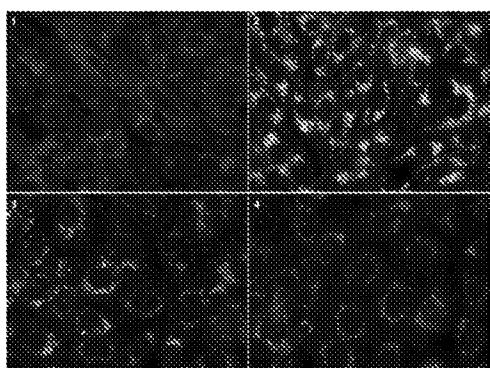
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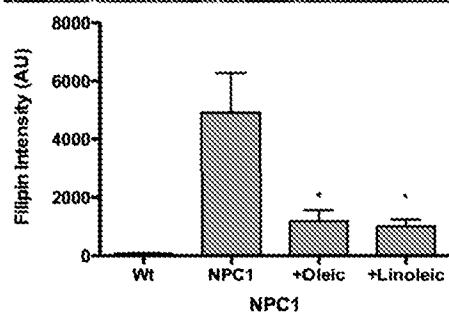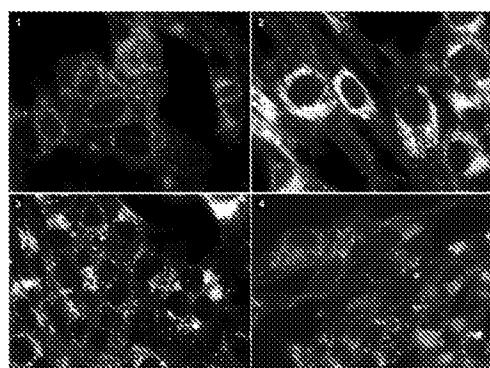
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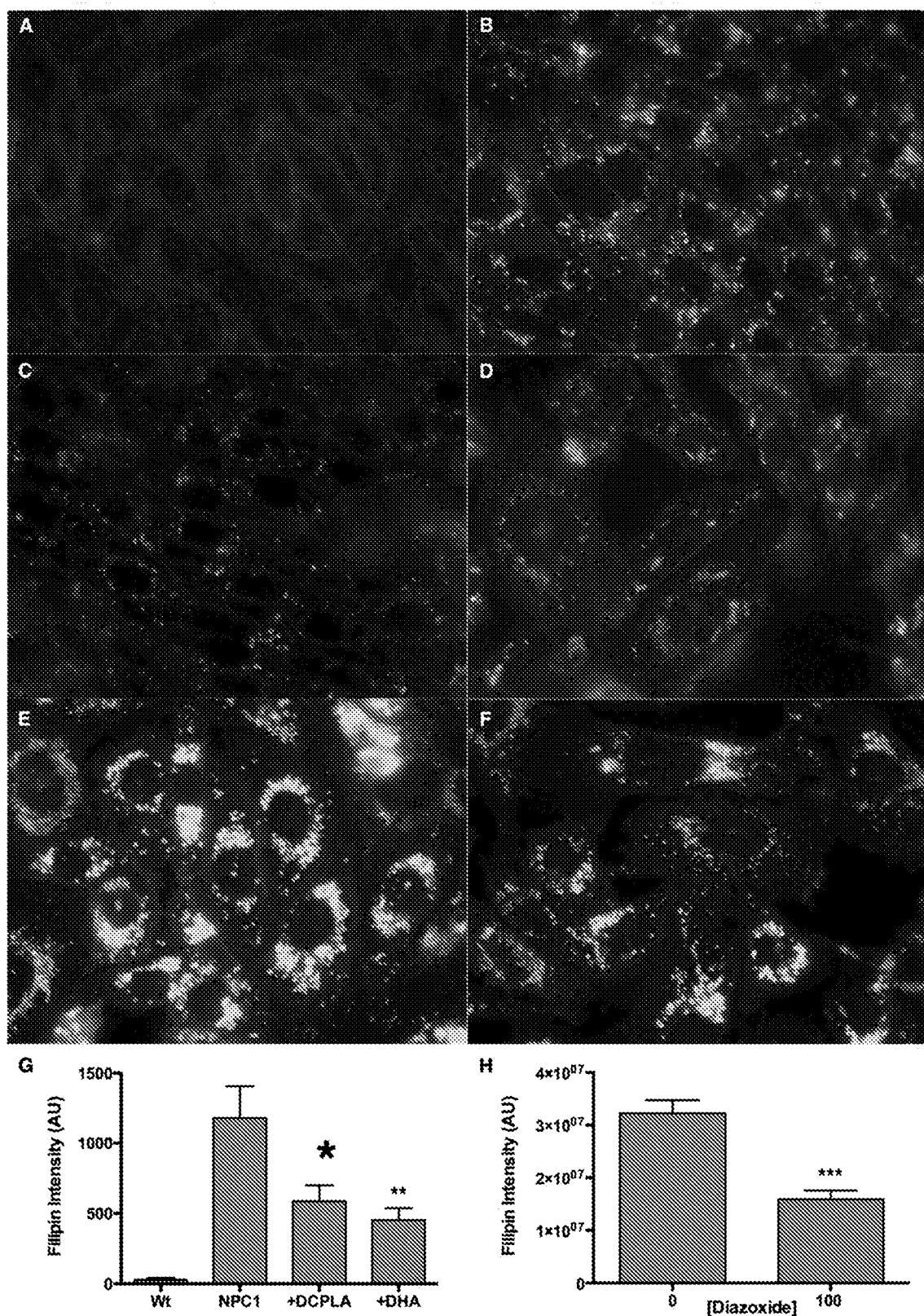
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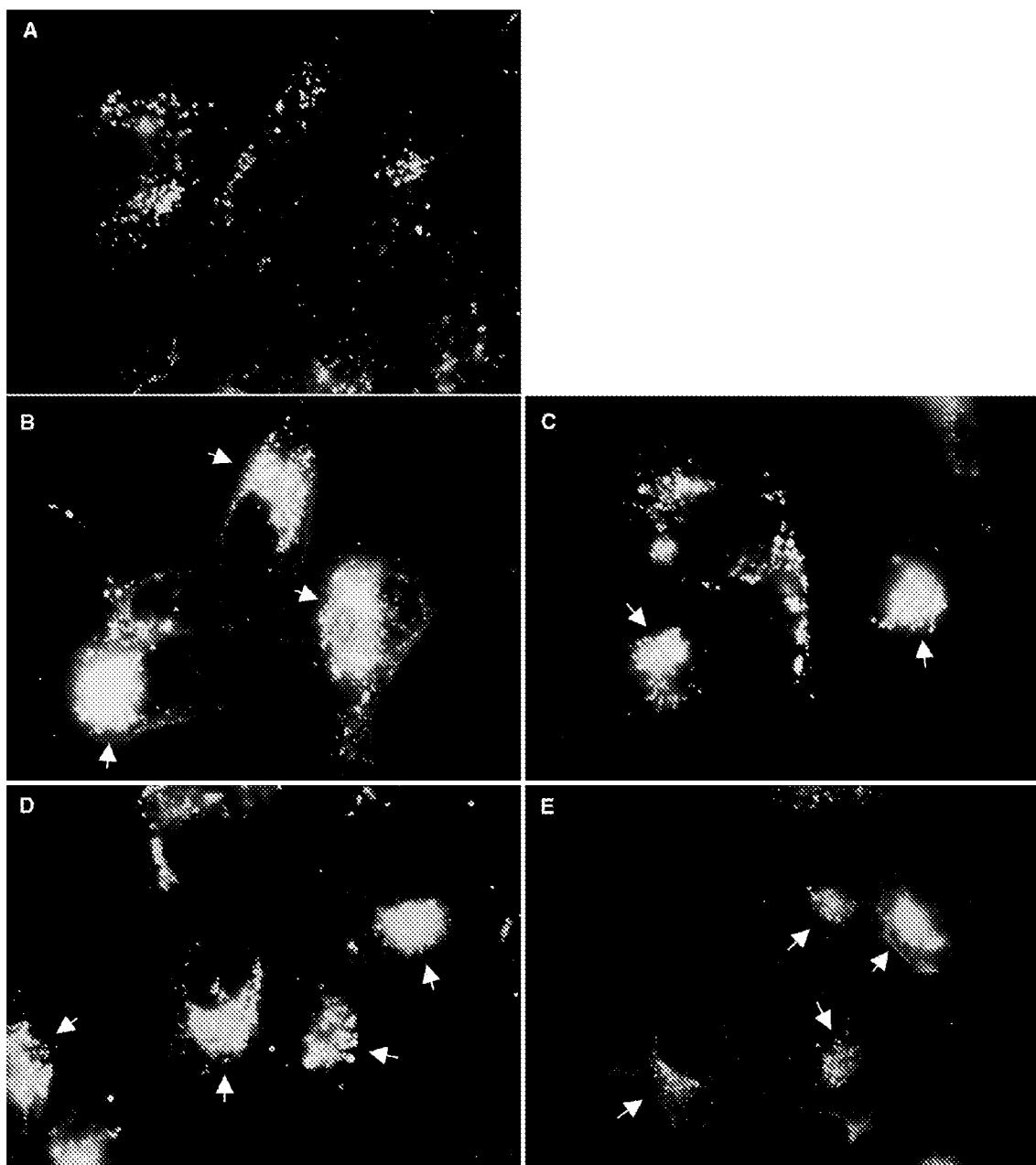
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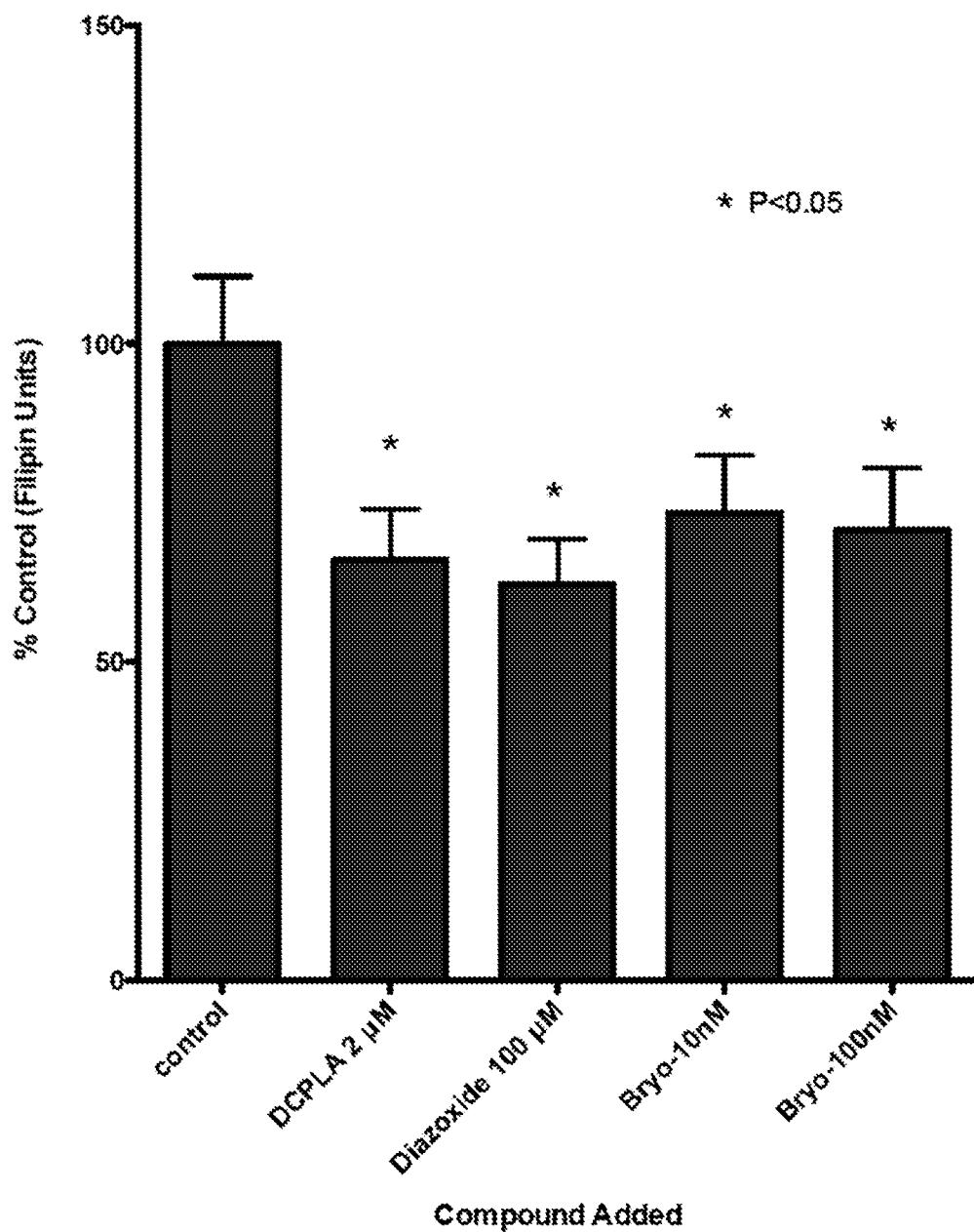
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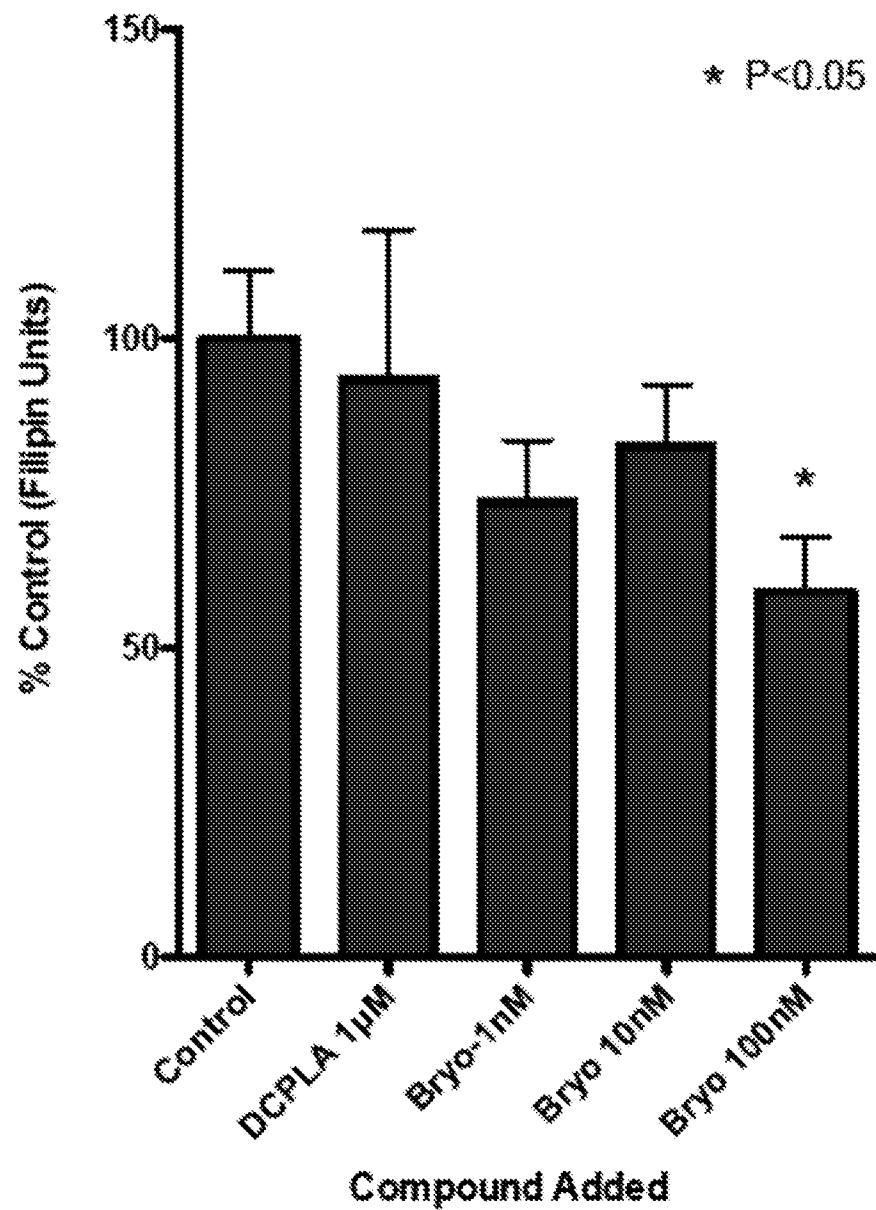
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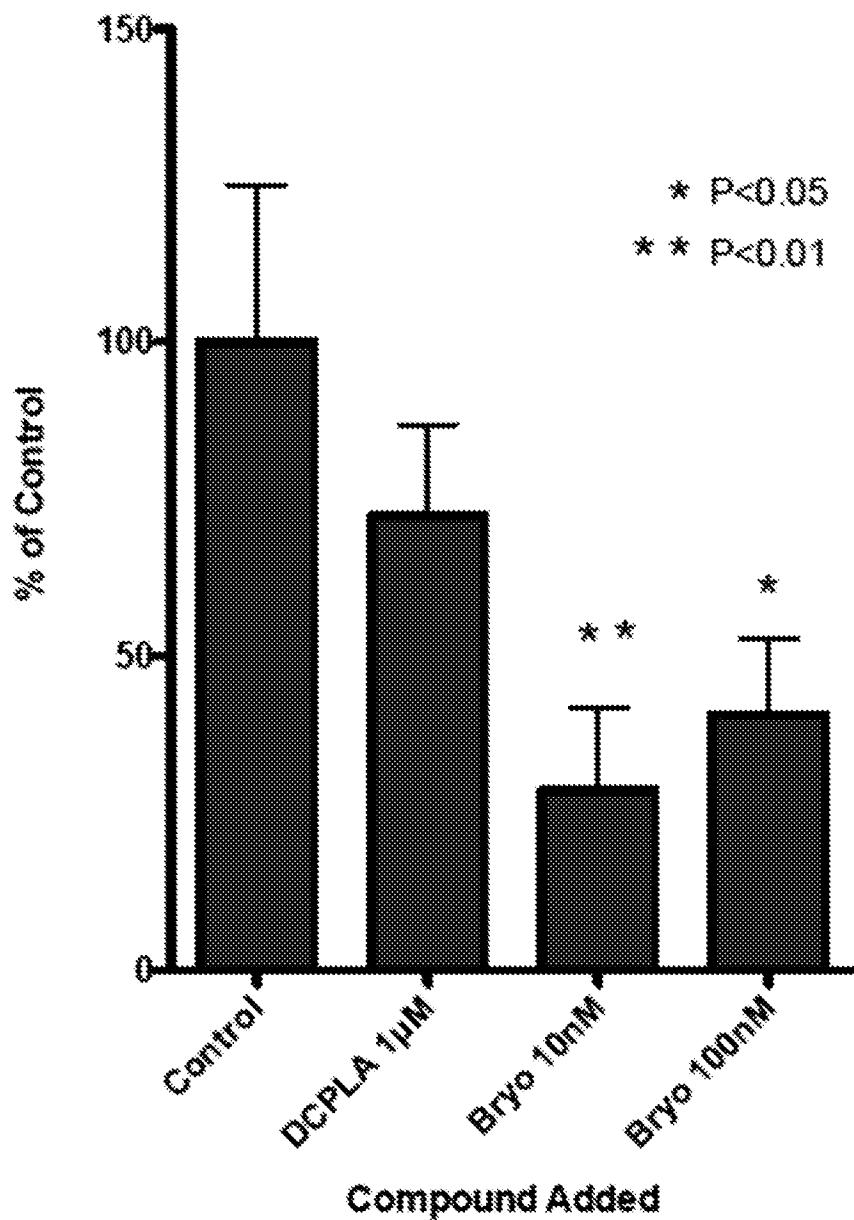
FIGURE 11

FIGURE 12

NPC3/CTB

Control

Bryo 10 nM

DCPLA 2μM

FIGURE 13

Cholesterol

* P<0.05
** P<0.005
*** P<0.0005

FIGURE 14

FIGURE 15

Cholesterol   Glycosphingolipids

DMSO

Bryostatin
0.1 nM

1 nM

10 nM

100 nM

FIGURE 16

Glycosphingolipid containing endosomes

Cholesterol containing endosomes

Cholera toxin (CTB)
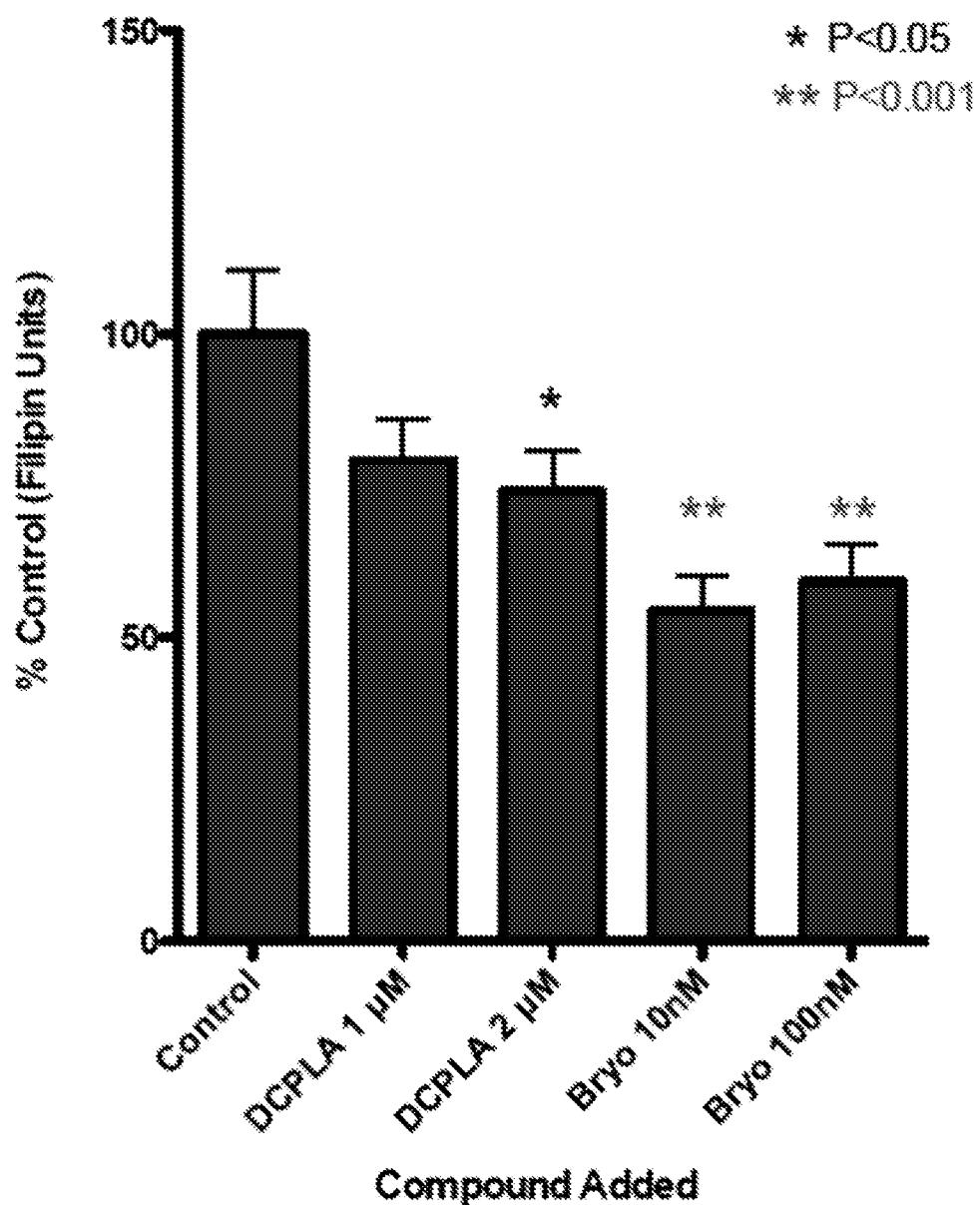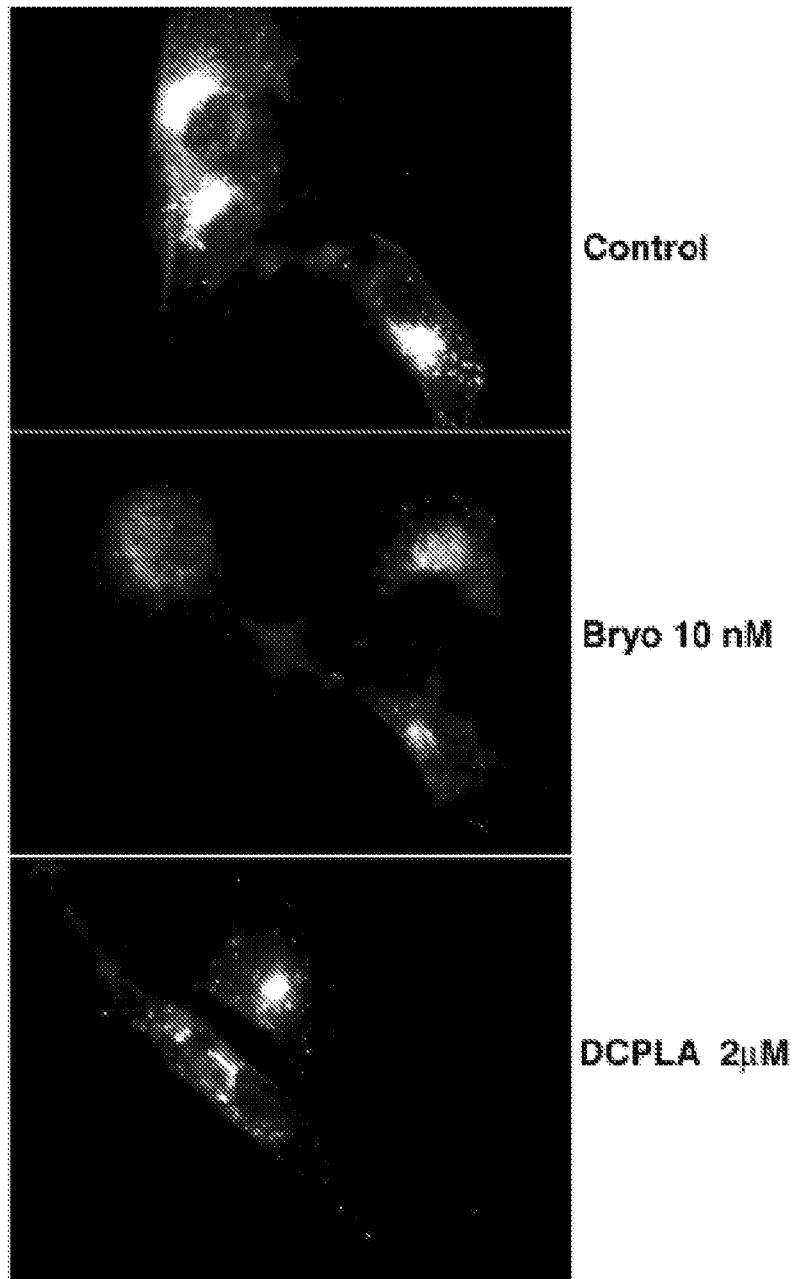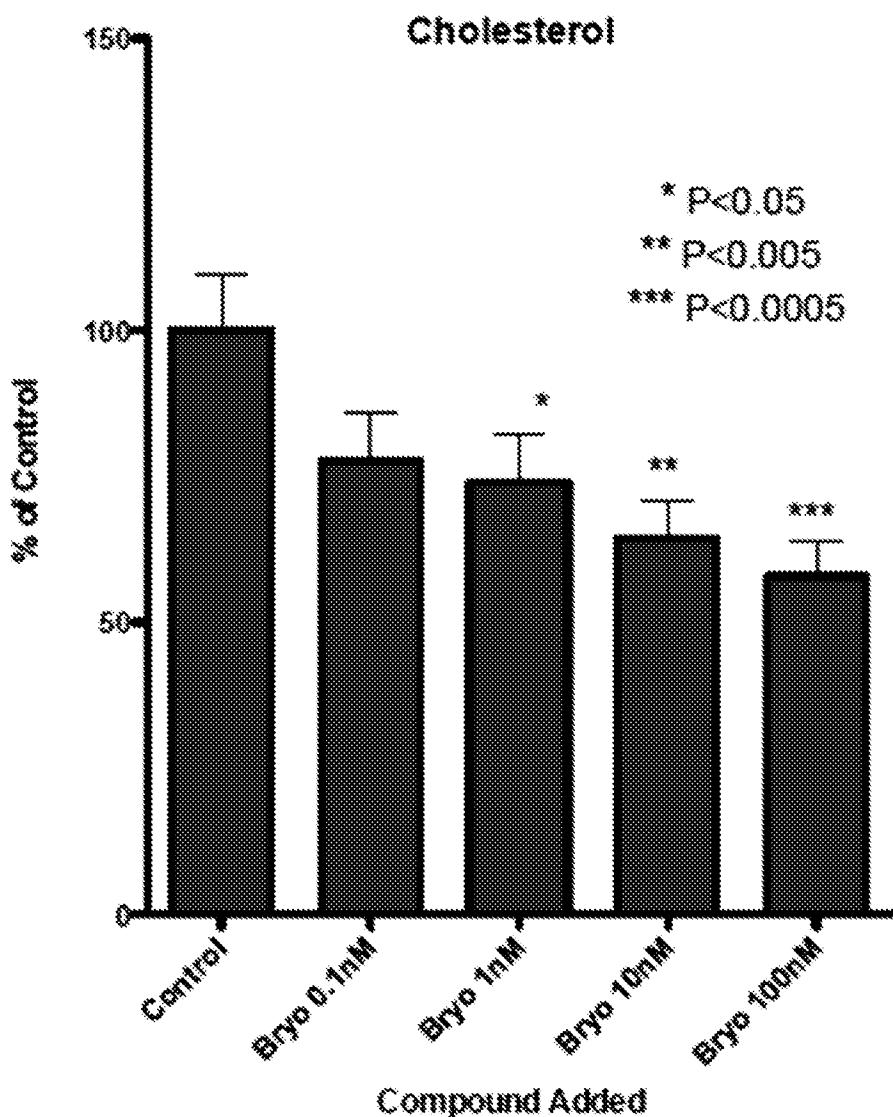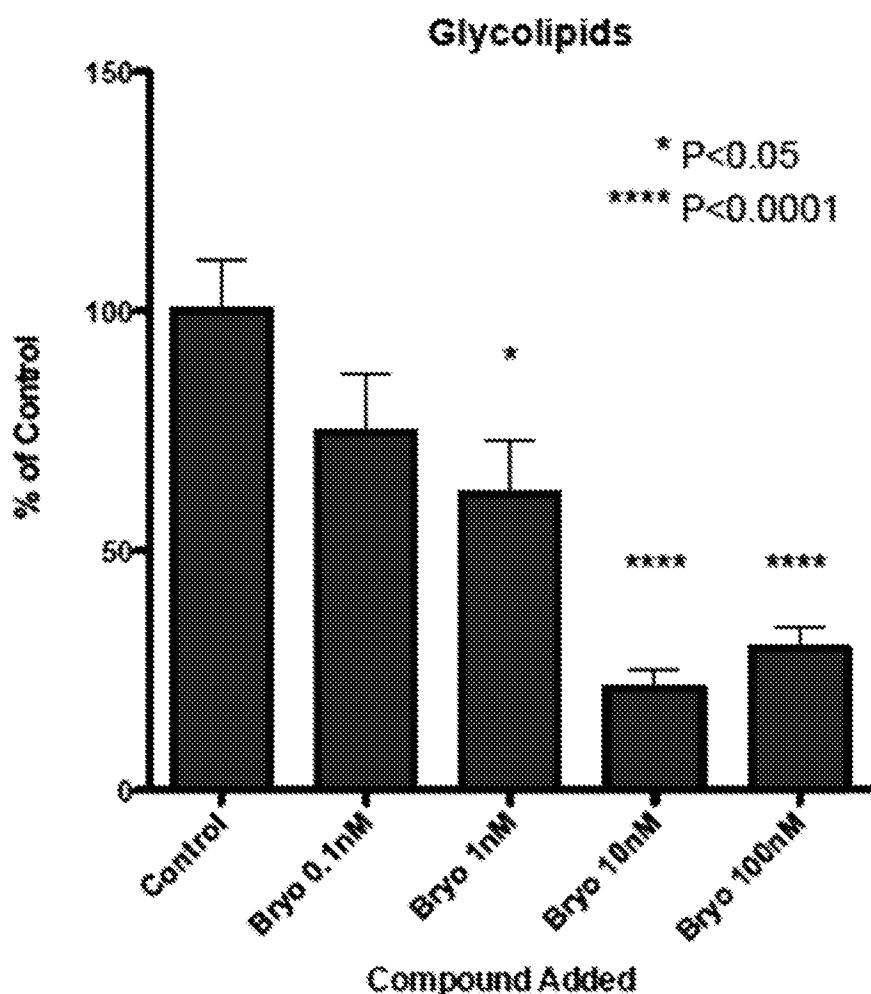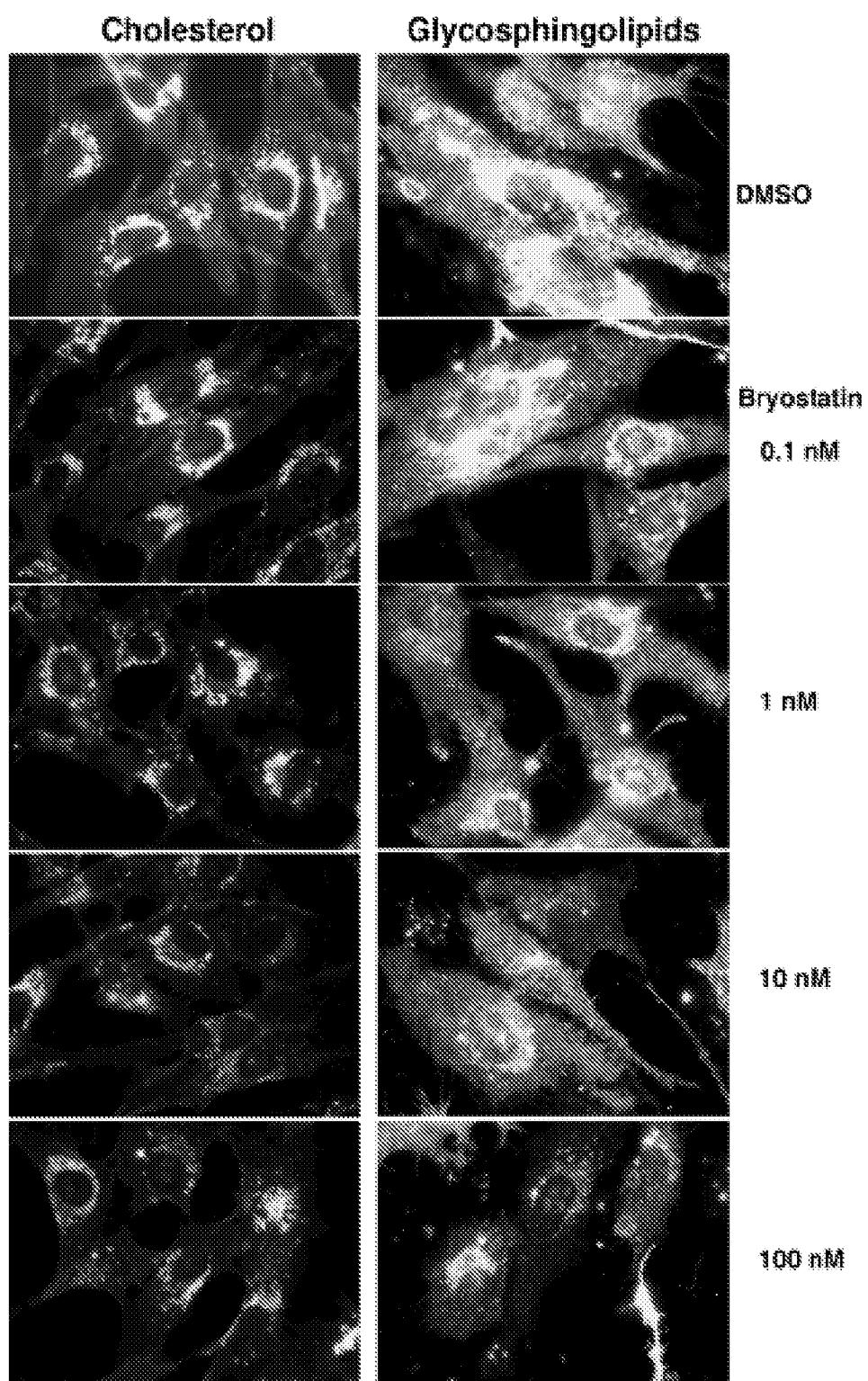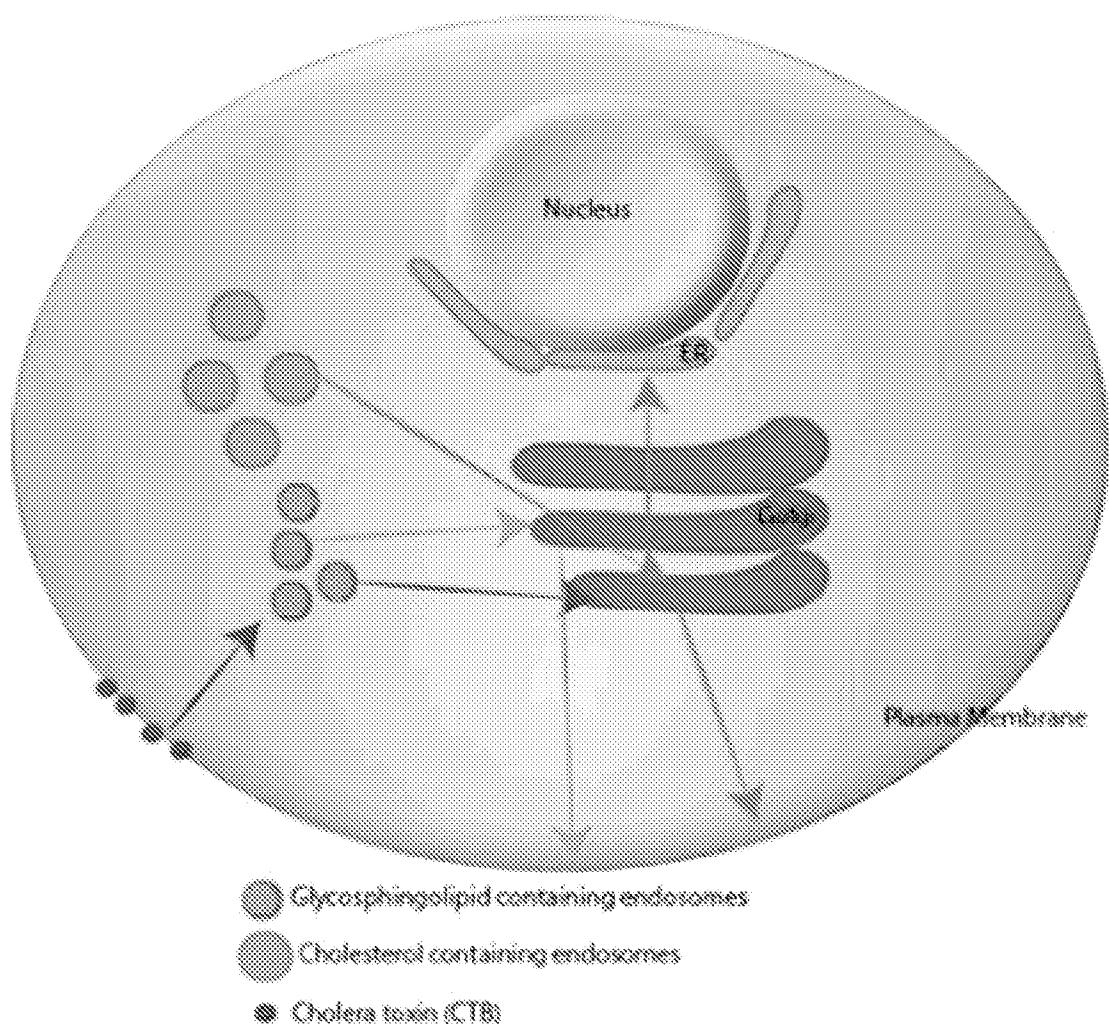
Figure 17

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - A61K 31/365 (2015.01)
CPC   - A61K 31/365 (2015.05)
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)
IPC(8) - A61K 31/365; A61P 3/00, 43/00; C07D 493/22 (2015.01)
CPC - A61K 31/365; C07D 409/12, 417/12, 493/22 (2015.05) (keyword delimited)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC - 424/94.61; 435/375 (keyword delimited)

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

PatBase, Google Patents, Google Scholar.
Search terms used: bryostatin niemann-pick type c disease macrolide lactones

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2009/099563 A2 (BLANCHETTE ROCKEFELLER NEUROSCIENCES INSTITUTE) 13 August 2009 (13.08.2009) entire document	1-5
A	US 2012/0156180 A1 (BONGARZONE) 21 June 2012 (21.06.2012) entire document	1-5

☐ Further documents are listed in the continuation of Box C.   ☐

*	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 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

"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	Date of mailing of the international search report
02 June 2015	0 1 JUL 2015

Name and mailing address of the ISA/US	Authorized officer:
Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Blaine R. Copenheaver PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774