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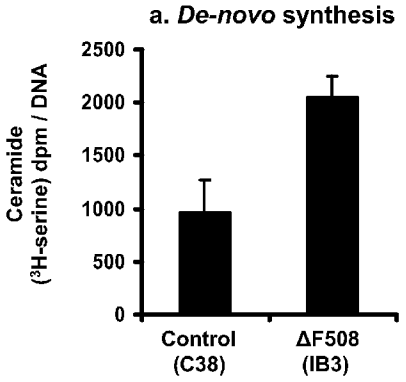
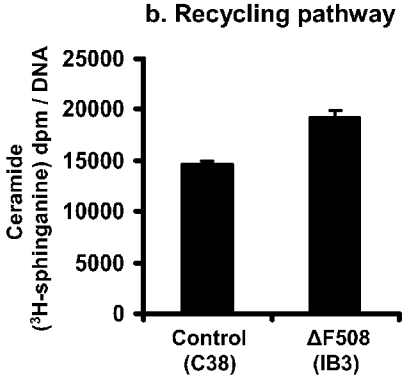
(54) Title: DEFECTIVE CYSTIC FIBROSIS TRANSDUCTION REGULATOR (CFTR) CAUSES INCREASED SPHINGOLIPID SYNTHESIS




(57) Abstract: The invention relates to methods and compositions for treating cystic fibrosis and *P. aeruginosa* infections by administering a therapeutic amount of one or more agents that inhibit *de novo* sphingolipid synthesis or recycling pathways.

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**Declarations under Rule 4.17:**

— *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))*

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# DEFECTIVE CYSTIC FIBROSIS TRANSDUCTION REGULATOR (CFTR) CAUSES INCREASED SPHINGOLIPID SYNTHESIS

## CROSS-REFERENCE TO RELATED APPLICATIONS

**[0001]** This application claims benefit of Provisional Appln. 60/818,351, filed 07/03/2006, the entire contents of which are hereby incorporated by reference as if fully set forth herein, under 35 U.S.C. §119(e).

## STATEMENT OF GOVERNMENTAL INTEREST

**[0002]** This invention was not made with Government support.

## BACKGROUND OF THE INVENTION

1. Field of the Invention

**[0003]** The present invention describes certain aspects of abnormal sphingolipid metabolism in cystic fibrosis, and provides therapeutic methods for treating cystic fibrosis and *P. aeruginosa* infections.

2. Description of the Related Art

**[0004]** Cystic fibrosis (CF) is the most frequent severe autosomal recessive disease in Caucasians where it occurs in one of 2500 live births[8]. It is caused by mutations in CFTR, a chloride channel. [1] The protein is part of a large family of ABC transporters that is activated by ATP and phosphorylated by AMP. The most frequent disease causing mutation, ΔF508, is in the ATP binding domain and leads to two types of functional defects: a trafficking defect causing retention in the endoplasmic reticulum and a gating defect resulting in decreased time in the open state. There are more than 1000 known mutations that differ in severity[1].

**[0005]** The *cftr* gene is expressed in epithelial lung and intestinal cells and at low levels in lung fibroblasts, cultured cells of lymphocytic lineage and freshly isolated blood mononuclear cells, monocytes, neutrophils and alveolar macrophages[9-11]. The mechanism by which mutations in the gene encoding the CFTR protein lead to the complex clinical phenomenon associated with cystic fibrosis are unclear. Explanations must take into account that this apical chloride channel is involved in fluid secretion and that increased viscosity of ductal

fluids contributes to the pathology. However, a number of findings in cystic fibrosis are less obviously related to chloride channel function, such as the increased adherence of *P. aeruginosa* to lung epithelial cells, a susceptibility to chronic lung infections, and an excessive inflammatory state present in cystic fibrosis children already at an early age. *Pseudomonas aeruginosa* infections are the major cause of morbidity and mortality. Increased phospholipase A2 (PLA2) activity and NF-Kappa beta mediated interleukin-8 (IL-8) secretion are only two contributors that lead to an increased inflammatory state in CF. Other clinical findings show abnormal plasma fatty acid profiles and low plasma concentrations of high-density lipoprotein (HDL) cholesterol. A recent report demonstrated that wild-type CFTR facilitates the uptake of a class of differentiated sphingolipids (sphingolipid)[2], the entire contents of reference 2 are hereby incorporated by reference as if set forth fully herein. Sphingolipid uptake and synthesis are tightly regulated and their metabolites are relevant to CF. For example, ceramide-1-phosphate is an obligatory regulator of PLA2α that is highly increased in CF, and the glycosphingolipid GM1 functions as part of a receptor complex for *P. aeruginosa*[3-7].

**[0006]** Thus, there is still a great need to understand the abnormal metabolic pathways in cystic fibrosis patients, and to identify new means of therapeutic intervention.

**[0007]** The past approaches described in this section could be pursued, but are not necessarily approaches that have been previously conceived or pursued. Therefore, unless otherwise indicated herein, the approaches described in this section are not to be considered prior art to the claims in this application merely due to the presence of these approaches in this background section.

## SUMMARY OF THE INVENTION

**[0008]** Certain aspects of the invention are directed to methods for treating cystic fibrosis in an animal, by administering an agent that inhibits the synthesis of one or more sphingolipids in cells from the animal in an amount that ameliorates one or more symptoms of cystic fibrosis. Some agents are enzyme inhibitors that inhibit the activity of an enzyme that catalyzes part of the *de novo* sphingolipid pathway, including serine-palmitoyl transferase, ceramide synthase, sphingosine kinase, UDP-glucose ceramide glucosyltransferase and glucosylceramide synthase. Other enzymes that can be inhibited to treat cystic fibrosis include an enzyme that is a member selected from the group: SPTLC1 serine palmitoyltransferase, long chain base subunit 1, Degs1 degenerative spermatocyte homolog 1, SGMS1 sphingomyelin synthase 1, Sphingomyelin synthase 2, ASAH2 N-acylsphingosine amidohydrolase (non-lysosomal ceramidase) 2, LASS6 LAG1 homolog, ceramide synthase 6, LASS5 LAG1 homolog, ceramide synthase 5, LASS4 LAG1 homolog, ceramide synthase 4, LASS3 LAG1 homolog, ceramide synthase 3, LASS2 LAG1 homolog, ceramide synthase 2, and LASS2 LAG1 homolog, ceramide synthase 1. Some therapeutic enzyme inhibitors that can be used in this aspect of the invention include (a) myriocin; (b) cycloserine; (c) Fumonisin B1; (d) PPMP; (e) compound D609; (f) methylthiodihydroceramide; (g) propanolol; and (h) resvaratrol. (i) Dimethyl sphingosine, (j) dihydroceramide saturase (GT 11), (k) n-butyldeoxynojirimycin, and (l) N-oleyl-ethanolamine.

**[0009]** In those aspects of the invention where sphingolipid synthesis is decreased through feedback mechanisms, the agent is a member of the group by ceramide, dihydroceramide, fenretinide, 4-oxo-HPR or a derivative, variant or fragment thereof, and an unsaturated fatty acid that is a member of the group by linoleic acid, linolenic acid, cholesterol DHA, eicosapentaenoic acid, oleic acid, and arachidonic acid. In certain other aspects *de novo* sphingolipid synthesis is reduced using a therapeutic agent that is an isolated antisense nucleic acid or small interfering RNA that is sufficiently complementary to the sense strand of the gene or to an mRNA encoding an enzyme in the sphingolipid synthesis pathway to permit hybridization to the sense strand of the gene or to the mRNA of the respective enzyme, and wherein the hybridization event prevents expression of the enzyme thereby decreasing the amount of sphingolipids produced in the cell. These enzymes are listed above.

**[0010]** In some aspects the agent is administered at a daily dose of between about 0.1 nanograms per kilogram body weight per day and about 20 milligrams per kilogram body weight per day, or between about 1 nanogram per kilogram body weight per day and about 10 milligrams per kilogram body weight per day. In other aspects the agent is administered to achieve a serum level of between about 1 nanogram per milliliter and about 10 micrograms per milliliter in the patient, or from between about 1 nanogram per milliliter and about 7 micrograms per milliliter. In some aspects the daily dose of the agent is between about 0.1 nanograms per kilogram body weight per day and about 20 milligrams per kilogram body weight per day, or between about 1 nanogram per kilogram body weight per day and about 10 milligrams per kilogram body weight per day.

**[0011]** In other aspects of the invention a therapeutic amount of an agent that reduces sphingolipid synthesis in a biological sample taken from the animal is determined by :

a. taking a first biological sample from the animal before administering the agent,

b. determining a sphingolipid level in the first sample,

c. administering an amount of the agent,

d. taking a second biological sample from the animal after administering the agent,

e. determining a sphingolipid level in the second sample,

f. if the sphingolipid level in the second sample is significantly lower than in the first sample, then concluding that the amount of the agent is a therapeutic amount, and

g. if the sphingolipid level in the second sample is not lower than the level in the first sample, then repeating steps a-f.

**[0012]** The level of sphingolipid synthesis in the biological sample can be determined using any method known in the art, including by measuring the level of sphingolipid mass using mass spectrometry or the amount of an individual sphingolipid that is a member of the group by sphinganine, C16, C18, C20, C22, C24, C 26, dihdyroceramide, sphingosine, sphingosine–s-phosphate, and sphingomyelin. Preferably the animal is a human and the biological sample is peripheral blood mononuclear cells, bronchial lavage or lung epithelial cells.

**[0013]** In certain other aspects of the invention *P. aeruginosa* infections in an animal are treated as described above for cystic fibrosis by administering an agent that inhibits

the synthesis of one or more sphingolipids in cells from the animal in an amount that ameliorates one or more of the symptoms of the disease.

**[0014]** Certain other aspects are directed to methods for identifying enzyme inhibitors that reduce the synthesis of a compound in the sphingolipid synthesis pathway. For example, a method of identifying serine palmitoyl transferase inhibitors in an animal cell-based assay using cells expressing defective CFTR or no CFTR and overexpressing serine palmitoyl transferase, by:

a) providing test cells overexpressing serine palmitoyl transferase;

b) contacting the test cells with a test compound;

c) determining the level of ketosphinganine or sterol-regulatory element-binding protein produced by the test cells,

d) comparing the determined level in the test cells to a level of ketosphinganine or sterol-regulatory element-binding protein in control cells that are not exposed to the test compound, and

e) determining that the test compound is a serine palmitoyl transferase inhibitor if the level of ketosphinganine or sterol-regulatory element-binding protein is significantly lower in test cells compared to control cells. In a preferred embodiment the expression of sterol-regulatory element-binding protein is indicated by a sterol-regulatory element reporter gene, including luciferase, green fluorescent protein and lacz. In certain preferred aspects the cell used in the drug screening assay is a member selected from the group including an IB3 cell, an epithelial cell from an animal having cystic fibrosis, a cell transduced to express defective CFTR or no CFTR, an A549 cell.

**[0015]** Other aspects are directed to pharmacological compositions including any combination of agent described above for decreasing sphingolipid synthesis through *de novo* synthesis or recycling pathways, including ceramide and DHA, ceramide and EPA and ceramide plus EPA and DPA; ceramide and a compound selected from the group by fenretinide and 4-oxo-HPR; ceramide and a compound selected from the group by (a) myriocin; (b) cycloserine; (c) Fumonisin B1; (d) PPMP; (e) compound D609; (f) nethylthiodihydroceramide; (g) propanolol; and (h) resvaratrol. (i) Dimethyl sphingosine, dihydroceramide saturase (GT 11), and n-butyldeoxynojirimycin. pharmacological compositions include an isolated nucleic acid that is a

member of the group by an antisense DNA, antisense RNA, and small interfering RNA, which nucleic acid is sufficiently complementary to the gene or mRNA encoding an enzyme in the sphingolipid *de novo* synthesis pathway to permit specific hybridization to the gene or mRNA, respectively.

**[0016]** Other aspects include methods to treat cystic fibrosis using an agent that reduces NF-kappa B and phospholipase A2, including fenretinide and ceramide and other agents known in the art.

## BRIEF DESCRIPTION OF THE DRAWINGS

The present invention is illustrated by way of example, and not by way of limitation, in the figures of the accompanying drawings in which:

**[0017]** FIG. 1 is a diagram of sphingolipid synthesis through *de novo* and recycling pathways.

**[0018]** FIG. 2 shows the metabolic pathway caused by defective CFTR. Defective CFTR $\rightarrow \uparrow$ Sphingolipid Synthesis $\rightarrow \uparrow$ Ceramide-1-phosphate and $\uparrow$ aGM1 synthesis.

**[0019]** FIG. 3(a) shows the increased synthesis of ceramide from $^{3}$H-serine that reflects *de-novo* synthesis by SPT, and 3(b) ceramide synthesis from $^{3}$H-sphinganine that reflects synthesis through the recycling pathway; standard deviations shown are of measurements in triplicates.

**[0020]** FIG. 4 (a) shows that SRE-mediated gene transcription is increased in the IB3 cells having defective CFTR compared to the C38 controls and 4(c) shows that SRE-mediated gene expression is increased in A 549 cells that do not express CFTR and that SRE-mediated gene transcription is decreased when CFTR is overexpressed in A549 cells. 4(c) shows CFTR expression in A549 cells transfected with AdCFTR confirmed by Western analysis.

**[0021]** Fig. 5 shows that ceramide and Doxosahexaenoic acid (DHA) increase linoleic acid mass in C38 control cells and in IB3 cells expressing mutant CFTR.

**[0022]** FIG. 6 shows mass spectrometry data confirming that sphingolipid synthesis and mass is increased in cells expressing defective CFTR.

**[0023]** FIG. 7 shows the time and a concentration curve of fenretinide in CHO fibroblast cells.

**[0024]** FIG. 8 shows the effect of fenretinide and palmitic acid on SRE-mediated gene expression and transcription in IB3 cells (expressing $\Delta$F508, the most prevalent (~90%) mutation in CF) compared to C38 control cells.

**[0025]** FIG. 9 shows that fenretinide decreases SRE-mediated gene transcription in cells that express no CFTR to levels seen in cells that express normal CFTR. These data were obtained in A 549 cells and A549 cells transduced to express CFTR.

**[0026]** FIG. 10 shows that fenretinide and ceramide increased cellular linoleic acid levels in control C38 cells (black bar) and in Ib3 cells expressing defective CFTR.

**[0027]** FIG. 11 shows that inhibition of *de-novo* sphingolipid synthesis by treatment with ceramide, EPA or myriocin decreased *P. aeruginosa*-GFP adhesion to CF-model lung epithelial cells.

**[0028]** Fig. 12 DHA and EPA alone or in combination decrease interleukin-8 secretion in immortalized human in lung epithelial cells (1HAEo).

**[0029]** FIG. 13 Assessment of the *de-novo* (a) and reverse or recycling pathways of (b) sphingolipid synthesis pathways in human peripheral blood mononuclear cells. $^{3}$H-serine was used to evaluate *de-novo* sphingolipid synthesis (a). $^{3}$H-sphingosine was used to assess sphingolipid synthesis through recycling pathways. (b). *De-novo* sphingolipid synthesis correlates inversely with low plasma HDL cholesterol (<40 mg/dl).

**[0030]** FIG. 14 The results show that incubation in the presence of 10 μM fenretinide significantly ($p<0.05$) decreases phosphatidylcholine specific PLA2 activity .

**[0031]** FIG. 15 Results demonstrate that NF-Kappa B is increased in control condition (1% BSA) and that Fenretinide decreases NF-Kappa B activity in IB3 cells comparably to Parthenolide which is known to decrease NF-Kappa B activity in CFTR defective cells

**[0032]** FIG. 16 Results demonstrate that inhibition of dihydroceramide desaturase using the specific Dihydroceramide desaturase inhibitor GT 11 decreases SRE-mediated gene transcription in CHO fibroblasts .

## DEFINITIONS

**[0033]** A therapeutically effective amount of a protein or polypeptide (i.e., an effective dosage) or nucleic acid (such as antisense nucleotides), is an amount that achieves the desired therapeutic result. For example, a therapeutically effective amount includes an amount that ameliorates one or more symptoms of the disease, or produces at least one effect selected from the group comprising a reduction of sphingolipid levels, gm1 gangliosides, a reduction of expression or activity of one or more of the enzymes serine palmitoyl transferase, sphingosine kinase, ceramide synthase/desaturase, or glucosylceramide synthase or a reduction of *P. aeruginosa* infection in cystic fibrosis patients or other patients having *P. aeruginosa* infections.

**[0034]** Significantly lower means that the difference is statistically significant.

**[0035]** "Specifically inhibiting" sphingolipid *de novo* synthesis includes, without limitation, (i) inhibiting sphingolipid *de novo* synthesis without inhibiting all other synthetic pathways, (ii) inhibiting sphingolipid *de novo* synthesis more than most or any other synthetic pathway, and/or (iii) inhibiting sphingolipid *de novo* synthesis without inhibiting any other synthetic pathway.

**[0036]** As used herein, by "protein" or "polypeptide" or "peptide" is meant any chain of amino acids, regardless of length or post-translational modification (e.g., glycosylation or phosphorylation). All "proteins" or "polypeptides" or "peptides" described herein include variants, derivatives and fragments and modifications thereof.

**[0037]** Peptide or protein variants" means polypeptides that may contain one or more substitutions, additions, deletions and/or insertions such that the therapeutic, antigenic and/or immunogenic properties of the peptides encoded by the variants are not substantially diminished, relative to the corresponding peptide. Such modifications may be readily introduced using standard mutagenesis techniques, such as oligonucleotide directed site-specific mutagenesis as taught, for example, by Adelman et al. (DNA, 2:183, 1983). Preferably, the antigenicity or immunogenicity of a peptide variant is not substantially diminished. Variants also include what are sometimes referred to as "fragments." Fragments also include peptides that may contain one or more amino acid substitutions, additions, deletions and/or insertions, such that the therapeutic, antigenic and/or immunogenic properties of the peptide variants are not substantially diminished, relative to the corresponding peptide.

**[0038]** "Protein derivatives" means that a protein has been derivatized. Derivatization is a technique used in chemistry which transforms a chemical compound into a product of similar chemical structure, called a derivative. Generally, a specific functional group of the compound participates in the derivatization reaction and transforms the educt to a derivate of deviating reactivity, solubility, boiling point, melting point, aggregate state, or chemical composition. Resulting new chemical properties can be used for quantification or separation of the educt or can be used to optimize the compound as a therapeutic agent. Derivatives include the protein modifications described herein.

**[0039]** By "operably linked" is meant that a gene and a regulatory sequence are connected in such a way as to permit expression of the gene product under the control of the regulatory sequence.

**[0040]** By "reporter gene" is meant any gene which encodes a product whose expression is detectable. A reporter gene product may have one of the following attributes, without restriction: fluorescence (e.g., green fluorescent protein), enzymatic activity (e.g., lacZ or luciferase), or an ability to be specifically bound by a second molecule (e.g., biotin or an antibody-recognizable epitope).

**[0041]** By "protein activity" or "biological activity" is meant the functional activity of a given protein in a standardized quantity of tissue or cells. The activity of a protein, as a whole, in such a sample can be modified as a result of a change in the quantity of the given protein present (e.g., as a result of change in gene expression) or as a result of a change in the function of each protein molecule present in the sample (e.g., as a result of an alteration in amino acid sequence).

**[0042]** By a "mature form" protein is meant the protein form that results from complete, eukaryotic, post-translational processing.

**[0043]** As used herein, the term "nucleic acid" refers to both RNA and DNA, including cDNA, genomic DNA, mRNA and synthetic (e.g., chemically synthesized, non-naturally occurring sequences) DNA including antisense nucleic acids, small interfering RNA and isolated nucleic acids. The nucleic acid can be double-stranded or single-stranded (i.e., a sense or an antisense single strand).

**[0044]** cDNA means a single-stranded DNA synthesized in the laboratory using messenger RNA as a template and the enzyme reverse transcriptase.

**[0045]** An "isolated nucleic acid" refers to a nucleic acid that is separated from other nucleic acid molecules that are present in a mammalian genome, including nucleic acids that normally flank one or both sides of the nucleic acid in a mammalian genome. The term "isolated" as used herein with respect to nucleic acids also includes any non-naturally-occurring nucleic acid sequence, since such non-naturally-occurring sequences are not found in nature and do not have immediately contiguous sequences in a naturally-occurring genome. An isolated nucleic acid can be, for example, a DNA molecule, provided one of the nucleic acid sequences

normally found immediately flanking that DNA molecule in a naturally-occurring genome is removed or absent. Thus, an isolated nucleic acid includes, without limitation, a DNA molecule that exists as a separate molecule (e.g., a chemically synthesized nucleic acid, or a cDNA or genomic DNA fragment produced by PCR or restriction endonuclease treatment) independent of other sequences. It also includes DNA that is incorporated into a vector, an autonomously replicating plasmid, a virus (e.g., a retrovirus, lentivirus, adenovirus, or herpes virus), or into the genomic DNA of a prokaryote or eukaryote. In addition, an isolated nucleic acid includes an engineered nucleic acid such as a DNA molecule that is part of a hybrid or fusion nucleic acid. A nucleic acid existing among hundreds to millions of other nucleic acids within, for example, cDNA libraries or genomic libraries, or gel slices containing a genomic DNA restriction digest, is not to be considered an isolated nucleic acid.

**[0046]** As used herein, the term "subject" includes mammals, e.g., humans, dogs, cows, horses, kangaroos, pigs, sheep, goats, cats, mice, rabbits, rats, and transgenic non-human animals. The preferred subject is a human.

**[0047]** "Biological samples" include solid and body fluid samples. The biological samples of the present invention may include tissue, organs, cells, protein or membrane extracts of cells, blood or biological fluids such as blood, serum, bronchial lavage, ascites fluid or brain fluid (e.g., cerebrospinal fluid). Preferred samples are peripheral blood mononuclear cells and lung epithelium.

**[0048]** Abbreviations: AA, arachidonic acid (20:4); ACC, acetyl-CoA carboxylase; Ad, Adenovirus; aGM1, asialylated GM1;CF, cystic fibrosis; CFTR, cystic fibrosis transduction regulator; DHA, docosahexaenoic acid (22:6); EPA, eicosapentaenoic acid (20:5); FAS; fatty acid synthase; GC; gas-chromatography, GFP, green fluorescent protein; HDL, high-density lipoprotein; SPT, serine-palmitoyl transferase; IL-8, interleukin-8; LC-MS/MS; liquid chromatography tandem mass spectrometry, *P. aeruginosa*, *Pseudomonas aeruginosa*; PLA2, Phospholipase A2; SRE, Sterol regulatory element, SREBP; SRE-binding protein; sphingolipid, sphingolipid; sphingolipid synthesis, sphingolipid synthesis; SMS, sphingomyelin synthase; SK, sphingosine kinase; siRNA, small interfering RNA; TLC, thin-layer chromatography.

## DETAILED DESCRIPTION.

**[0049]** In the following description, for the purposes of explanation, numerous specific details are set forth in order to provide a thorough understanding of the present invention. It will be apparent, however, to one skilled in the art that the present invention may be practiced without these specific details.

**[0050]** We have discovered that cells expressing defective CFTR (a model for cystic fibrosis) or no CFTR have elevated levels of sphingolipid synthesis compared to normal cells. Various methods are described for treating cystic fibrosis by administering one or more agents that reduce or inhibit sphingolipid (sphingolipid) synthesis through either the *de novo* or recycling (reverse) pathways or both.

**[0051]** Sphingolipid uptake and synthesis are known to be tightly regulated and their metabolites are relevant to CF. Using *in vitro* studies we show that sphingolipid synthesis (sphingolipid synthesis) through the de-novo pathway is abnormally high (about two times normal levels) in cells expressing defective CFTR. Increased sphingolipid synthesis leads to a 50 % higher sphingolipid mass. This increased sphingolipid synthesis can lead to increases in Gm1 gangliosides that facilitate the attachment of *P. aeruginosa* to lung epithelial cells. Another metabolite of sphingolipid synthesis is ceramide-1-phosphate that is known to be an activator of phospholipase A2, which in turn generates arachidonic acid (AA). AA is a key precursor of inflammatory eicosanoids. Increased NF-kappa B is a hallmark of cystic fibrosis that leads to increased IL-8 secretion. We show that cells expressing defective CFTR with abnormally high sphingolipid synthesis can be treated with various enzyme inhibitors or other agents/drugs that decrease sphingolipid synthesis causing it to return to levels seen in cells expressing wild-type CFTR. These same agents can be administered therapeutically to cystic fibrosis patients to normalize sphingolipid. fatty acid metabolism and normalize, in turn, increased activity of NF-KB and PLA2 .

**[0052]** Certain embodiments of the present invention are directed to methods for treating a cystic fibrosis patient by administering one or more agents that inhibit enzymes in the sphingolipid *de novo* synthesis pathway, including but not limited to: serine palmitoyl transferase (such as myriocin (0.1-10 μM), cycloserine (0.5-5 mM)), sphingosine kinase (such as dimethyl

sphingosine), ceramide synthase/desaturase (such as fumonisin B1 (0.1-40 μM)), ceramidase, and glucosylceramide synthase (such as PPMP or n-butyldeoxynojirimycin), and UDP-glucose ceramide glucosyltransferase. These enzymes can also be inhibited by antisense nucleotides and si RNA.

**[0053]** Other methods for treating a cystic fibrosis patient include administering an amount of one or more agents that decrease sphingolipid synthesis through an end product feedback mechanism, such as ceramide (including c6 ceramide and dihydroceramide) (2-20 μM), 4-HPR (fenretinide, 1-10 μM), glucosylceramide synthase inhibitors PPMP, Dihydroceramide desaturase inhibitors (i.e. GT11, 1-4 ug/ml) (NOTE: I had this in the other patent with SREBP already), including derivatives, variants, fragments or modifications thereof. Unsaturated fatty acids such as oleic acid, AA, DHA, EPA, cholesterol, oleic acid, linoleic acid, linolenic acid or combinations thereof can also be used to decrease sphingolipid synthesis by negative feedback.

**[0054]** Other embodiments include treating a cystic fibrosis patient with antisense nucleic acids or small interfering RNAs that reduce sphingolipid synthesis by inhibiting expression of one or more of the relevant enzymes, including serine palmitoyl transferase, sphingosine kinase, (dihydro)ceramide synthase/desaturase, UDP-glucose ceramide glucosyltransferase, and glucosylceramide synthase to decrease sphingolipid synthesis. The nucleic acid sequences for the cDNA for these enzymes and some others are set forth in the Sequence Listing at the end of the application with SEQ ID NOs. 1-14. They are:

SEQ ID NO. 1, Serine palmitoyltransferase Long Chain Base Subunit 1, Amino Acid Sequence, Homo sapiens;

SEQ ID NO. 2, Serine palmitoyltransferase Long Chain Base Subunit 1, Nucleotide Sequence, Homo sapiens;

SEQ ID NO. 3, Serine palmitoyltransferase Amino Acid Sequence Long Chain Base Subunit 2, Homo sapiens;

SEQ ID NO. 4, Serine palmitoyltransferase Amino Acid Sequence Long Chain Base Subunit 2, Homo sapiens;

SEQ ID NO. 5, Sphingosine kinase 1, Homo sapiens, Amino Acid Sequence; SEQ ID NO. 6, Sphingosine kinase 1, Homo sapiens, Nucleic Acid Sequence; SEQ ID NO. 7, Sphingosine kinase 2, Homo sapiens, Amino Acid Sequence; SEQ ID NO. 8, Sphingosine kinase 2, Homo sapiens;

SEQ ID NO. 9, UDP-glucose ceramide glucosyltransferase, Amino Acid Sequence, Homo sapiens;

SEQ ID NO. 10, UDP-glucose ceramide glucosyltransferase, Nucleotide Sequence, Homo sapiens;

SEQ ID NO. 11, Fatty acid desaturase (ceramide synthase) Homo sapiens, Gene Name DEGS2, Amino Acid Sequence;

SEQ ID NO. 12, Fatty acid desaturase (ceramide synthase), Homo sapiens, Gene Name DEGS2, Nucleotide sequence;

SEQ ID NO. 13, Fatty acid desaturase, Gene Name DEGS1, Amino Acid Sequence, Homo Sapiens; and

SEQ ID NO. 14, Fatty acid desaturase, Gene Name DEGS1, Nucleic Acid Sequence, Homo Sapiens.

Other genes in the sphingolipid synthesis pathway that are targets for enzyme inhibitors and antisense or siRNA inhibition include:

SPTLC1 serine palmitoyltransferase, long chain base subunit 1 [ *Homo sapiens* ]

Official Symbol SPTLC1

Degs1 degenerative spermatocyte homolog 1

Official Symbol Degs1

SGMS1 sphingomyelin synthase 1 [ *Homo sapiens* ]

Official Symbol SGMS1

Sphingomyelin synthase 2 [*Homo sapiens*]
Official Symbol SGMS2

ASAH2 N-acylsphingosine amidohydrolase (non-lysosomal ceramidase) 2 [ *Homo sapiens* ] Official Symbol ASAH2

LASS6 LAG1 homolog, ceramide synthase 6 (S. cerevisiae) [ *Homo sapiens* ] Official Symbol LASS6

LASS5 LAG1 homolog, ceramide synthase 5 (S. cerevisiae) [ *Homo sapiens* ]

Official Symbol LASS5

LASS4 LAG1 homolog, ceramide synthase 4 (S. cerevisiae) [ *Homo sapiens* ]

Official Symbol LASS4

LASS3 LAG1 homolog, ceramide synthase 3 (S. cerevisiae) [ *Homo sapiens* ]

Official Symbol LASS3

LASS2 LAG1 homolog, ceramide synthase 2 (S. cerevisiae) [ *Homo sapiens* ]

Official Symbol LASS2

LASS1 LAG1 homolog, ceramide synthase 1 (S. cerevisiae) [ *Homo sapiens* ]

Official Symbol LASS1

**[0055]** The sequences for the genes and mRNA encoding these enzymes are available in on line databases. The lass genes are ceramide synthases with different fatty acid preferences. We have unpublished data showing that Lass 4, 5 and 6 with C 16:0 preference are specifically high in cystic fibrosis patients. GT 11 is the first specific inhibitor of dihydroceramide desaturase identified. Sphingomyelin synthases 1 and 2 Inhibition of alkaline and neutral ceramidase with inhibitors decreases Sphingomyelin synthases 1 and 2 are valuable targets because their inhibition will increase ceramide and cause feedback inhibition. Inhibition of alkaline and neutral ceramidases with will also decrease sphingolipid synthesis.

**[0056]** Changes in sphingolipids can be monitored by determining sphingolipid mass or the amount of individual sphingolipids (including sphinganine, C16/C18/C20/C22/C24/C 26, (Dihdyro) ceramide, sphingosine, sphingosine –s-phosphate, and sphingomyelin) in a biological sample taken from the patient as is described in the examples. Preferred biological samples include peripheral blood mononuclear cells or lung and intestinal epithelium.

**[0057]** Certain other embodiments of the present invention are directed to *in vitro* drug screening methods to identify compounds that: inhibit serine palmitoyl transferase, (dihydro)ceramide synthase/desaturase, ceramidase, glucosylceramide synthase, sphingosine kinase and/or sphingomyelinase; or inhibit the production or accumulation of sphingolipids, Gm1 gangliosides, or ceramide-1-phosphate in a cell, preferably a cell expressing defective CFTR or no CFTR. The term "inhibit" as used herein means to decrease or reduce enzyme activity or enzyme expression compared to pretreatment levels, or decrease synthesis of a substance being determined.

**[0058]** Cystic fibrosis patients exhibit a state of severe and chronic inflammation, characterized by increased phospholipase A2 and N-kB activity as well as chronic lung infection, particularly with P. aeruginosa. We have further discovered that decreasing sphingolipid synthesis in lung epithelial cells causes a decrease or inhibition of *P. aeruginosa* attachment to the cells. Certain embodiments are directed to methods for treating *P. aeruginosa* infections in a patient, preferably a cystic fibrosis patient, preferably a human, by administering a therapeutic amount of one (or more) agents that decrease sphingolipid synthesis, in an amount that ameliorates one or more of the symptoms of cystic fibrosis and/or the *P. aeruginosa* infection.

**[0059]** Sphingolipids (sphingolipid) are ubiquitous and form a large family of lipid second messengers. sphingolipid are synthesized de-novo or through recycling pathways of end products (FIG. 1). In the endoplasmic reticulum, serine-palmitoyl transferase (SPT) generates ketosphinganine from serine and palmitoyl-CoA. SPT is the rate limiting enzyme of de-novo sphingolipid synthesis. Ceramide is a central intermediary that is metabolized to sphingosine, ceramide-1-phosphate, sphingo-myelin or glucosylceramides. An equally important synthetic pathway occurs through recycling of long chain bases derived from these end products (recycling pathway).

**[0060]** Ceramide is metabolized to sphingomyelin, glucosylceramides, ceramide-1-phosphate or sphingosine. Of particular interest to cystic fibrosis are the two sphingolipid metabolites (1) ceramide-1-phosphate, which directly activates cPLA2. (2) ganglioside αGM1, part of the *P. aeruginosa* receptor complex in epithelial cells and (3) sphingolipid synthesis, i.e. ceramide and DAG )diacyl glycerol) related increased NF-Kappa B mediated IL-8 secretion. In cells expressing wild-type CFTR, sphingolipid synthesis is inhibited by its end-product ceramide, glucosylceramide, sphingosine, sphinganine, and by unsaturated fatty acids. Unsaturated fatty acids regulate sphingolipid synthesis through both the *de novo* and recycling pathways. Saturated fatty acids, i.e. palmitic acid increase de-novo sphingolipid synthesis. Lauric acid (12:0), a substrate for reverse ceramidases, and sphingosine, increase sphingolipid synthesis through recycling pathways[47]. We previously showed that sphingolipid synthesis correlates positively with the post-transcriptional regulation of sterol-regulatory element-binding protein (SREBP) and described details of the relevant methods [32], and U.S. Patent Application No. 20050182020, the entire contents of which are hereby incorporated by reference as if set forth fully herein. Therefore, in some of the experiments we describe below, SRE-mediated luciferase activity, the reporter gene that reflects SREBP levels and SRE-mediated gene transcription, is used to monitor sphingolipid synthesis.

**De-novo sphingolipid synthesis is Increased in cells expressing defective CFTR**

**[0061]** It is known that normal cells expressing wild-type CFTR take up long sphingoid bases, and that expression of mutant CFTR correlates with decreased uptake of sphingoid base phosphates.[2] Recently it was reported that murine epithelial cells transfected with functional

CFTR take up, in a dose-dependent manner, significantly more sphingosine-1-phosphate, dihydrosphingosine-1-phosphate and lysophosphatidic acid than cells transfected with mutated (ΔF508 / C127) CFTR. [2] sphingolipid uptake and synthesis are known to be tightly regulated. Clinical findings show that cystic fibrosis patients have altered fatty acid profiles, low plasma HDL-cholesterol and increased cholesterol synthesis. Our lab showed that increased sphingolipid synthesis increased the levels of ceramide-1-phosphate and glucosylceramide, which are both intrinsically related to increased phospholipase A2 (PLA2) activity increased *P. aeruginosa* adherence to epithelial cells and increased NF-Kappa B activity.

**[0062]** While cells that express normal CFTR take up long base sphingoid bases that lead to feedback inhibition of sphingolipid synthesis (FIG. 2a), expression of mutant CFTR correlates with decreased uptake of sphingoid base phosphates[2] (FIG. 2b), causing a lack of end-product inhibition, for example by sphingoid base phosphates, which in turn leads to increased sphingolipid synthesis. Thus sphingolipid mass is increased in cells expressing defective, decreased or no CFTR.

**[0063]** The experiments described below show that: (1) sphingolipid synthesis in lung epithelial cells expressing defective CFTR is increased about two times the normal wild type levels, and (2) there is a correlation with the regulation of SRE-mediated gene transcription and restoration of altered fatty acid profiles to control profiles.

**[0064]** Three different human lung epithelial cell lines were used. (1) IB3 cells (ATCC CRL-2777) that express mutant CFTR (ΔF508/W1282X), and C38 control cells (ATCC CRL-2778) that were derived from the IB3 cells by transfection with episomal copies of wild-type CFTR[51]; (2) 16HBE cells that are a human bronchial epithelial cell line that maintains tight junctions and stably expresses episomes encoding CFTR in the sense or antisense orientation. Kube D, Sontich U, Fletcher D, Davis PB. Proinflammatory cytokine responses to *P. aeruginosa* infection in human airway epithelial cell lines. /Am J Physiol Lung Cell Mol Physiol/ 2001;280:L493–L502.

**[0065]** The cells expressing the CFTR antisense construct show the expected absence of CFTR function and fail to secrete chloride ($Cl^-$) in response to reagents that stimulate cAMP production[52]; and (3) A549 cells derived from a human pulmonary adenocarcinoma cell line that does not express CFTR[53, 54]. A 549 cells were transduced with Ad-CFTR to evaluate the effect of CFTR expression. Details of cell culturing are set forth in Example 1.

**[0066]** C38 and IB3 cells were grown in 6-well plates and incubated for 1.5h in serine-free medium in the presence of either $^{3}$H-serine, used to assess de-novo sphingolipid synthesis, or $^{3}$H-sphinganine, used to determine sphingolipid synthesis through recycling pathways. We discovered that the synthesis of ceramide from $^{3}$H-serine reflecting de-novo synthesis by SPT, is twice as high in IB3 cells as in C38 control cells (FIG. 3a). By contrast, synthesis through the recycling pathway, reflected by $^{3}$H-sphinganine incorporation into ceramide, is not different from controls (FIG. 3b).

**[0067]** In previous work we showed that sphingolipid synthesis correlates with SRE-mediated gene transcription[32]. Specifically, increased cellular ceramide decreases mSREBP protein levels and SRE-mediated gene transcription. Inhibition of ceramide *de novo* synthesis decreases SRE-mediated gene transcription. Worgall, T. S., Johnson, R. A., Seo, T., Gierens, H., and Deckelbaum, R. J. (2002) J Biol Chem 277, 3878-85), Worgall, et al., U/S. Application Serial No. 20050182020. Both exogenous and endogenous ceramide exert a negative feed-back mechanism on its own synthesis. Therefore, SRE-mediated gene transcription is a good indirect and surrogate marker for sphingolipid synthesis that can be easily assessed using a promoter/reporter gene assay.

**[0068]** We investigated the relationship between CFTR and SRE-mediated gene transcription in C38/IB3 and A549 cells. First, cells were transduced with an adenovirus (Ad) vector expressing the SRE-promoter reporter gene and an unregulated β-gal control gene (Ad-SRE-luc/β-gal). Because A549 do not express CFTR, they were additionally transduced (i.e. infected) with an Ad-vector that expresses CFTR (Ad-CFTR) or a control Ad-vector (Ad-null). These experimental conditions allowed us to assess SRE-mediated gene transcription in the presence and absence of CFTR expression. Example 2.

**[0069]** We found that sphingolipid synthesis (SRE-mediated gene transcription) was increased in IB3 cells having defective CFTR compared to the C38 controls with normal CFTR (FIG. 4a). As expected, SRE-mediated gene expression was low in A549 cells that have CFTR expression (FIG. 4b). Addition of cholesterol or oleic acid to IB3 cells decreased SRE-mediated gene transcription, indicating that post-transcriptional regulatory mechanisms of SREBP were intact in all examined cell lines. Furthermore, these results are consistent with increased fatty acid and cholesterol synthesis that has been observed in CFTR defective cells, because SREBP is

the key regulator of rate-limiting enzymes of cholesterol and fatty acid synthesis[33], the contents of all of the references cited herein are hereby incorporated by reference as if set forth fully herein.

**Reducing Sphingolipid Synthesis Corrects Abnormal Fatty Acid Profiles in Cells Expressing Defective CFTR or No CFTR**

**[0070]** Cystic fibrosis is associated with altered fatty acid profiles that show a deficiency of essential fatty acids and increased ratios of saturated to unsaturated fatty acids. The most significant differences compared to controls are: palmitic acid (16:0), which is a product of endogenous fatty acid synthase, and lauric acid (12:0). Plasma levels of unsaturated fatty acids, in particular linoleic (18:2,n-6) and linolenic acid (18:3,n-3), are also decreased in individuals with cystic fibrosis compared to controls[25-27]. It has been shown, however, that individuals with cystic fibrosis absorb supplemented fatty acids as efficiently as controls[28]. It has been suggested that defective long-chain polyunsaturated fatty acid synthesis contributes to the cystic fibrosis phenotype[27, 29, 30]. However, no differences in long chain fatty acid synthesis were found in cftr knock-out mice[31].

**[0071]** Some end products of endogenous fatty acid synthesis are palmitic (16:0) and stearic acid (18:0). Fatty acid synthesis occurs through the fatty acid synthase (FAS) complex which requires malonyl CoA for which acetyl-CoA carboxylase (ACC) is the rate limiting enzyme. Key transcriptional regulators of the rate-limiting enzymes of fatty acid synthesis as well as of rate limiting enzymes of cholesterol and phospholipid synthesis are the sterol-regulatory element binding proteins (SREBPs). SREBPs are expressed in all cells and significant regulation occurs on a post-transcriptional level. It is known that cholesterol synthesis is increased in CF, although the clinical relevance of this finding is not well established[33, 34]. Even though cholesterol is elevated in cystic fibrosis patients, it does not exert a negative feedback effect because sphingolipid synthesis is high and because the increased sphingolipids sequester cholesterol and do not make it available as a regulator within the membrane of the endoplasmic reticulum.

**[0072]** The next experiment shows that fatty acid profiles in the experimental cell lines reflect the abnormal plasma fatty acid profiles found in cystic fibrosis patients, and assesses whether inhibition of sphingolipid synthesis affects fatty acid profiles. The level of linoleic and

linolenic acid in cystic fibrosis patients is typically decreased over normal levels by about 50% or even less. Cawood and Calder, Curr Opin Clin Nutr Metab Care :8: 153-159, 2005). We used ceramide to decrease sphingolipid synthesis by end product feed back inhibition. The polyunsaturated fatty acid (DHA) doxosahexaenoic acid was used as a positive control, as it was previously shown to correct abnormal plasma fatty acid profiles in cftr-/- mice[55,30, 56]. C38 controls and IB3 cells were incubated for 9h in the presence of control conditions (1% BSA) or the experimental conditions ceramide (20 μM) or DHA (0.3 mM). Then cells were washed and fatty acid mass was determined by gas - chromatography[25]. The results shown in FIG. 5 demonstrate that end product feed back inhibition of sphingolipid synthesis by ceramide and DHA increased linoleic and linolenic acid concentrations in IB3 cells by about 30% (FIG. 5) compared to untreated cells. The ceramide and DHA normalized the fatty acid mass of 18:2 in IB3 cells, showing that the fatty acid composition in IB3 cells reflects the altered pattern found in cystic fibrosis patients and that reducing sphingolipid synthesis using ceramide or DHA corrected the altered fatty acid profiles. Unfortunately there is no good in vivo model for CF. but these results validate the use of our in vitro model for CF.

**[0073]** Certain embodiments of the invention are therefore directed to methods for treating cystic fibrosis in a human being by reducing sphingolipid synthesis, including by administering ceramide (including c6-ceramide or dihydroceramide, or fragments, variants or derivatives thereof), or ceramide combined with DHA. DHA is presently used clinically for treating cystic fibrosis. Other embodiments are directed to a pharmaceutical composition for use in treating cystic fibrosis or *P. aeruginosa* infections that includes a therapeutic amount of ceramide, or ceramide plus DHA. All of the therapeutic agents described herein can be modified to enhance uptake, prevent degradation, target delivery, or increase half life using methods known in the art and described herein. Therapeutic amounts or doses of ceramide and DHA and other therapeutic agents discussed herein are described below.

**[0074]** C6-Ceramide has been used for treating ovarian cancer in amounts of about 100 mg/kg administered intravenously either in aqueous solution or as a nanoparticle in biodegradable poly(ethylene oxide)-modified poly (epsilon-caprolactone). Cancer Ther. Paclitaxel and ceramide co-administration in biodegradable polymeric nanoparticulate delivery system to overcome drug resistance in ovarian cancer, Devalapally, et al. On Line 7 June 2007,

incorporated herein by reference. Although ceramide has some adverse side effects, an attending physician can determine if the benefits outweight the adverse effects in treating a cystic fibrosis patient. A range of doses and frequency of administration can be varied to find the optimum therapy, as described below.

**[0075]** FIG. 6 shows mass spectrometry data confirming that sphingolipid synthesis and mass are both increased in cells expressing defective CFTR. Experiments were carried out in three models of the cystic fibrosis defect: No CFTR expression (A549 vs A549 Ad-CFTR), decreased expression (Sense/ Antisense), and cells expressing the most prevalent mutation Δ F 508 (C38/IB3).

**[0076]** Another compound known to have a negative feed back effect that decreases sphingolipid synthesis through the recycling pathway is fenretinide, a synthetic retinoid that has been used as an anticancer agent to treat neuroblastoma as well as other cancers. Fenretinide was recently shown to be an inhibitor of dihydroceramide desaturate, that increases cellular dihydroceramide concentration in Du145 cells. Zheng, W et al BBA 1758 (2006) 1864-1884. We have previously showed that, like ceramide, dihydroceramide decreases sphingolipid synthesis in regular fibroblasts (Worgall, JBC, 2002). See FIG 16 that shows the effect of GT11, a specific inhibitor of dihydroceramide desaturase on SRE-mediated gene transcription in CHO-fibroblasts. GT11 is a target for enzyme inhibition to decrease sphingolipid synthesis in cystic fibrosis and *P. aeruginosa* infections.

**[0077]** To evaluate the effect of fenretinide on SRE-mediated gene transcription, cystic fibrosis model and control cells were contacted with different concentrations of fenretinide. FIG. 7 shows that at six hours (lowest line) fenretinide decreased SRE-mediated gene transcription by as much as 33% in cystic fibrosis model cells compared to controls (1% BSA) in a dose dependent manner from 1-10 μM fenretidine. There was no major difference between 10 and 20 μM fenretinide. Like ceramide, fenretinide can be used therapeutically to treat cystic fibrosis patients by normalizing sphingolipid synthesis. Certain embodiments of the invention are directed to treatment of cystic fibrosis by administering a therapeutic amount of fenretinide or of a combination of ceramide and fenretinide. Other embodiments are directed to a pharmaceutical composition for use in treating cystic fibrosis or *P. aeruginosa* infections, that includes a therapeutic amount of ceramide plus fenretinide.

**[0078]** Retinoids are natural and synthetic derivatives of vitamin A (retinol), which modulate different cellular processes, including proliferation, differentiation and apoptosis. Fenretinide, or N-(4-hydroxyphenyl) retinamide (4-HPR), is a synthetic retinoid (an amide of all-trans retinoic acid), which, in preclinical models, proved to be less toxic than many other retinoids while maintaining a significant biological activity. (Moon et al., Cancer Res., 1979, 39:1339-1346). Fenretinide has been successfully used at a dose of about 200 mg PO daily for up to 5 years (two capsules, 100 mg each, at dinner) to treat stage I breast cancer or ductal carcinoma-in-situ. Journal of Clinical Oncology, Vol 19, Issue 6 (March), 2001: 1664-167. These amounts can be administered to treat cystic fibrosis and *P. aeruginosa* infections. To improve the oral delivery of 4-HPR, and possibly increase plasma and tissue levels in vivo, 4-HPR is sometimes complexed with the novel lipid matrix Lym-X-Sorb$^{TM}$ (LXS) by Avanti. 4-oxo-HPR is a metabolite of 4-HPR that is more potent and that has a wider range of tumor cell growth inhibitory activity than the parent drug. It can also be used to treat cystic fibrosis or *P. aeruginosa* infections by reducing sphingolipid synthesis. US Application Serial No. Formelli, et al., 20060264514. 4-oxo-HPR enhances the effects of fenretinide, therefore certain embodiments are directed to methods for treating cystic fibrosis (or *P. aeruginosa* infections) by administering a therapeutic amount of fenretinide or 4-oxo-HPR or both, and to a pharmaceutical compositions comprising fenretinide and ceramide; 4-oxo-HPR and ceramide; and fenretinide, 4-oxo-HPR and ceramide for the treatment of cystic fibrosis and PA.

**[0079]** In another experiment we found that SRE-mediated gene transcription was increased in CFTR defective IB3 cells in control conditions (1% BSA) and when exposed to palmitic acid (0.3 mM). By contrast, control C38 cells showed no change upon exposure to palmitic acid. FIG. 8 Palmitic acid is a substrate for serine-palmitoyl transferase (the rate limiting enzyme of sphingolipid de-novo synthesis) as well as a substrate for LASS 5 ceramide/dihydroceramide synthase. Thus palmitic acid increased *de novo* sphingolipid synthesis in cells expressing defective CFTR, but had no effect on sphingolipid synthesis in normal controls. By contrast fenretinide (10 μM) again decreased SRE-mediated gene transcription in both control and IB3 cells to about 40 % of the level seen in the respective cells under control conditions. Unlike palmitic acid, fenretinide also decreased sphingolipid synthesis by about 50% in control C38 cells.

**[0080]** We next looked at the effect of fenretinide (10 μM) on A549 human bronchoalveolar cells that do not express CFTR (No CFTR) in comparison to A549 cells that were transduced to express normal CFTR. In control medium (1% BSA), a lack of CFTR expression correlated with increased SRE-mediated gene transcription (black bar on the left), while expression of CFTR decreased SRE-mediated gene transcription (black bar on the right). Incubation in the presence of fenretinide (10 μM) decreased SRE-mediated gene transcription to almost the same extent in both the NO CFTR cells (gray bar on the left) and in CFTR expressing cells.

**[0081]** FIG. 10 shows the effect of ceramide (20 uM) and fenretinide (10 uM) on cellular linoleic (18:2) fatty acid content in control cells (C38, black bar) and cells expressing defective CFTR (IB3, white bar). Both agents increased linoleic fatty acid content in both control and in IB3 cells showing that both ceramide and fenretinide can be used to reduce sphingolipid synthesis and normalize fatty acid profiles in cystic fibrosis cells and in cystic fibrosis patients.

**[0082]** To summarize, certain embodiments are directed to decreasing sphingolipid synthesis in cells expressing defective CFTR by inhibiting enzymes in the sphingolipid synthesis biosynthetic pathway. This includes new methods for treating cystic fibrosis by decreasing the amount of sphingolipid produced (or synthesized) in a cell, preferably a cell that expresses defective CFTR, by administering a therapeutic amount of an inhibitor of an enzyme in the sphingolipid synthesis *de novo* synthesis pathway including: serine palmitoyl transferase (such as myriocin or cycloserine), sphingosine kinase (such as dimethyl sphingosine(DMS)), ceramide synthase/desaturase (such as fumonisin B1), glucosylceramide synthase (such as PPMP or n-butyldeoxynojirimycin), dihydroceramide saturase (GT 11), or combinations thereof. Other ways of treating cystic fibrosis by decreasing sphingolipid synthesis include administering agents that have a negative feedback effect on sphingolipid synthesis through recycling pathways, including ceramide, fenretinide, sphingosine, sphinganine, glucosylceramide, dihydroceramide and unsaturated fatty acids, including eicosapentaenoic acid (EPA),and docosahexaenoic acid (DHA), or combinations thereof. Agents that decrease *de novo* sphingolipid synthesis can be administered together with agents that have a negative feedback effect on sphingolipid synthesis through recycling pathways. The agents can be administered at the same time on the same day or at different times on the same day, or on different days. They can be formulated and administered as a single pharmaceutical composition. A preferred route of administration for

cystic fibrosis is by inhalation since the lungs are heavily involved in the disease. However, any route of administration may be used as is discussed in more detail below.

**DHA and EPA decrease interleukin-8 expression in lung epithelial cells.**

**[0083]** IL-8, a member of the neutrophil-specific CXC subfamily of chemokines, is a potent neutrophil chemotactic and activating factor. It has been reported that lL-8 levels are constituitively higher in non-infected cystic fibrosis infants compared to culture positive controls[43-45]. We have discovered that DHA and EPA decrease IL-8 secretion from 12 HAEo cells, which are an SV-40 transformed human airway cell line that constitutively expresses IL-8. Assessment of IL-8 secretion was carried out in low serum conditions because serum itself stimulates IL-8 secretion. Cells were preincubated for 16h in the presence of 0.1 % serum. Then, cells were incubated for 2h in the presence of control conditions (1% BSA) or in the presence of the experimental conditions, DHA (22:6, n-3), EPA (20:5, n-3) or a DHA together with EPA (0.15 mM each). Secretion of IL-8 was determined in the supernatant using standard ELISA techniques and data were normalized to cell protein content. IL-8 concentration was decreased by both DHA or EPA (Figure 12). Both DHA and EPA are presently used to improve fatty acid patterns in cystic fibrosis. Certain embodiments of the invention are directed to methods for treating cystic fibrosis or ameliorating one or more of its symptoms in an animal by administering ceramide, including c6-ceramide, combined for example with EPA, or EPA plus DHA. Other embodiments are directed to a pharmaceutical composition for use in treating cystic fibrosis or *P. aeruginosa* infections that includes a therapeutic amount of ceramide plus EPA, or EPA plus DHA, or ceramide plus both EPA and DPA.

**[0084]** Another relevant observation is that most cystic fibrosis patients have low plasma HDL cholesterol levels in cystic fibrosis patients[48]. Low HDL cholesterol is an independent risk factor for coronary artery disease. In the general population, a major regulator of plasma HDL levels is the expression of the ABCA1 protein at the plasma membrane. This protein functions as a cholesterol efflux receptor and is, like CFTR, part of the ABC transporter family. A recent observation is that ceramide or inhibition of glucosylceramide synthase increases ABCA1 expression[49, 50]. It is not known whether sphingolipid have a role in the modulation of plasma HDL-cholesterol in CF.

**Inhibition of Sphingolipid Synthesis decreases Phosphatidylcholine specific PLA2 activity, and NF-Kappa B activity in cells expressing defective CFTR**

**[0085]** The inflammatory PLA2s are potent mediators of inflammatory processes and are highly expressed in serum and synovial fluids of patients with inflammatory disorders as well as in cystic fibrosis. The secretory phospholipase A2 (PtA2) superfamily comprises a number of heterogeneous enzymes whose common feature is to hydrolyze the sn-2 fatty acid acyl ester bond of phospholipids. For example phosphatidylcholine is a substrate for the PLA2 which is increased in cystic fibrosis. Hydrolysis of phosphatidylcholine (PC) to lyso-PC affords a chemoattractant for circulating monocytes and arachidonic acid. Arachidonic acid, is the key eicosanoids precursor for the production of thromboxanes, prostaglandins and leukotrienes. Furthermore, leukotriene-B4 is known to function in a feedback loop which further increases PLA2 activity (Wijkander, J. et al. (1995) J. Biol. Chem. 270:26543-26549).

**[0086]** We have discovered that inhibition of sphingolipid synthesis decreases phosphatidylcholine specific PLA2 activity and NF-Kappa B activity in cells expressing defective CFTR. FIG. 14 shows the effect of fenretinide on phosphatidylcholine specific PLA2 activity. CFTR control (Sense) and CFTR minus (Antisense) cells were incubated for 16 h in the presence of Fenretinide (10 μM). Cells were harvested and lysed by passing through a 22 gauge needle. The cell extract was reacted with a phospholipase A2 specific phosphatidylcholine probe (Echelon- inc, Gift from CEO Prestwich). Fluorescence of this substrate is quenched in the unreacted state. Fluorescence is emitted when arachidonic is cleaved at the sn-2 position. This probe is specific for phosphatidylcholine. The results show that incubation in the presence of 10 μM fenretinide significantly ($p<0.05$) decreases phosphatidylcholine specific PLA2 activity. Thus the methods described herein for reducing sphingolipid synthesis through *de novo* and recycling pathways can be used to treat other diseases associated with elevated PHA2. Other known phospholipase A2 inhibitors include mepacrine (Rao et al., 1993), and bromphenylbromide (BPB) (Peppelenbosch et al., 1993, dexamethasone and pentoxifylline. These agents can also be used therapeutically to reduce inflammation in cystic fibrosis patients.

**[0087]** Cystic fibrosis (CF) is characterized by prolonged and excessive inflammatory responses in the lung and increased activation of NF- kappa B. NF-kappa B is an ubiquitously expressed transcription factor that controls the expression of a diverse range of genes involved in

inflammation, immune response, lymphoid differentiation, growth control and development. NF-kappa B resides in the cytoplasm as an inactive dimer consisting of p50 and p65 subunits, bound to an inhibitory protein known as I kappa B. The latter becomes phosphorylated and degraded in response to various environmental stimuli, such as pro-inflammatory cytokines, viruses, lipopolysaccharides, oxidants, UV light and ionizing radiation. This allows NF-kappa B to translocate to the nucleus where it activates genes that play a key role in the regulation of inflammatory and immune responses, including genes that encode pro-inflammatory cytokines (IL-1.beta., TNF, GM-CSF, IL-2, IL-6, IL-11, IL-17), chemokines (IL-8, RANTES, MIP-1.alpha., MCP-2), enzymes that generate mediators of inflammation (NO synthetase, cyclo-oxygenase), immune receptors (IL-2 receptor) and adhesion molecules (ICAM-1, VCAM-1, E-selectin). Some of these induced proteins can on their turn activate NF-kappa.B, leading to the further amplification and perpetuation of the inflammatory response. Inhibition of this enzyme is useful in the treatment of inflammatory NF-kappa.B related diseases including cystic fibrosis. Our results with fenretinide show that reducing sphingolipid synthesis will in turn reduce NF-kappa.B. Thus the methods described herein for reducing sphingolipid synthesis through *de novo* and recycling pathways can be used to treat other diseases associated with elevated NF-kappa B besides cystic fibrosis and *P. aeruginosa*.

**[0088]** FIG. 15 shows the effects of fenretinide and parthenolide on NF-Kappa B activity in CFTR control (C38 ) and CFTR defective cells transfected with an NF-Kappa B reporter gene construct together with β-gal transfection control. After 42 h cells were incubated for 16 h in the presence of parthenolide (15 μM) or Fenretinide (10μM). At the end of the incubation period, cells were harvested, lyzed. Luciferase and β-gal activity were determined using chemiluminescent detection. The results demonstrate that NF-Kappa B is increased in control condition (1% BSA). However, both parthenolide and fenretinide equally decreased NF-Kappa B activity in IB3 cells. Parthenolide is a sesquiterpene lactone derived from the plant feverfew, which has been used in folk medicine for anti-inflammatory activity. It has been used clinically to treat cystic fibrosis. Jain NK, Kulkarni SK. Antinociceptive and anti-inflammatory effects of Tanacetum parthenium L. extract in mice and rats. /J Ethnopharmacol/ 1999;68:251–259; Reuter U, Chiarugi A, Bolay H, Moskowitz MA. Nuclear factor-kappaB as a molecular target for migraine therapy. /Ann Neurol/ 2002;51:507–516). Parthenolide has been recently shown to

inhibit the NF-_B pathway, Saadane, A., et al. The agents described herein for reducing sphingolipid synthesis, including fenretinide, can be combined with parthenolide to treat cystic fibrosis or other inflammatory diseases.

**[0089]** FIG. 16 shows the effect of the dihydroceramide synthase inhibitor G11 on SRE-mediated gene transcription. GT11 is the only described specific dihydoceramide desaturase inhibitor so far (GenBank note). Gemma Fabrias and Gerhild von Echten, Specificity of the dihydroceramide desaturate inhibitor GT11 In primary cultured cerebellar Neurons, Molecular Pharmacology 66:1671-1678, 2004). CHO fibroblasts were incubated for 5 h in the presence of increasing concentrations of GT11. Luciferase activity was measured and normalized to cellular protein levels. The results demonstrate that inhibition of dihydroceramide desaturase decreases SRE-mediated gene transcription. As mentioned earlier inhibition of dihydroceramide synthase using inhibitors such as G11 or antisense nucleotides or siRNA can be used to treat cystic fibrosis or *P. aeruginosa* infections.

**DRUG SCREENING**

**[0090]** Certain embodiments of the invention are directed to cell-based and non-cell based methods of drug screening to identify candidate agents that:

1. decrease sphingolipid synthesis in cells expressing no CFTR or defective CFTR;
2. are enzyme inhibitors or reduce the activity or expression of certain enzymes in an the *de novo* sphingolipid synthesis pathway in an animal cell expressing defective CFTR, including serine palmitoyl transferase, ceramide synthase, sphingosine kinase, ceramidase, UDP-glucose ceramide glucosyltransferase , and glucosylceramide synthase, also referred to herein as "the various proteins;" and
3. inhibit the production of one or more sphingolipids in an animal cell expressing defective or no Cftr.

**[0091]** The subject assays are both non-cell based and cell-based. Non-cell based assays for identifying enzyme inhibitors or agents that affect gene expression are very well known. They generally involve (a) contacting a transformed or recombinant cell that has a mutant of a native allele encoding a reporter of gene expression of one (or more) of the various proteins, wherein the expression of the reporter is under the control of the native gene expression regulatory

sequences of the native allele, with a candidate agent under conditions whereby but for the presence of the agent, the reporter is expressed at a first expression level; and, (b) determining the expression of the reporter to obtain a second expression level, wherein a difference between the first and second expression levels indicates that the candidate agent modulates expression of one of the gene.

**[0092]** The mutant may result from replacement of a portion of the native allele with a sequence encoding the reporter. For example, the cell may be a progeny of a genetic knock-in cell made by homologous recombination of the native allele with a transgene comprising a sequence encoding the reporter flanked by flanking sequences capable of effecting the homologous recombination of the transgene with the native allele, a positive selectable marker positioned inside the flanking sequences and a negative selectable marker positioned outside the flanking sequences.

**Inhibition of Sphingolipid Synthesis Decreases Adhesion of *P. aeruginosa* to CF-Model Lung Epithelial Cells**

**[0093]** Lungs of cystic fibrosis patients become infected by bacterial pathogens shortly after birth, often followed by massive infiltration of neutrophils recruited by elevated cytokine and chemokine production in the small airways[35]. Even in the absence of clinically apparent lung disease, production of proinflammatory cytokines is excessive[36]. Infection with a variety of pathogens, such as Staphylococcus aureus and Haemophilus influenza occur with higher frequency in CF, but the major pathogen in cystic fibrosis is P. aeruginosa, a ubiquitous organism rarely associated with severe infections in normal hosts. Characteristic is an increased adherence of *P. aeruginosa* to cystic fibrosis lung epithelial cells, the formation of biofilms and failure of the host to eliminate P. aeruginosa[7, 37]. *P. aeruginosa* binds to the GalNAcβ1-4 Gal moiety of gangliosides and flagella activate airway cells through aGM1 and toll-like receptors 2 and 5[38]. *P. aeruginosa* is among many bacteria that bind to glucosylceramide derived glycosphingolipids[39]. Gangliosides are glucosylceramide-derived sialylated or fucosylated acid glycosphingolipids, found on the outside of cells (**Figure 1**). CFTR mutations are associated with the presence of an increased number of a GM1 gangliosides[7]. *P. aeruginosa* type IV pili and fimbriae bind to aGM1 and stimulate the expression of IL-8 by lung epithelial cells[40]. However,

others postulated that CFTR itself is the epithelial cellular receptor for *P. aeruginosa* resulting in the inability to ingest and clear *P. aeruginosa* in CFTR defective airway epithelial cells[41]. It is noteworthy that glycosphingolipid depletion therapy with PPMP has been experimentally shown to decrease the bacterial as well as viral load in several studies[42]. There are many strains of *P. aeruginosa* that differ with regard to expression of pili, other fimbriae and flagella. We used the well-characterized laboratory strain PAO1 [46]. Example 5.

**[0094]** Because *P. aeruginosa* infections are a constant problem for cystic fibrosis patients, we tested whether normalizing the level of sphingolipid synthesis in cystic fibrosis model lung epithelial cells would affect adhesion of *P. aeruginosa*. 16HBE 'sense' (expressing CFTR) and 'antisense' (that do not express CFTR) cells were grown to confluency in 96-well plates. They were then incubated for 2h in the presence of the control condition BSA or conditions that decrease sphingolipid synthesis: (1) C6-ceramide (20 μM). At this concentration c6-ceramide is not a substrate for metabolism to differentiated sphingolipid, but instead decreases sphingolipid synthesis through an end-product feedback mechanism. A wide range of concentrations can be used, including from about 5-100 micromolar can be used; (2) Eicosapentaenoic acid (EPA) (20:6, 0.3 mM), an unsaturated fatty acid that decreases sphingolipid synthesis; and (3) Myriocin (1μM), a specific inhibitor of SPT, the rate limiting enzyme of *de-novo* sphingolipid synthesis. Confluent cells were incubated for 1h with 50 μl of either the experimental GFP-labeled *P. aeruginosa* or the control *P. aeruginosa* that do not express GFP. The results demonstrate that ceramide decreased *P. aeruginosa* adhesion in both cell lines. Specifically, ceramide reduced bacterial adhesion in C38 control cells expressing normal CFTR by about 40%, and in antisense 16HBE cells (no CFTR) by about 64%. EPA and myriocin had no significant effect on *P. aeruginosa* adhesion in cells expressing normal CFTR because sphingolipid synthesis is not increased in these cells, but dramatically reduced adhesion by about 64% (EPA) and about 80% (myriocin) in cells expressing no CFTR.

**[0095]** Thus some embodiments of the invention are directed to a method for decreasing *P. aeruginosa* infection in a human or animal, preferably in a cystic fibrosis patient, by administering a therapeutic amount of an agent that decreases sphingolipid synthesis, particularly in lung epithelium. Examples include fenretinide, unsaturated fatty acids, GT 11, EPA, myriocin, dihydroceramide, ceramide, or a combination thereof. Any agent with acceptable toxicity that

reduces sphingolipid synthesis (through either *de novo* or recycling pathways) in an animal expressing no CFTR or defective CFTR can be administered alone or in combination with other agents to treat or prevent *P. aeruginosa* infection. The preferred route of administration is by inhalation since the main site of *P. aeruginosa* infection in cystic fibrosis patients is in the lungs. Without being bound by theory, we hypothesize that by decreasing sphingolipid synthesis, there is a reduction in gangliosides that facilitate *P. aeruginosa* adherence to lung epithelial cells.

**Table 1. Compounds, mechanisms and effects of SLS inhibitors**

| Compound | Mechanism | Conc. | Effect / Reference |
|---|---|---|---|
| Myriocin | Serine-palmitoyl transferase inhibitor | 0.5-1 μM | ↓ *de-novo* SLS[6] |
| Fumonisin B1 | Ceramide synthase inhibitor | 5-20 μM | ↓ SLS [6] |
| NOE (N-oleyl-ethanolamine) | Ceramidase inhibitor | 250 mM | ↑ Ceramide[6] |
| DMS (Dimethyl sphingosine) | Sphingosine kinase inhibitor | 5-20 μM | ↑ Sphingosine [63]<br>↑ recycling SLS |
| Ceramide | Negative feedback inhibition of SLS | 20 μM | ↓ *de-novo* SLS<br>↓ recycling SLS [6] |
| PPMP | Glucosylceramide synthase inhibitor | 5-20 μM | ↓ Glucosylceramide[59]<br>↑ Ceramide |
| NB-DNJ (N-Butyldeoxynojirimycin) | Glucosylceramide synthase inhibitor | 2-10 μM | ↓ Glucosylceramide[65]<br>= Ceramide (no effect) |

| Pathway | Regulators | Tracer |
|---|---|---|
| *De-novo* | ↑: Palmitoyl CoA<br>↓: Myriocin<br>↓: Ceramide, | $^3$H-Serine<br>$^3$H-Palmitoyl-CoA |
| Recycling | ↑: Sphinganine<br>↑: Saturated Fatty acids<br>↑: Sphingosine<br>↑: DMS<br>↓: Fumonisin<br>↓: Unsaturated fatty acids<br>↓: Ceramide | $^3$H-Sphingomyelin<br>$^3$H-Glucosylceramide<br>$^3$H-Sphinganine<br>$^3$H-Sphingosine |

**Table 2. Regulators and Tracers of Sphingolipid Synthesis**

**Bioactive Agents**

**[0096]** The term "bioactive agent" or "exogenous compound" as used herein includes any molecule, e.g., protein, oligopeptide, small organic molecule, polysaccharide, polynucleotide, lipid, etc., or mixtures thereof, with the capability of directly or indirectly altering the bioactivity of one of the various proteins. Some of the bioactive agents can be used therapeutically. Generally a plurality of assay mixtures is run in parallel with different bioactive agent concentrations to obtain a differential response to the various concentrations. Typically, one of these concentrations serves as a negative control, i.e., at zero concentration or below the level of detection.

**[0097]** Bioactive agent agents encompass numerous chemical classes, though typically they are organic molecules, preferably small organic compounds having a molecular weight of more than 100 and less than about 2,500 daltons. Bioactive agent agents comprise functional groups necessary for structural interaction with proteins, particularly hydrogen bonding, and typically include at least an amine, carbonyl, hydroxyl or carboxyl group, preferably at least two of the functional chemical groups. The bioactive agent agents often comprise cyclical carbon or heterocyclic structures and/or aromatic or polyaromatic structures substituted with one or more of the above functional groups. Bioactive agent agents are also found among biomolecules including peptides, saccharides, fatty acids, steroids, purines, pyrimidines, derivatives, structural analogs or combinations thereof. Particularly preferred are peptides.

**[0098]** Bioactive agent agents are obtained from a wide variety of sources including libraries of synthetic or natural compounds. For example, numerous means are available for random and directed synthesis of a wide variety or organic compounds and biomolecules, including expression of randomized oligonucleotides. Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant and animal extracts are available or readily produced. Additionally, natural or synthetically produced libraries and compounds are readily modified through conventional chemical, physical and biochemical means. Known pharmacological agents may be subjected to directed or random chemical modifications, such as acylation, alkylation, esterification, amidification to produce structural analogs.

**[0099]** In certain embodiments, the bioactive agent is a protein. By "protein" herein is meant at least two covalently attached amino acids, which includes proteins, polypeptides,

oligopeptides and peptides. The protein may be made up of naturally occurring amino acids and peptide bounds, or synthetic peptidomimetic structures. Thus "amino acid", or "peptide residue", as used herein means both naturally occurring and synthetic amino acids. For example, homo-phenylalanine, citrulline and noreleucine are considered amino acids for the purposes of the invention. "Amino acids" also includes imino acid residues such as proline and hydroxyproline. The side chains may be in either the (R) or the (S) configuration. In the preferred embodiment, the amino acids are in the (S) or L-configuration. If non-naturally occurring side chains are used, non-amino acid substituents may be used, for example to prevent or retard *in vivo* degradations.

**[0100]** In another embodiment, the bioactive agent is a naturally occurring protein or fragment or variant of a naturally occurring protein. Thus, for example, cellular extracts containing proteins, or random or directed digests of proteinaceous cellular extracts, may be used. In this way libraries of prokaryotic and eukaryotic proteins may be made for screening against one of the various proteins. Particularly preferred in this embodiment are libraries of bacterial, fungal, viral, and mammalian proteins, with the latter being preferred, and human proteins being especially preferred.

**[0101]** In a preferred embodiment, the bioactive agent are peptides of from about 5 to about 30 amino acids, with from about 5 to about 20 amino acids being preferred, and from about 7 to about 15 being particularly preferred. The peptides may be digests of naturally occurring proteins as is outlined above, random peptides, or "biased" random peptides. By "randomized" or grammatical equivalents herein is meant that each peptide consists of essentially random amino acids. Since generally these random peptides are chemically synthesized, they may incorporate any amino acid at any position. The synthetic process can be designed to generate randomized proteins, to allow the formation of all or most of the possible combinations over the length of the sequence, thus forming a library of randomized bioactive agent bioactive proteinaceous agents.

**[0102]** In one embodiment, the library is fully randomized, with no sequence preferences or constants at any position. In a preferred embodiment, the library is biased. That is, some positions within the sequence are either held constant, or are selected from a limited number of possibilities. For example, in a preferred embodiment, the amino acid residues are randomized within a defined class, for example, of hydrophobic amino acids, hydrophilic residues, sterically

biased (either small or large) residues, towards the creation of cysteines, for cross-linking, prolines for SH-3 domains, serines, threonines, tyrosines or histidines for phosphorylation sites, etc., or to purines, etc.

**[0103]** In a certain embodiments, the bioactive agent is an isolated nucleic acid, preferably antisense, siRNA, or cDNA" that binds to either the gene encoding the protein of interest, or its mRNA to block gene expression or mRNA translation, respectively. By "nucleic acid" or "oligonucleotide" or grammatical equivalents herein means at least two nucleotides covalently linked together. A nucleic acid of the present invention will generally contain phosphodiester bonds, although in some cases, as outlined below, nucleic acid analogs are included that may have alternate backbones, comprising, for example, phosphoramide (Beaucage et al., Tetrahedron 49)10):1925 (1993) and references therein; Letsinger, J. Org. Chem. 35:3800 (1970); Sprinzl et al., Eur. J. Biochem. 81:579 (1977); Letsinger et al., Nucl. Acids Res. 14:3487 (1986); Sawai et al, Chem. Lett. 805 (1984), Letsinger et al., J. Am. Chem. Soc. 110:4470 (1988); and Pauwels et al., Chemica Scripta 26:141 91986)), pohsphorothioate (Mag et al., Nucleic Acids Res. 19:1437 (1991); and U.S. Pat. No. 5,644,048), phosphorodithioate (Briu et al., J. Am. Chem. Soc. 111:2321 (1989), O-methylphophoroamidite linkages (see Eckstein, Oligonucleotides and Analogues: A Practical Approach, Oxford University Press), and peptide nucleic acid backbones and linkages (see Egholm, J. Am. Chem. Soc. 114:1895 (1992); Meier et al., Chem. Int. Ed. Engl. 31:1008 (1992); Nielsen, Nature, 365:566 (1993); Carlsson et al., Nature 380:207 (1996), all of which are incorporated by reference).

**[0104]** Other analog nucleic acids include those with positive backbones (Denpcy et al., Proc. Natl. Acad. Sci. USA 92:6097 (1995); non-ionic backbones (U.S. Pat. Nos. 5,386,023, 5,637,684, 5,602,240, 5,216,141 and 4,469,863; Kiedrowshi et al., Angew. Chem. Intl. Ed. English 30:423 (1991); Letsinger et al., J. Am. Chem. Soc. 110:4470 (1988); Letsinger et al., Nucleoside & Nucleoside 13:1597 (1994); Chapters 2 and 3, ASC Symposium Series 580, "Carbohydrate Modifications in Antisense Research", Ed. Y. S. Sanghui and P. Dan Cook; Mesmaeker et al., Bioorganic & Medicinal Chem. Lett. 4:395 (1994); Jeffs et al., J. Biomolecular NMR 34:17 (1994); Tetrahedron Lett. 37:743 (1996)) and non-ribose backbones, including those described in U.S. Pat. Nos. 5,235,033 and 5,034,506, and Chapters 6 and 7, ASC Symposium Series 580, "Carbohydrate Modifications in antisense Research", Ed. Y. S. Sanghui

and P. Can Cook. Nucleic acids containing one or more carbocyclic sugars are also included within the definition of nucleic acids (see Jenkins et al., Chem. Soc. Rev. (1995) pp169-176). Several nucleic acid analogs are described in Rawls, C & E News Jun. 2, 1997 page 35. All of these references are hereby expressly incorporated by reference. These modifications of the ribose-phosphate backbone may be done to facilitate the addition of additional moieties such as labels, or to increase the stability and half-life of such molecules in physiological environments. In addition, mixtures of naturally occurring acids and analogs can be made. Alternatively, mixtures of different nucleic acid analogs, and mixtures of naturally occurring nucleic acids and analogs may be made. The nucleic acids may be single stranded or double stranded, as specified, or contain portions of both double stranded or single stranded sequence. The nucleic acid may be DNA, both genomic and cDNA, RNA or a hybrid, where the nucleic acid contains any combination of deoxyribo- and ribo-nucleotides, and any combination of bases, including uracil, adenine, thymine, cytosine, guanine, inosine, xanthine hypoxathine, isocytosine, isoguanine, etc.

**[0105]** As described above generally for proteins, nucleic acid bioactive agents may be naturally occurring nucleic acids, random nucleic acids, or "biased" random nucleic acids. For example, digests of prokaryotic or eukaryotic genomes may be used as is outlined above for proteins.

**[0106]** In a preferred embodiment, the bioactive agents are obtained from combinatorial chemical libraries, a wide variety of which are available in the literature. By "combinatorial chemical library" herein is meant a collection of diverse chemical compounds generated in a defined or random manner, generally by chemical synthesis. Millions of chemical compounds can be synthesized through combinatorial mixing.

**[0107]** The determination of the binding of the bioactive agent to one of the various proteins may be done in a number of ways. In a preferred embodiment, the bioactive agent is labeled, and binding determined directly. For example, this may be done by attaching all or a portion of one of the various proteins to a solid support, adding a labeled bioactive agent (for example a bioactive agent comprising a fluorescent label), washing off excess reagent, and determining whether the label is present on the solid support. Various blocking and washing steps may be utilized as is known in the art.

**[0108]** By "labeled" herein is meant that the bioactive agent is either directly or indirectly labeled with a label which provides a detectable signal, e.g. a radioisotope (such as $H^3$, $C^{14}$, $P^{32}$, $P^{33}$, $S^{35}$, or $I^{125}$), a fluorescent or chemiluminescent compound (such as fluorescein isothiocyanate, rhodamine, or luciferin), an enzyme (such as alkaline phosphatase, beta-galactosidase or horseradish peroxidase), antibodies, particles such as magnetic particles, or specific binding molecules, etc. Specific binding molecules include pairs, such as biotin and streptavidin, digoxin and antidigoxin etc. For the specific binding members, the complementary member would normally be labeled with a molecule which provides for detection, in accordance with known procedures, as outlined above. The label can directly or indirectly provide a detectable signal. In some embodiments, only one of the components is labeled. Alternatively, more than one component may be labeled with different labels.

**[0109]** In a preferred embodiment, the binding of the bioactive agent is determined through the use of competitive binding assays. In this embodiment, the competitor is a binding moiety known to bind to the target molecule (i.e. one of the various proteins), such as an antibody, peptide, binding partner, ligand, etc. Under certain circumstances, there may be competitive binding as between the bioactive agent and the binding moiety, with the binding moiety displacing the bioactive agent.

**[0110]** In one embodiment, the bioactive agent is labeled. Either the bioactive agent bioactive agent, or the competitor, or both, is added first to the protein for a time sufficient to allow binding, if present. Incubations may be performed at any temperature which facilitates optimal activity, typically between 4 degrees Centigrade and 40 degrees Centigrade. Incubation periods are selected for optimum activity, but may also optimized to facilitate rapid high through put screening. Typically between 0.1 and 1 hour will be sufficient. Excess reagent is generally removed or washed away. The second component is then added, and the presence or absence of the labeled component is followed, to indicate binding.

**[0111]** In a preferred embodiment, the competitor is added first, followed by the bioactive agent bioactive agent. Displacement of the competitor is an indication that the bioactive agent is binding to one of the various proteins and thus is capable of binding to, and potentially modulating, its activity. In this embodiment, either component can be labeled. Thus, for example, if the competitor is labeled, the presence of label in the wash solution indicates

displacement by the agent. Alternatively, if the bioactive agent is labeled, the presence of the label on the support indicates displacement.

**[0112]** In an alternative embodiment, the bioactive agent is added first, with incubation and washing, followed by the competitor. The absence of binding by the competitor may indicate that the bioactive agent is bound to one of the various proteins with a higher affinity. Thus, if the bioactive agent is labeled, the presence of the label on the support, coupled with a lack of competitor binding, may indicate that the bioactive agent is capable of binding to one of the various proteins.

**[0113]** In a preferred embodiment, the methods comprise differential screening to identify bioactive agents that are capable of modulating the activity of one of the various proteins. In this embodiment, the methods comprise combining a protein and a competitor in a first sample. A second sample comprises a bioactive agent, a protein and a competitor. Addition of the bioactive agent is performed under conditions which allow the modulation of one of the various proteins. The binding of the competitor is determined for both samples, and a change or difference in binding between the two samples indicates the presence of an agent capable of binding to one of the various proteins and potentially modulating its activity. That is, if the binding of the competitor is different in the second sample relative to the first sample, the agent is capable of binding to one of the various proteins.

**[0114]** Positive controls and negative controls may be used in the assays. Preferably all control and test samples are performed in at least triplicate to obtain statistically significant results. Incubation of all samples is for a time sufficient for the binding of the agent to the protein. Following incubation, all samples are washed free of non-specifically bound material and the amount of bound, generally labeled agent determined. For example, where a radiolabel is employed, the samples may be counted in a scintillation counter to determine the amount of bound compound.

**[0115]** A variety of other reagents may be included in the screening assays. These include reagents like salts, neutral proteins, e.g. albumin, detergents, etc which may be used to facilitate optimal protein-protein binding and/or reduce non-specific or background interactions. Also reagents that otherwise improve the efficiency of the assay, such as protease inhibitors,

nuclease inhibitors, anti-microbial agents, etc., may be used. The mixture of components may be added in any order that provides for the requisite binding.

**[0116]** Screening for agents that modulate the activity of one of the various proteins may also be done. In a preferred embodiment, methods for screening for a bioactive agent capable of modulating the activity of one of the various proteins comprise the steps of adding a bioactive agent to a sample of one of the various proteins, as above, and determining an alteration in the biological activity of one of the various proteins. "Modulating the activity of one of the various proteins" includes an increase in activity, a decrease in activity, or a change in the type or kind of activity present. Thus, in this embodiment, the bioactive agent should both bind to the protein (although this may not be necessary), and alter its biological or biochemical activity as defined herein. The methods include both *in vitro* screening methods, as are generally outlined above, and *in vivo* screening of cells for alterations in the presence, distribution, activity or amount of one of the various proteins.

**Pharmaceutical compositions**

**[0117]** The invention thus provides methods for treating cystic fibrosis and *P. aeruginosa* infections in an animal. In one embodiment, the method involves administering a therapeutic agent that includes compounds that decrease sphingolipid synthesis including ceramide, dihydroceramide, dihydroceramide synthase inhibitors such as fenretinide or GT11 (Triola et al, Molecular Pharmacology 2004, 1671-1678), inhibitors of glucosylceramide synthase , sphingomyelin synthesis, sphingosine synthesis, given that these substances increase ceramide and thus inhibit de-novo synthesis, and enzyme inhibitors or antisense nucleic acids or si RNA that reduce the expression or activity of serine-palmitoyl transferase, dihydroceramide desaturase, sphingomyelin synthase, sphingosine synthase, glucosylceramide synthase in an amount sufficient to treat cystic fibrosis or P. a infections. Efficacy of the treatment can be monitored by determining whether the agent ameliorates any of the symptoms of the disease. Alternatively, one can monitor the level of SRE-mediated gene transcription, or sphingolipid synthesis.

**[0118]** The invention encompasses use of the polypeptides, nucleic acids, small molecules, antisense nucleic acids and si RNA and other therapeutic agents described herein

formulated in pharmaceutical compositions to administer to a subject or to target cells or tissues, preferably to the lung, in a subject whose cells express abnormal amounts of CFTR or defective CFTR, such as one of the mutations in CFTR in cystic fibrosis patients. The therapeutic agents (also referred to as "active compounds") can be incorporated into pharmaceutical compositions suitable for administration to a subject, preferably a human. Such compositions typically comprise the nucleic acid molecule, protein, modulator (enzyme inhibitor), or small molecule and a pharmaceutically acceptable carrier. It is understood however, that administration can also be to cells *in vitro* as well as to *in vivo* model systems such as human or non-human transgenic animals.

**[0119]** The term "administer" is used in its broadest sense and includes any method of introducing the compositions of the present invention into a subject; preferably inhalation therapy that delivers the agent to the lung. As used herein the language "pharmaceutically acceptable carrier" is intended to include any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like, compatible with pharmaceutical administration. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active compound, such media can be used in the compositions of the invention. Supplementary active compounds or therapeutic agents can also be incorporated into the compositions. A pharmaceutical composition of the invention is formulated to be compatible with its intended route of administration. Examples of routes of administration include parenteral, e.g., intravenous, intradermal, subcutaneous, oral including by inhalation, which is a method that is preferred for treating lung infections with *P. aeruginosa* and cystic fibrosis, transdermal (topical), transmucosal, and rectal administration. Solutions or suspensions used for parenteral, intradermal, or subcutaneous application can include the following components: a sterile diluent such as water for injection, saline solution, fixed oils, polyethylene glycols, glycerine, propylene glycol or other synthetic solvents; antibacterial agents such as benzyl alcohol or methyl parabens; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as ethylene diamante tetra acetic acid; buffers such as acetates, citrates or phosphates and agents for the adjustment of tonicity such as sodium chloride or dextrose. pH can be adjusted with acids or bases, such as hydrochloric acid or sodium hydroxide.

The parenteral preparation can be enclosed in ampoules, disposable syringes or multiple dose vials made of glass or plastic.

**[0120]** Pharmaceutical compositions suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. For intravenous administration, suitable carriers include physiological saline, bacteriostatic water, Cremophor ELTM (BASF, Parsippany, N.J.) or phosphate buffered saline (PBS). In all cases, the composition must be sterile and should be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prevention of the action of microorganisms can be achieved by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, ascorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as mannitol, sorbitol, sodium chloride in the composition. Prolonged absorption of the injectable compositions can be brought about by including in the composition an agent which delays absorption, for example, aluminum monostearate and gelatin.

**[0121]** Sterile injectable solutions can be prepared by incorporating the active compound in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle which contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and freeze-drying which yields a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

**[0122]** Oral compositions generally include an inert diluent or an edible carrier. They can be enclosed in gelatin capsules or compressed into tablets. Depending on the specific conditions

being treated, pharmaceutical compositions of the present invention for treatment of atherosclerosis or the other elements of metabolic syndrome can be formulated and administered systemically or locally. Techniques for formulation and administration can be found in "Remington: The Science and Practice of Pharmacy" (20.sup.th edition, Gennaro (ed.) and Gennaro, Lippincott, Williams & Wilkins, 2000). For oral administration, the agent can be contained in enteric forms to survive the stomach or further coated or mixed to be released in a particular region of the GI tract by known methods. For the purpose of oral therapeutic administration, the active compound can be incorporated with excipients and used in the form of tab lets, troches, or capsules. Oral compositions can also be prepared using a fluid carrier for use as a mouthwash, wherein the compound in the fluid carrier is applied orally and swished and expectorated or swallowed. Pharmaceutically compatible binding agents, and/or adjuvant materials can be included as part of the composition. The tablets, pills, capsules, troches and the like can contain any of the following ingredients, or compounds of a similar nature: a binder such as microcrystalline cellulose, gum tragacanth or gelatin; an excipient such as starch or lactose, a disintegrating agent such as alginic acid, Primogel, or corn starch; a lubricant such as magnesium stearate or Sterotes; a glidant such as colloidal silicon dioxide; a sweetening agent such as sucrose or saccharin; or a flavoring agent such as peppermint, methyl salicylate, or orange flavoring.

**[0123]** For administration by inhalation, the compounds are delivered in the form of an aerosol spray from pressured container or dispenser, which contains a suitable propellant, e.g., a gas such as carbon dioxide, or a nebulizer. Systemic administration can also be by transmucosal or transdermal means. For transmucosal or transdermal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art, and include, for example, for transmucosal administration, detergents, bile salts, and fusidic acid derivatives. Transmucosal administration can be accomplished through the use of nasal sprays or suppositories. For transdermal administration, the active compounds are formulated into ointments, salves, gels, or creams as generally known in the art.

**[0124]** If appropriate, the compounds can also be prepared in the form of suppositories (e.g., with conventional suppository bases such as cocoa butter and other glycerides) or retention enemas for rectal delivery.

**[0125]** In one embodiment, the active compounds are prepared with carriers that will protect the compound against rapid elimination from the body, such as a controlled release formulation, including implants and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Methods for preparation of such formulations will be apparent to those skilled in the art. The materials can also be obtained commercially from Alza Corporation and Nova Pharmaceuticals, Inc. Liposomal suspensions (including liposomes targeted to infected cells with monoclonal antibodies to viral antigens) can also be used as pharmaceutically acceptable carriers. These can be prepared according to methods known to those skilled in the art, for example, as described in U.S. Pat. No. 4,522,811.

**[0126]** It is especially advantageous to formulate oral or parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. "Dosage unit form" as used herein refers to physically discrete units suited as unitary dosages for the subject to be treated; each unit containing a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms of the invention are dictated by and directly dependent on the unique characteristics of the active compound and the particular therapeutic effect to be achieved, and the limitations inherent in the art of compounding such an active compound for the treatment of individuals.

**[0127]** A therapeutically effective amount of protein or polypeptide or nucleic acid (antisense or si RNA) (i.e., an effective dosage) has been defined. This amount typically varies and can be an amount sufficient to achieve serum therapeutic agent levels typically of between about 1 nanogram per milliliter and about 10 micrograms per milliliter in the subject, or an amount sufficient to achieve serum therapeutic agent levels of between about 1 nanogram per milliliter and about 7 micrograms per milliliter in the subject. Expressed as a daily dose, this amount can be between about 0.1 nanograms per kilogram body weight per day and about 20 milligrams per kilogram body weight per day, or between about 1 nanogram per kilogram body weight per day and about 10 milligrams per kilogram body weight per day. A therapeutic amount can be determined as an amount that ameliorates one or more symptoms of the disease, or that decreases sphingolipid synthesis in a biological sample taken from the patient, such as peripheral

blood mononuclear cells (described in the Examples) or lung epithelium. However, the skilled artisan will appreciate that certain factors may influence the dosage required to effectively treat a subject, including but not limited to the severity of the condition, previous treatments, the general health and/or age of the subject, and other disorders or diseases present. Moreover, treatment of a subject with a therapeutically effective amount of a protein, polypeptide, or nucleotide can include a single treatment or, preferably, can include a series of treatments.

**[0128]** It will also be appreciated that the effective dosage of, protein, or polypeptide used for treatment may increase or decrease over the course of a particular treatment. Changes in dosage may result and become apparent from monitoring the level of sphingolipids in a biological sample. If the therapeutic agent is administered by inhalation, higher amounts of the agent may be administered than if the agent were to be administered systemically. In studies by others C6-ceramide has been administered at a dose of about 100mg/kg by intravenous injection; the associated benefits were considered to outweigh the adverse effects. Other drugs like myriocin that have toxic side effects can be used. As with anticancer agents that have adverse side effects and high toxicity, localized delivery is advantageous. Moreover, like anticancer agents, the benefits often outweigh the risks. So is it with seriously ill patients having cystic fibrosis. Myriocin has been used either intraperitoneally or orally at doses around 1 mg/kg ( in mice ). The drug FTY720, a derivative of myriocin, has been used at doses of 0. 5 -2.5 mg/dose in human adults orally. Fumonisin B1 has been given subcutaneously at 2.5 mg/kg in mice. Fenretinide is used from 200 mg/day orally or at higher doses.

**[0129]** An agent may, for example, be a small molecule. Such small molecules include, but are not limited to, peptides, peptidomimetics, amino acids, amino acid analogs, polynucleotides, polynucleotide analogs, nucleotides, nucleotide analogs, organic or inorganic compounds (i.e., including heteroorganic and organometallic compounds) having a molecular weight less than about 10,000 grams per mole, organic or inorganic compounds having a molecular weight less than about 5,000 grams per mole, organic or inorganic compounds having a molecular weight less than about 1,000 grams per mole, organic or inorganic compounds having a molecular weight less than about 500 grams per mole, and salts, esters, and other pharmaceutically acceptable forms of such compounds.

**[0130]** It is understood that appropriate doses of small molecule agents depends upon a number of factors within the ken of the ordinarily skilled physician, veterinarian, or researcher. The dose(s) of the small molecule will vary, for example, depending upon the identity, size, and condition of the subject or sample being treated, further depending upon the route by which the composition is to be administered, if applicable, and the effect which the practitioner desires the small molecule to have upon the nucleic acid or polypeptide of the invention. It is furthermore understood that appropriate doses of a small molecule depend upon the potency of the small molecule with respect to the expression or activity to be modulated. Such appropriate doses may be determined using the assays described herein. When one or more of these small molecules is to be administered to an animal (e.g., a human) in order to modulate expression or activity of a polypeptide or nucleic acid of the invention, a physician, veterinarian, or researcher may, for example, prescribe a relatively low dose at first, subsequently increasing the dose until an appropriate response is obtained. In addition, it is understood that the specific dose level for any particular animal subject will depend upon a variety of factors including the activity of the specific compound employed, the age, body weight, general health, gender, and diet of the subject, the time of administration, the route of administration, the rate of excretion, any drug combination, and the degree of expression or activity to be modulated.

**Antisense Nucleic Acids**

**[0131]** Other embodiments of the present invention are directed to the use of antisense nucleic acids (either DNA or RNA) or small interfering RNA to inhibit expression of the various enzymes associated with sphingolipid synthesis in cells expressing defective CFTR, including serine palmitoyl transferase, sphingosine kinase, ceramide synthase/fatty acid desaturase, UDP-glucose ceramide glucosyltransferase and glucosylceramide synthase (hereafter "the various enzymes"). The SEQ ID NOs. for these enzymes are set forth below. The antisense nucleic acid can be antisense RNA, antisense DNA or small interfering RNA. Based on these known sequences, antisense DNA or RNA that hybridize sufficiently to the respective gene or mRNA encoding the various enzymes to turn off expression can be readily designed and engineered using methods known in the art.

**[0132]** Nucleic acids that interfere with transcription or translation of a gene product including these various enzymes include DNA that stops transcription of the sense strand of the gene encoding the targeted enzyme, such as antisense DNA. Identification of antisense nucleotides (RNA or DNA) is straightforward since the gene sequence encoding the enzymes in humans is known. RNA nucleic acids that stop or otherwise interfere with efficient transcription or translation of the gene or messenger RNA for the various enzymes can also be used therapeutically to interfere with expression; these include antisense RNA or small interfering RNA. The sequence of messenger RNA for the various enzymes can be determined form the gene sequence, and antisense nucleic acid s that would bind to the messenger RNA and block translation can be determined using routine methods. Antisense technology and small interfering RNAs are well known in the art and are described in detail below.

**[0133]** Antisense-RNA and anti-sense DNA have been used therapeutically in mammals to treat various diseases. See for example Agrawal, S. and Zhao, Q. (1998) Curr. Opi. Chemical Biol. Vol. 2, 519-528; Agrawal, S and Zhang, R. (1997) CIBA Found. Symp. Vol. 209, 60-78; and Zhao, Q, et al., (1998), Antisense Nucleic Acid Drug Dev. Vol 8, 451-458; the entire contents of which are hereby incorporated by reference as if fully set forth herein. Antisense oligodeoxyribonucleotides (antisense-DNA) and oligoribonucleotides (antisense-RNA) can base pair with a gene, or its transcript. An antisense PS-oligodeoxyribonucleotide for treatment of cytomegalovirus retinitis in AIDS patients is the first antisense oligodeoxyribonucleotiede approved for human use in the US. Anderson, K.O., et al., (1996) Antimicrobiol. Agents Chemother. Vol. 40, 2004-2011, the entire contents of which are hereby incorporated by reference as if fully set forth herein.

**[0134]** U.S. Patent No. 6, 828, 151 by Borchers, et al. entitled Antisense modulation of hematopoietic cell protein tyrosine kinase expression describes methods for making and using antisense-nucleic acid s and their formulation. The entire contents of U.S. Patent No. 6, 828, 151 are hereby incorporated by reference as if fully set forth herein. Others have shown that antisense nucleic acid s complementary to the gene for glutamine synthetase mRNA in Mtb effectively enter the bacteria, complex with the mRNA and inhibit glutamine synthetase expression, the amount of the poly-L-glutamate/glutamine component in the cell wall, and bacterial replication

*in vitro*. Harth, G., et al., PNAS Jan. 4, 2000, Vol. 97, No. 1, P 418-423, the entire contents of which are hereby incorporated by reference as if fully set forth herein.

**[0135]** As used herein, the terms "target nucleic acid" encompass DNA encoding the various enzymes, RNA (including pre-mRNA and mRNA) transcribed from such DNA, and also cDNA derived from such RNA. The specific hybridization of a nucleic acid oligomeric compound with its target nucleic acid interferes with the normal function of the target nucleic acid. This modulation of function of a target nucleic acid by compounds which specifically hybridize to it is generally referred to as "antisense". The functions of DNA to be interfered with include replication and transcription. The functions of RNA to be interfered with include all vital functions such as, for example, translocation of the RNA to the site of protein translation, translation of protein from the RNA, and catalytic activity which may be engaged in or facilitated by the RNA. The overall effect of such interference with target nucleic acid function is modulation of the expression of the various enzymes . In the context of the present invention, "modulation" means (inhibition) in the expression of the genes or mRNA of the various proteins (enzymes). In the context of the present invention, inhibition is the preferred form of modulation of gene expression and mRNA is a preferred target.

**[0136]** The targeting process includes determination of a site or sites within the target gene (or mRNA) encoding the various enzymes for the antisense interaction to occur to achieve the desired inhibitory effect. Within the context of the present invention, a preferred intragenic site is the region encompassing the translation initiation or termination codon of the open reading frame (ORF) of the gene. Since, as is known in the art, the translation initiation codon is typically 5'-AUG (in transcribed mRNA molecules; 5'-ATG in the corresponding DNA molecule), the translation initiation codon is also referred to as the "AUG codon," the "start codon" or the "AUG start codon". A minority of genes have a translation initiation codon having the RNA sequence 5'-GUG, 5'-UUG or 5'-CUG, and 5'-AUA, 5'-ACG and 5'-CUG have been shown to function *in vivo*. Thus, the terms "translation initiation codon" and "start codon" can encompass many codon sequences, even though the initiator amino acid in each instance is typically methionine (in eukaryotes) or formylmethionine (in prokaryotes). It is also known in the art that eukaryotic and prokaryotic genes may have two or more alternative start codons, any one of which may be preferentially utilized for translation initiation in a particular cell type or tissue, or under a particular set of conditions. In the context of the invention, "start codon" and

"translation initiation codon" refer to the codon or codons that are used *in vivo* to initiate translation of an mRNA molecule transcribed from a gene.

**[0137]** It is also known in the art that a translation termination codon (or "stop codon") of a gene may have one of three sequences, i.e., 5'-UAA, 5'-UAG and 5'-UGA (the corresponding DNA sequences are 5'-TAA, 5'-TAG and 5'-TGA, respectively). The terms "start codon region" and "translation initiation codon region" refer to a portion of such an mRNA or gene that encompasses from about 25 to about 50 contiguous nucleotides in either direction (i.e., 5' or 3') from a translation initiation codon. Similarly, the terms "stop codon region" and "translation termination codon region" refer to a portion of such an mRNA or gene that encompasses from about 25 to about 50 contiguous nucleotides in either direction (i.e., 5' or 3') from a translation termination codon.

**[0138]** The open reading frame (ORF) or "coding region," which is known in the art to refer to the region between the translation initiation codon and the translation termination codon, is also a region which may be targeted effectively. Other target regions include the 5' untranslated region (5'UTR), known in the art to refer to the portion of an mRNA in the 5' direction from the translation initiation codon, and thus including nucleotides between the 5' cap site and the translation initiation codon of an mRNA or corresponding nucleotides on the gene, and the 3' untranslated region (3'UTR), known in the art to refer to the portion of an mRNA in the 3' direction from the translation termination codon, and thus including nucleotides between the translation termination codon and 3' end of an mRNA or corresponding nucleotides on the gene.

**[0139]** It is also known in the art that variants can be produced through the use of alternative signals to start or stop transcription and that pre-mRNAs and mRNAs can possess more that one start codon or stop codon. Variants that originate from a pre-mRNA or mRNA that use alternative start codons are known as "alternative start variants" of that pre-mRNA or mRNA. Those transcripts that use an alternative stop codon are known as "alternative stop variants" of that pre-mRNA or mRNA. One specific type of alternative stop variant is the "polyA variant" in which the multiple transcripts produced result from the alternative selection of one of the "polyA stop signals" by the transcription machinery, thereby producing transcripts that terminate at unique polyA sites.

**[0140]** Once one or more target sites have been identified, nucleic acids are chosen which are sufficiently complementary to the target, i.e., hybridize sufficiently well and with sufficient specificity, to give the desired effect of inhibiting gene expression and transcription.

**[0141]** In the context of this invention, "hybridization" means hydrogen bonding, which may be Watson-Crick, Hoogsteen or reversed Hoogsteen hydrogen bonding, between complementary nucleoside or nucleotide bases. For example, adenine and thymine are complementary nucleobases which pair through the formation of hydrogen bonds. "Complementary," as used herein, refers to the capacity for precise pairing between two nucleotides. For example, if a nucleotide at a certain position of a nucleic acid is capable of hydrogen bonding with a nucleotide at the same position of a DNA or RNA molecule, then the nucleic acid and the DNA or RNA are considered to be complementary to each other at that position. The nucleic acid and the DNA or RNA are complementary to each other when a sufficient number of corresponding positions in each molecule are occupied by nucleotides which can hydrogen bond with each other. Thus, "specifically hybridizable" and "complementary" are terms which are used to indicate a sufficient degree of complementarity or precise pairing such that stable and specific binding occurs between the nucleic acid and the DNA or RNA target. It is understood in the art that the sequence of an antisense compound need not be 100% complementary to that of its target nucleic acid to be specifically hybridizable. An antisense compound is specifically hybridizable when binding of the compound to the target DNA or RNA molecule interferes with the normal function of the target DNA or RNA to cause a loss of utility, and there is a sufficient degree of complementarity to avoid non-specific binding of the antisense compound to non-target sequences under conditions in which specific binding is desired, i.e., under physiological conditions in the case of *in vivo* assays or therapeutic treatment, and in the case of *in vitro* assays, under conditions in which the assays are performed.

**[0142]** Antisense and other compounds of the invention which hybridize to the target and inhibit expression of the target are identified through routine experimentation, and the sequences of these compounds are herein below identified as preferred embodiments of the invention. The target sites to which these preferred sequences are complementary are herein below referred to as "active sites" and are therefore preferred sites for targeting. Therefore another embodiment of the invention encompasses compounds which hybridize to these active sites.

**[0143]** Antisense nucleic acid drugs, including ribozymes, have been safely and effectively administered to humans and numerous clinical trials are presently underway. It is thus established that nucleic acid s can be useful therapeutic modalities that can be configured to be useful in treatment regimes for treatment of cells, tissues and animals, especially humans, for example to regulate expression of the various enzymes involved in sphingolipid synthesis in cystic fibrosis patients.

**[0144]** Nucleic acid in the context of this invention includes "oligonucleotide", which refers to an oligomer or polymer of ribonucleic acid (RNA) or deoxyribonucleic acid (DNA) or mimetics thereof. This term includes oligonucleotides composed of naturally-occurring nucleobases, sugars and covalent internucleoside (backbone) linkages as well as oligonucleotides having non-naturally-occurring portions which function similarly. Such modified or substituted oligonucleotides are often preferred over native forms because of desirable properties such as, for example, enhanced cellular uptake, enhanced affinity for nucleic acid target and increased stability in the presence of nucleases.

**[0145]** While antisense nucleic acids are a preferred form of antisense compound, the present invention comprehends other oligomeric antisense compounds, including but not limited to oligonucleotide mimetics. The antisense compounds in accordance with this invention preferably comprise from about 8 to about 50 nucleobases (i.e. from about 8 to about 50 linked nucleosides). Particularly preferred antisense compounds are antisense nucleic acids, even more preferably those comprising from about 12 to about 30 nucleobases. Antisense compounds include ribozymes, external guide sequence (EGS) nucleic acid s (oligozymes), and other short catalytic RNAs or catalytic nucleic acid s which hybridize to the target nucleic acid and modulate its expression.

**[0146]** The antisense compounds used in accordance with this invention may be conveniently and routinely made through the well-known technique of solid phase synthesis. Equipment for such synthesis is sold by several vendors including, for example, Applied Biosystems (Foster City, Calif.). Any other means for such synthesis known in the art may additionally or alternatively be employed. It is well known to use similar techniques to prepare nucleic acid s such as the phosphorothioates and alkylated derivatives.

**[0147]** The antisense compounds of the invention are synthesized *in vitro* and do not include antisense compositions of biological origin, or genetic vector constructs designed to direct the *in vivo* synthesis of antisense molecules. The compounds of the invention may also be admixed, encapsulated, conjugated or otherwise associated with other molecules, molecule structures or mixtures of compounds, as for example, liposomes, receptor targeted molecules, oral, rectal, topical or other formulations, for assisting in uptake, distribution and/or absorption. Representative United States patents that teach the preparation of such uptake, distribution and/or absorption assisting formulations include, but are not limited to, U.S. Pat. Nos.: 5,108,921; 5,354,844; 5,416,016; 5,459,127; 5,521,291; 5,543,158; 5,547,932; 5,583,020; 5,591,721; 4,426,330; 4,534,899; 5,013,556; 5,108,921; 5,213,804; 5,227,170; 5,264,221; 5,356,633; 5,395,619; 5,416,016; 5,417,978; 5,462,854; 5,469,854; 5,512,295; 5,527,528; 5,534,259; 5,543,152; 5,556,948; 5,580,575; and 5,595,756, each of which is herein incorporated by reference.

**[0148]** The antisense compounds of the present invention can be utilized for diagnostics, therapeutics, prophylaxis and as research reagents and kits. For therapeutics, an animal, preferably a human, suspected of having a disease or disorder which can be treated by modulating the expression of the various enzymes, such as cystic fibrosis, is treated by administering antisense compounds in accordance with this invention. The compounds of the invention can be utilized in pharmaceutical compositions by adding an effective amount of an antisense compound to a suitable pharmaceutically acceptable diluent or carrier. Use of the antisense compounds and methods of the invention may also be useful prophylactically, e.g., to prevent or delay *P. aeruginosa* infection.

**[0149]** The present invention also includes pharmaceutical compositions and formulations which include the antisense compounds of the invention that are administered to return the level of sphingolipid synthesis in cystic fibrosis patients to normal, especially in lung and colon epithelial cells. The pharmaceutical compositions of the present invention may be administered in a number of ways depending upon whether local or systemic treatment is desired and upon the area to be treated. Administration may be topical, e.g., by inhalation or insufflation of powders or aerosols, including by nebulizer; intratracheal, intranasal, epidermal and transdermal), oral or parenteral. Parenteral administration includes intravenous, intraarterial,

subcutaneous, intraperitoneal or intramuscular injection or infusion; or intracranial, e.g., intrathecal or intraventricular, administration.

**Small Interfering RNA**

**[0150]** US Patent Application 20040023390 (the entire contents of which are hereby incorporated by reference as if fully set forth herein ) teaches that double-stranded RNA (dsRNA) can induce sequence-specific posttranscriptional gene silencing in many organisms by a process known as RNA interference (RNAi). However, in mammalian cells, dsRNA that is 30 base pairs or longer can induce sequence-nonspecific responses that trigger a shut-down of protein synthesis and even cell death through apoptosis. Recent work shows that RNA fragments are the sequence-specific mediators of RNAi (Elbashir et al., 2001). Interference of gene expression by these small interfering RNA (siRNA) is now recognized as a naturally occurring strategy for silencing genes in C. elegans, Drosophila, plants, and in mouse embryonic stem cells, oocytes and early embryos (Cogoni et al., 1994; Baulcombe, 1996; Kennerdell, 1998; Timmons, 1998; Waterhouse et al., 1998; Wianny and Zernicka-Goetz, 2000; Yang et al., 2001; Svoboda et al., 2000).

**[0151]** In mammalian cell culture, a siRNA-mediated reduction in gene expression has been accomplished by transfecting cells with synthetic RNA nucleic acid s (Caplan et al., 2001; Elbashir et al., 2001). The 20040023390 application, the entire contents of which are hereby incorporated by reference as if fully set forth herein, provides methods using a viral vector containing an expression cassette containing a pol II promoter operably-linked to a nucleic acid sequence encoding a small interfering RNA molecule (siRNA) targeted against a gene of interest.

**[0152]** As used herein RNAi is the process of RNA interference. A typical mRNA produces approximately 5,000 copies of a protein. RNAi is a process that interferes with or significantly reduces the number of protein copies made by an mRNA. For example, a double-stranded short interfering RNA (siRNA) molecule is engineered to complement and match the protein-encoding nucleotide sequence of the target mRNA to be interfered with. Following intracellular delivery, the siRNA molecule associates with an RNA-induced silencing complex (RISC). The siRNA-associated RISC binds the target mRNA (such as mRNA encoding “the

various enzymes" the sequences of which are set forth herein) through a base-pairing interaction and degrades it. The RISC remains capable of degrading additional copies of the targeted mRNA. Other forms of RNA can be used such as short hairpin RNA and longer RNA molecules. Longer molecules cause cell death, for example by instigating apoptosis and inducing an interferon response. Cell death was the major hurdle to achieving RNAi in mammals because dsRNAs longer than 30 nucleotides activated defense mechanisms that resulted in non-specific degradation of RNA transcripts and a general shutdown of the host cell. Using from about 20 to about 29 nucleotide siRNAs to mediate gene-specific suppression in mammalian cells has apparently overcome this obstacle. These siRNAs are long enough to cause gene suppression but not of a length that induces an interferon response.

**Protein Modifications**

**[0153]** Uncarboxylated osteocalcin can be modified according to known methods in medicinal chemistry to increase its stability, half-life, uptake or efficacy. Known modifications include, but are not limited to, acetylation, acylation, ADP-ribosylation, amidation, covalent attachment of flavin, covalent attachment of a heme moiety, covalent attachment of a nucleotide or nucleotide derivative, covalent attachment of a lipid or lipid derivative, covalent attachment of phosphatidylinositol, cross-linking, cyclization, disulfide bond formation, demethylation, formation of covalent crosslinks, formation of cystine, formation of pyroglutamate, formylation, glycosylation, GPI anchor formation, hydroxylation, iodination, methylation, myristoylation, oxidation, proteolytic processing, phosphorylation, prenylation, racemization, selenoylation, sulfation, transfer-RNA mediated addition of amino acids to proteins such as arginylation, and ubiquitination.

**[0154]** Acylation of the N-terminal amino group can be accomplished using a hydrophilic compound, such as hydroorotic acid or the like, or by reaction with a suitable isocyanate, such as methylisocyanate or isopropylisocyanate, to create a urea moiety at the N-terminus. Other agents can also be N-terminally linked that will increase the duration of action of the SRIF analog as known in this art.

**[0155]** Reductive amination is the process by which ammonia is condensed with aldehydes or ketones to form imines which are subsequently reduced to amines. For drugs

bearing one or more amino groups, reductive amination is a potentially useful method for conjugation to PEG. Covalent linkage of poly(ethylene glycol) (PEG) to drug molecules results in water-soluble conjugates with altered bioavailability, pharmacokinetics, immunogenic properties, and biological activities. For drugs bearing one or more amino groups, reductive amination is a potentially useful method for conjugation to PEG. Bentley et al., J Pharm Sci. 1998 Nov;87(11):1446-9.

**[0156]** Proteins like osteocalcin can also be modified to create peptide linkages that are susceptible to proteolytic enzymes. For instance, alkylation of cysteine residues with P-haloethylamines yields peptide linkages that are hydrolyzed by trypsin. Such modifications are well-known to those of skill in the art and have been described in great detail in the scientific literature. Several particularly common modifications, glycosylation, lipid attachment, sulfation, hydroxylation and ADP-ribosylation, for instance, are described in most basic texts, such as Proteins--Structure and Molecular Properties, 2nd ed., T. E. Creighton, W. H. Freeman and Company, New York (1993). Many detailed reviews are available on this subject, such as by Wold, F., Posttranslational Covalent Modification of Proteins, B. C. Johnson, Ed., Academic Press, New York 1-12 (1983); Seifter et al. (1990) Meth. Enzymol. 182: 626-646) and Rattan et al. (1992) Ann. NY: Acad. Sci. 663:48-62).

**[0157]** As is also well known, polypeptides are not always entirely linear. For instance, polypeptides may be branched as a result of ubiquitination, and they may be circular, with or without branching, generally as a result of post-translation events, including natural processing events and events brought about by human manipulation which do not occur naturally. Circular, branched and branched circular polypeptides may be synthesized by non-translational natural processes and by synthetic methods.

**[0158]** Modifications can occur anywhere in a polypeptide, including the peptide backbone, the amino acid side-chains and the amino or carboxyl termini. Blockage of the amino or carboxyl group in a polypeptide, or both, by a covalent modification, is common in naturally-occurring and synthetic polypeptides. For instance, the amino terminal residue of polypeptides made in E. coli, prior to proteolytic processing, almost invariably will be N-formylmethionine.

**[0159]** The modifications can be a function of how the protein is made. For recombinant polypeptides, for example, the modifications will be determined by the host cell posttranslational

modification capacity and the modification signals in the polypeptide amino acid sequence. Accordingly, when glycosylation is desired, a polypeptide should be expressed in a glycosylating host, generally a eukaryotic cell. Insect cells often carry out the same posttranslational glycosylations as mammalian cells, and, for this reason, insect cell expression systems have been developed to efficiently express mammalian proteins having native patterns of glycosylation. Similar considerations apply to other modifications. The same type of modification may be present in the same or varying degree at several sites in a given polypeptide. Also, a given polypeptide may contain more than one type of modification.

**[0160]** Isolated osteocalcin can be purified from cells that naturally express it, e.g., osteoblasts, purified from cells that naturally express it but have been modified to overproduce osteocalcin, e.g., purified from cells that have been altered to express it (recombinant), synthesized using known protein synthesis methods, by modifying cells that naturally encode osteocalcin to express it, or from bacteria modified to overexpress it.

**[0161]** A brief description of various protein modifications that come within the scope of this invention are set forth below:

| **Protein Modification** | **Description** |
|---|---|
| **Acetylation** | Acetylation of N-terminus or e-lysines. Introducing an acetyl group into a protein, specifically, the substitution of an acetyl group for an active hydrogen atom.<br><br>A reaction involving the replacement of the hydrogen atom of a hydroxyl group with an acetyl group ($CH_3CO$) yields a specific ester, the acetate. Acetic anhydride is commonly used as an acetylating agent, which reacts with free hydroxyl groups.<br><br>Acylation may facilitate addition of other functional groups. A common reaction is acylation of e.g., conserved lysine residues with a biotin appendage. |
| **ADP-ribosylation** | Covalently linking proteins or other compounds via an arginine-specific reaction. |
| **Alkylation** | Alkylation is the transfer of an alkyl group from one molecule to another. The alkyl group may be transferred as an alkyl |

| Protein Modification | Description |
|---|---|
| | carbocation, a free radical or a carbanion (or their equivalents). Alkylation is accomplished by using certain functional groups such as alkyl electrophiles, alkyl nucleophiles or sometimes alkyl radicals or carbene acceptors. A common example is methylation (usually at a lysine or arginine residue). |
| **Amidation** | Reductive animation of the N-terminus. Methods for amidation of insulin are described in U.S. 4,489,159. |
| **Carbamylation** | Nigen et al. describes a method of carbamylating hemoglobin. |
| **Citrullination** | Citrullination involves the addition of citrulline amino acids to the arginine residues of a protein, which is catalyzed by peptidylarginine deaminase enzymes (PADs). This generally converts a positively charged arginine into a neutral citrulline residue, which may affect the hydrophobicity of the protein (and can lead to unfolding). |
| **Condensation of amines with aspartate or glutamate** | Such reactions, may be used, e.g., to attach a peptide to other proteins labels. |
| **Covalent attachment of flavin** | Flavin mononucleotide (FAD) may be covalently attached to serine and/or threonine residues. May be used, e.g., as a light-activated tag. |
| **Covalent attachment of heme moiety** | A heme moiety is generally a prosthetic group that consists of an iron atom contained in the center of a large heterocyclic organic ring, which is referred to as a porphyrin. The heme moiety may be used, e.g., as a tag for the peptide. |
| **Attachment of a nucleotide or nucleotide derivative** | May be used as a tag or as a basis for further derivatising a peptide. |
| **Cross-linking** | Cross-linking is a method of covalently joining two proteins. Cross-linkers contain reactive ends to specific functional groups (primary amines, sulfhydryls, etc.) on proteins or other |

| Protein Modification | Description |
|---|---|
| | molecules. Several chemical groups may be targets for reactions in proteins and peptides. For example, Ethylene glycol bis[succinimidylsuccinate, Bis[2-(succinimidooxycarbonyloxy)ethyl]sulfone, and Bis[sulfosuccinimidyl] suberate link amines to amines. |
| **Cyclization** | For example, cyclization of amino acids to create optimized delivery forms that are resistant to, e.g., aminopeptidases (e.g., formation of pyroglutamate, a cyclized form of glutamic acid). |
| **Disulfide bond formation** | Disulfide bonds in proteins are formed by thiol-disulfide exchange reactions, particularly between cysteine residues (e.g., formation of cystine). |
| **Demethylation** | See, e.g., U.S. 4,250,088 (Process for demethylating lignin). |
| **Formylation** | The addition of a formyl group to, e.g., the N-terminus of a protein. See, e.g., U.S. Patent Nos. 4,059,589, 4,801,742, and 6,350,902. |
| **Glycylation** | The covalent linkage of one to more than 40 glycine residues to the tubulin C-terminal tail. |
| **Glycosylation** | Glycosylation may be used to add saccharides (or polysaccharides) to the hydroxy oxygen atoms of serine and threonine side chains (which is also known as O-linked Glycosylation). Glycosylation may also be used to add saccharides (or polysaccharides) to the amide nitrogen of asparagine side chains (which is also known as N-linked Glycosylation), e.g., via oligosaccharyl transferase. |
| **GPI anchor formation** | The addition of glycosylphosphatidylinositol to the C-terminus of a protein. GPI anchor formation involves the addition of a hydrophobic phosphatidylinositol group - linked through a carbohydrate containing linker (e.g., glucosamine and mannose linked to phosphoryl ethanolamine residue) - to the C-terminal amino acid of a protein. |
| **Hydroxylation** | Chemical process that introduces one or more hydroxyl groups |

| Protein Modification | Description |
|---|---|
| | (-OH) into a protein (or radical). Hydroxylation reactions are typically catalyzed by hydroxylases. Proline is the principal residue to be hydroxylated in proteins, which occurs at the $C^{\gamma}$ atom, forming hydroxyproline (Hyp). In some cases, proline may be hydroxylated at its $C^{\beta}$ atom. Lysine may also be hydroxylated on its $C^{\delta}$ atom, forming hydroxylysine (Hyl). These three reactions are catalyzed by large, multi-subunit enzymes known as prolyl 4-hydroxylase, prolyl 3-hydroxylase and lysyl 5-hydroxylase, respectively. These reactions require iron (as well as molecular oxygen and α-ketoglutarate) to carry out the oxidation, and use ascorbic acid to return the iron to its reduced state. |
| **Iodination** | See, e.g., U.S. 6,303,326 for a disclosure of an enzyme that is capable of iodinating proteins. U.S. 4,448,764 discloses, e.g., a reagent that may be used to iodinate proteins. |
| **ISGylation** | Covalently linking a peptide to the ISG15 (Interferon-Stimulated Gene 15) protein, for, e.g., modulating immune response. |
| **Methylation** | Reductive methylation of protein amino acids with formaldehyde and sodium cyanoborohydride has been shown to provide up to 25% yield of N-cyanomethyl ($-CH_2CN$) product. The addition of metal ions, such as $Ni^{2+}$, which complex with free cyanide ions, improves reductive methylation yields by suppressing by-product formation. The N-cyanomethyl group itself, produced in good yield when cyanide ion replaces cyanoborohydride, may have some value as a reversible modifier of amino groups in proteins. (Gidley et al.) Methylation may occur at the arginine and lysine residues of a protein, as well as the N- and C-terminus thereof. |
| **Myristoylation** | Myristoylation involves the covalent attachment of a myristoyl group (a derivative of myristic acid), via an amide bond, to the alpha-amino group of an N-terminal glycine residue. This addition is catalyzed by the N-myristoyltransferase enzyme. |
| **Oxidation** | -Oxidation of cysteines.<br>-Oxidation of N-terminal Serine or Threonine residues |

| Protein Modification | Description |
|---|---|
| | (followed by hydrazine or aminooxy condensations).<br>-Oxidation of glycosylations (followed by hydrazine or aminooxy condensations). |
| **Palmitoylation** | Palmitoylation is the attachment of fatty acids, such as palmitic acid, to cysteine residues of proteins. Palmitoylation increases the hydrophobicity of a protein. |
| **(Poly)glutamylation** | Polyglutamylation occurs at the glutamate residues of a protein. Specifically, the gamma-carboxy group of a glutamate will form a peptide-like bond with the amino group of a free glutamate whose alpha-carboxy group may be extended into a polyglutamate chain. The glutamylation reaction is catalyzed by a glutamylase enzyme (or removed by a deglutamylase enzyme). Polyglutamylation has been carried out at the C-terminus of proteins to add up to about six glutamate residues. Using such a reaction, Tubulin and other proteins can be covalently linked to glutamic acid residues. |
| **Phosphopantetheinylation** | The addition of a 4'-phosphopantetheinyl group. |
| **Phosphorylation** | A process for phosphorylation of a protein or peptide by contacting a protein or peptide with phosphoric acid in the presence of a non-aqueous apolar organic solvent and contacting the resultant solution with a dehydrating agent is disclosed e.g., in U.S. 4,534,894. Insulin products are described to be amenable to this process. See, e.g., U.S. 4,534,894. Typically, phosphorylation occurs at the serine, threonine, and tyrosine residues of a protein. |
| **Prenylation** | Prenylation (or isoprenylation or lipidation) is the addition of hydrophobic molecules to a protein. Protein prenylation involves the transfer of either a farnesyl (linear grouping of three isoprene units) or a geranyl-geranyl moiety to C-terminal cysteine(s) of the target protein. |
| **Proteolytic Processing** | Processing, e.g., cleavage of a protein at a peptide bond. |
| **Selenoylation** | The exchange of, e.g., a sulfur atom in the peptide for selenium, using a selenium donor, such as selenophosphate. |

| Protein Modification | Description |
|---|---|
| **Sulfation** | Processes for sulfating hydroxyl moieties, particularly tertiary amines, are described in, e.g., U.S. 6,452,035. A process for sulphation of a protein or peptide by contacting the protein or peptide with sulphuric acid in the presence of a non-aqueous apolar organic solvent and contacting the resultant solution with a dehydrating agent is disclosed. Insulin products are described to be amenable to this process. See, e.g., U.S. 4,534,894. |
| **SUMOylation** | Covalently linking a peptide a SUMO (small ubiquitin-related Modifier) protein, for, e.g., stabilizing the peptide. |
| **Transglutamination** | Covalently linking other protein(s) or chemical groups (e.g., PEG) via a bridge at glutamine residues |
| **tRNA-mediated addition of amino acids (e.g., arginylation)** | For example, the site-specific modification (insertion) of an amino acid analog into a peptide. |
| **Ubiquitination** | The small peptide ubiquitin is covalently linked to, e.g., lysine residues of a protein. The ubiquitin-proteasome system can be used to carryout such reaction. See, e.g., U.S. 2007-0059731. |

## EXAMPLES

**[0162]** The invention is illustrated herein by the experiments described above and by the following examples, which should not be construed as limiting. The contents of all references, pending patent applications and published patents, cited throughout this application are hereby expressly incorporated by reference. Those skilled in the art will understand that this invention may be embodied in many different forms and should not be construed as limited to the embodiments set forth herein. Rather, these embodiments are provided so that this disclosure will fully convey the invention to those skilled in the art. Many modifications and other embodiments of the invention will come to mind in one skilled in the art to which this invention pertains having the benefit of the teachings presented in the foregoing description. Although specific terms are employed, they are used as in the art unless otherwise indicated.

**Example 1: Cell cultures of C38 and IB3 cells**

**[0163]** C38 and IB3 cells were grown in 6-well plates and incubated for 1.5h in serine-free medium in the presence of $^{3}$H-serine to assess *de-novo* sphingolipid synthesis or $^{3}$H-sphinganine to determine sphingolipid synthesis through recycling pathways. At the end of the incubation period, lipids were extracted with chloroform:methanol and separated by thin-layer chromatography (TLC). Ceramide spots were analyzed by scintillation counting. Dpm associated with ceramide reflect synthesis. The synthesis of ceramide from $^{3}$H-serine reflects *de-novo* synthesis by SPT.

**Example 2: CFTR and SRE-mediated gene transcription in C38/IB3 and A549 cells**

**[0164]** CFTR expression was confirmed by Western analysis (FIG. 4c). Forty-eight hours later, cells were incubated for 16h in the presence of 1 % BSA, the sterol-free control medium known to increase SRE-mediated gene transcription. This condition was used to compare SRE-mediated gene transcription between cell lines. We also incubated cells in the presence of cholesterol and oleic acid, known inhibitors and direct regulators of SREBP, to assess whether regulation of SREBP is physiologically intact in our cell lines.

**[0165]** C38/IB3 cells were transduced with Ad-SRE-luc/βgal (FIG. 4a). A549 cells, that do not express CFTR, were transduced with SRE-luc/βgal together with Ad-CFTR or the control vector Ad-null (b). Western analysis in A549 demonstrates CFTR expression in A549 cells transduced with Ad-CFTR (c). Cells were incubated for 48h in growth medium to allow for transgene expression. Then, cells were incubated for 16h in sterol depleted control medium (1 % BSA) to compare the effect of CFTR expression on SRE-mediated gene transcription. Cells were also incubated in the presence of known inhibitors of SREBP (cholesterol or oleic acid) to assess the physiological regulation of SREBP. SRE-mediated luciferase activity, the reporter gene that reflects SREBP levels and SRE-mediated gene transcription, is significantly (*= $p<0.05$) decreased in cells that express CFTR (C38 cells, black bars in a), and A549 cells infected with Ad-CFTR (white bars in b). Cholesterol and oleic acid significantly ($p<0.05$) decreased SRE-mediated gene transcription in all conditions, reflecting intact regulation of SREBP.

**Example 3**

All the following were tested in fibroblasts:

**[0166]** We used myriocin to inhibit SPT and *de-novo* sphingolipid synthesis. Fumonisin B1 was used to decrease sphingolipid synthesis in general. NOE inhibits ceramidases in fibroblasts, decreases de-novo synthesis and promotes synthesis of sphingomyelin and glucosylceramide. DMS inhibits sphingosine kinase by increasing sphingolipid synthesis through recycling pathways by promoting synthesis of ceramide using sphingosine as a substrate. PPMP and NB-DNJ inhibit glucosylceramide synthase. PPMP also increases ceramide levels and decreases *de-novo* sphingolipid synthesis. [59]. SPT activity can be decreased by siRNA-mediated knock-down. All expression plasmids are available in the laboratory. The control plasmid Lc is a scrambled version of L394. We also looked at LC394, LC1176 and LC control.

**[0167]** Lipofectamine was used to transiently transfect cells with these plasmids; this is a standard approach. We also used an adenovirus vector as transfection agent.. To assure sufficient transgene expression, transfections were carried out 48h before experimental conditions was added. Controls were transfected with control vectors.

**[0168]** Lipofectamine was used to transiently transfect cells with these plasmids; this is a standard approach. We also used an adenovirus vector as transfection agent.. To assure sufficient transgene expression, transfections were carried out 48h before experimental conditions was added. Controls were transfected with control vectors.

**Example 4 Sphingolipid Synthesis and Turnover**

**[0169]** Sphingolipid synthesis was determined using direct radiotracer incorporation, pulse-chase. Microsomal assays well non in the art can also be used. Cells were plated in 12-well plates, grown to confluency and incubated for 2-16h with inhibitors and stimulators of sphingolipid synthesis (Figure 9, Tables 1 and 2). Endpoint for all experiments was lipid extraction and separation by TLC (see *analysis of lipid synthesis*, below). For example, cells were incubated in the presence of palmitic acid to increase *de-novo* sphingolipid synthesis or lauric acid to increase recycling pathways. Or, cells were incubated for 6h in the presence of myriocin or C6-ceramide to decrease *de-novo* sphingolipid synthesis. Recycling pathways will also be increased by incubation for 24h in sphingolipid free medium (Nutridoma BO). Cells were lyzed and cytosol was reacted with 3H-sphingomyelin in the presence of acidic and neutral

buffers specific for neutral and acidic sphingomyelinase. This approach demonstrated significantly increased sphingomyelin hydrolysis as a source for increased recycling pathways.

**[0170]** We carried out pulse-chase experiments to assess turnover. Cells were labeled for 24h with radioactive tracers, washed twice (PBS, 0.2 % BSA) and once with PBS alone followed by incubation for 2h and 4h in the presence of DMEM containing 1 % BSA and experimental conditions. At the end of the experiment, lipids were extracted and analyzed by TLC and scintillation counting.

**[0171]** **Microsomal assays** SPT activity can also be assessed in standard microsomal assays. For example, to prepare microsomes, 75 x $10^6$ cells will be pelleted, washed with cold PBS and resuspended in homogenization buffer (300 µl, HEPES, pH 7.4, EGTA, NAF, leupeptin, trypsin inhibitor). Cells will be disrupted with a tissue homogenizer, lysates centrifuged (800 x g, 5 min), postnuclear supernatant centrifuged (250,000 x g, 30 min) and the microsomal membrane pellet will be resuspended in homogenization buffer. SPT activity will be assessed by dissolving 100-300 µg of microsomal protein in 0.1 ml of reaction buffer (HEPES, DTT, EDTA, pyridoxal phosphate, palmitoyl-CoA) in the presence of $^3$H-serine or $^3$H-palmitoyl-CoA (1 µCi) for 10 min at 37°C. Controls will be microsomes extracts of palmitoyl-CoA (positive) or myriocin (negative) treated cells. Radioactivity associated with sphinganine and ceramide will be evaluated by TLC and scintillation counting.

**[0172]** **Analysis of lipid synthesis** In cystic fibrosis cells; sphingolipid synthesis was analyzed by TLC. Lipids were extracted with chloroform:methanol:water (1:1:0.9), subjected to alkaline hydrolysis (0.1 N methanolic KOH, 60 min) re-extracted and separated by TLC (chloroform: methanol:0.22 % CaCl (65:8:2)). Standards for sphinganine, sphingosine, ceramide and sphingomyelin were run in parallel. Spots corresponding to these lipid standards were visualized in iodine vapors and quantified by scintillation counting.

**[0173]** **Lipid mass analysis** We will use enzymatic methods and mass spectrometry to measure lipid mass. Ceramide mass was assessed by the diacyl-glycerol (DAG) kinase assay. Extracted lipids were solubilized by bath-sonication (30 min, 37°C) in the presence of di-oleoyl-phosphatidylglycerol and β-octylglucoside (825 mM) to generate micelles, incubated for 30 min with $^{32}$P-γATP (0.1 µl, 3000 Ci/mmol), DAG kinase (1 U) and carrier ATP (50 mM), followed by extraction and separation on TLC (chloroform:methanol:acetic acid (130:30:10)). Ceramide

standards (0.5-20 nmol) were run in parallel. In this solvent system, ceramide stays at the origin and ceramide-1-phosphate migrates up. Mass was quantified by densitometry of radioactive spots and correlation to known standards. Sphingomyelin mass was determined in a quantitative high-throughput 96-well assay[68]. After incubation of cells in the presence or absence of sphingomyelinase (10 μU/ml, 30 min) to generate phosphorylcholine, lipids were extracted, subjected to alkaline phosphatase (1U/ml) and choline oxidase (10μU/ml) to generate hydrogen peroxide. Hydrogen peroxide was reacted with peroxidase (200 mU) and Amplex Red reagent (20 nmol) to generate resorufin The assay is read in a fluorescence plate reader (ex. λ560 nm, em. λ 587nm).

**[0174]** We have determined ceramides, sphinganine, sphingosine and sphingosine 1-phosphate mass by liquid chromatography tandem mass spectrometry[69, 70]. A new HPLC-tandem quadropole mass spectrometer (Waters, Quattro micro API). with a mass analyzer spectrum range of m/z 2-2000 is available to the PI (see letter Dr. S. Spitalnik). Procedures for sphingolipid mass analysis are currently established. Precursor ion scans were used to distinguish various species of sphingolipid synthesis in crude extracts by their unique molecular decomposition products. Specific headgroup, sphingoid base, and fatty acid chain combinations can be readily determined by this method. Quantitation was achieved by multiple reaction monitoring in conjunction with HPLC. Samples were extracted with chloroform and methanol in the presence of internal standards. Internal standards, 0.5 nmol each of $C_{12}$-SM, $C_8$-GlcCer, and $C_{12}$-Cer, and 0.25 nmol $C_{20}$-sphingosine, $C_{20}$-sphinganine and $C_{17}$-sphingosine-1-phosphate, were obtained from Avanti Polar lipids. After addition of standards, cells were sonicated and incubated overnight at 48°C. The next day, alkaline hydrolysis was carried out and, after a neutralization and a reconstitution step, lipids were injected into the LC-MS/MS. sphingolipid was identified from crude extracts by analysis of their unique molecular decomposition products using precursor ion scans[69].

**[0175]** **Data analysis.** All experiments were carried out in triplicate at least five different times on different days. We will compare data between experiments on the bases of 'percentage of control' as well as by raw data expressed as a function of a common denominator such as cell DNA content (Hoechst 33258 fluorescent DNA quantitation), protein measurement (Biorad), or β-gal expression (Tropix, BD-Bioscience). We will perform Student's t-tests to assess statistical

significance. Determination of the absolute iodine transport rates, the time course of intracellular iodide concentration was computed from the time course of LMQ fluorescence using the Stern-Volmer equation. Fluorescence intensities in the absence ($F_0$) and presence (F) of quencher anion (Q) was determined to give the Stern-Volmer constant (Kq) as defined by: $F_0/F = 1 + Kq (Q)$.

**[0176]** **Fatty acid synthesis and mass** Fatty acid synthesis and mass was assessed by incorporation of radiotracers and TLC analysis. Fatty acid mass was assessed by gas-chromatography (GC) and enzymatic based kits. These methods are established in the laboratory. Fatty acid and triglyceride synthesis will assessed by incubation for 4h in the presence of $^3$H-acetyl-CoA (0.5 μCi/ml, fatty acids and triglycerides) or $^3$H-oleic acid (triglycerides only) tracers and in the presence and absence of cerulenin, an inhibitor of fatty acid synthesis (500 μM,). All lipids were extracted with hexane:isopropanol (3:2), dried under $N_2$, re-suspended, spotted on TLC plates, separated in hexane:ethyl-ether:glacial acetic acid (70:30:1), and visualized in iodine vapors. Spots corresponding to phospholipids, free cholesterol, fatty acids, triglycerides and cholesteryl-ester were identified by co-migrating standards. Radioactivity (dpm) was expressed normalized to cell protein. Analysis of fatty acid profiles by GC was carried out by direct transesterification[75]. Samples were boiled for 1h in the presence of acetyl-chloride and methanol, followed by neutralization with $K_2C0_3$ and centrifugation. The upper layer was directly injected into the GC. Pentadecanoic and heptadecanoic phospholipids were used as internal standards. Mass was calculated in relationship to internal standards and expressed as mol % of total fatty acids analyzed. Total fatty acid mass was determined using enzymatic assays (WAKO-kits).

**[0177]** **Data analysis** . All experiments were carried out in triplicate at least five different times on different days. We will compare data between experiments on the bases of 'percentage of control' as well as by raw data expressed as a function of a common denominator such as cell protein, or β-gal expression. Statistical significance was assessed by student's t-tests for paired and unpaired samples and per experiment.

## Example 5 *P. aeruginosa* Adherence

**[0178]** The laboratory strain PAO1 that has been stably transfected with GFP. Negative controls were the same strain transfected with an empty vector. To confirm pertinent findings, we will use a panel of five well characterized *P. aeruginosa* clinical isolates from cystic fibrosis

patients (see letter Dr. A. Prince). For select experiments we will use $^{35}$S-labeled *P. aeruginosa*. $^{35}$S-labeling was carried out by adding 50 μCi of $^{35}$S-methionine/ml of culture for 30 min, followed by washing in PBS and resuspension in MEM9 growth medium without antibiotics. Specific activity was determined by assessing CPM per colony forming units (cfu). Desirable concentrations of the labeled organisms are $10^6$-$10^8$ cfu/ml. *P. aeruginosa*-GPF was cultured in MEM9 medium containing piperacillin (200 μg/ml) until late logarithmic growth is reached. *P. aeruginosa* controls were cultured in MEM9 alone. Logarithmic phase was determined by measurement of the optical density at λ 600 nm. Numbers of bacteria were confirmed by determination of the cfu of diluted aliquots on MacConkey agar plates.

**[0179]** **Cell monolayers**. All experiments were carried out in the lung epithelial cell lines evaluated in d.1. Cells were plated in 96-well plates, and used only when confluent monolayers have formed that enable the formation of tight junctions. Care was taken that cells have the same passage number. Empty control wells were used to assess background fluorescence.

**[0180]** **Experimental conditions** Cells were incubated in the presence and absence of conditions that increase and decrease sphingolipid synthesis. For example, cells were incubated for 16h in the presence of increasing concentrations of glucosylceramide synthase inhibitors PPMP or NB-DNJ to determine the effect of glucosphingolipid synthesis on *P. aeruginosa* adherence. Using these two inhibitors permits one to evaluate whether increased concentrations of ceramide, mediated by PPMP affect *P. aeruginosa* adherence. We will incubate cells with palmitic acid, a stimulator of sphingolipid synthesis, or myriocin, the inhibitor of SPT, to assess the role of increased or decreased *de-novo* sphingolipid synthesis on *P. aeruginosa* adhesion. We will incubate cells with lauric acid or DMS to evaluate the role of increased recycling pathways on *P. aeruginosa* adhesion. We will transfect cells with siRNA-SPT to decrease sphingolipid synthesis or transfect cells with pSMS or pSK to increase sphingolipid synthesis. To differentiate between the dependence on glucosylceramide synthesis and sphingolipid synthesis, cells were preincubated with conditions that decrease *de-novo* synthesis (i.e. myriocin or unsaturated fatty acids) together with conditions that increase glucosylceramide synthesis such as acetylsphingosine or sphingomyelinase. To assess whether sphingolipid synthesis alone, independent of CFTR affects *P. aeruginosa* adherence, we will use A549 cells that do not express CFTR, modulate sphingolipid synthesis and measure *P. aeruginosa*-adherence. To

evaluate whether inhibition of CFTR function affects *P. aeruginosa* adherence, cells that express functional CFTR was incubated in the presence or absence of the CFTR inhibitor GlyH-101, followed by assessment of *P. aeruginosa* adherence. In parallel we will assess ceramide and glycosphingolipid synthesis using radioactive tracer incorporation and TLC separation as outlined in d.1.b.

**[0181] Assessment of *P. aeruginosa* adherence to CF-model lung epithelial cell lines** In standard assays, increasing concentrations of bacteria (5-50 μl / well of a bacterial suspension, with an optical density λ 600 nm equal to 1.0) was added for 0.5h - 4h to cells plated in 96-well plates in a total volume of 100 μl. We will use 72 wells of a 96-well plate. Remaining wells were used as empty controls and for the determination of protein levels. Half of the remaining wells were incubated with *P. aeruginosa*-GFP, while the other half was incubated with *P. aeruginosa*-control. At the end of the incubation period, we will visually inspect monolayers to assure that cells remained adherent during the experiment. Then, cells were washed extensively with 150 μl/ well PBS, 1 mM $CaCl_2$ and 1 mM $MgSO_4$. Adherent organisms were identified using a fluorescence plate reader (GFP excitation λ395 nm, emission λ 509 nm). In parallel, we will determine fluorescence in the washes to assure data validity. We will assess cell survival by determination of LDH in cellular supernatants. We will assess cell survival by trypan blue exclusion and MTT assays, as outlined above. We will repeat pertinent experiments using $^{35}S$-labeled bacteria to control for the use of GFP-labeled *P. aeruginosa*.

**[0182] Assessment of *P. aeruginosa* uptake** We will use confocal microscopy and $^{35}S$-labeled *P. aeruginosa* to assess internalization and differentiate between intracellular and extracellular organisms. To assess uptake by confocal microscopy, cells were plated in 35 mm cell Matek microscopy culture dishes. Confluent monolayers were incubated with *P. aeruginosa*-GFP for the desired time. At the end of the experiment, cells were washed and fixed for 15 min in 3% paraformaldehyde. Cells will then be mounted and wide field images were collected on a DMIRB inverted Leica microscope with standard GFP optics. Images were collected on a Zeiss LSM510 laser scanning confocal unit equipped with an image capture device. Excitation was produced with a 24m W-Argon laser (excitation λ 488 nm, emission λ 505 nm, long pass filter). First, cells were visualized with transmitted light to ensure that they are intact. Next, fluorescence was visualized and intensity was determined using MetaMorph image analysis

software (Universal Imagine). We will analyze a minimum of 50 cells per individual condition. Experiments were performed at least 5 different times for individual conditions and the mean of the ratios were determined. In the second methods we will assess uptake using $^{35}$S-labeled bacteria. Confluent monolayers were incubated at the end of the incubation period with cidal concentrations (> 50 μg/ml) of gentamicin to kill extracellular bacteria. The cells will then be washed extensively and lysed using 0.1 NaOH. Aliquots of cell lysates were analyzed by scintillation counting.

**Example 6 Monitoring sphingolipid synthesis in peripheral blood mononuclear cells**

**[0183]** To demonstrate the feasibility of the of monitoring sphingolipid synthesis in peripheral blood mononuclear cells, we looked at individuals with low, normal and high plasma HDL cholesterol levels, but not affected by CF. One ml of whole EDTA blood was obtained from healthy subjects with different plasma HDL cholesterol levels. Mononuclear cells were separated by Ficoll gradient centrifugation. Cells were incubated for 5 days in growth medium in the presence of phytohemagglutinin (PHA, 10μg/ml) to stimulate cell growth. Cells were then quantitated and equal numbers were incubated for 2h with radioactive tracers of *de-novo* sphingolipid synthesis and recycling pathways. $^{3}$H-serine was used to assess *de-novo* sphingolipid synthesis. $^{3}$H-sphingosine was used to assess sphingolipid synthesis though recycling pathways. At the end of the experiment, lipids were extracted by chloroform/methanol, separated by TLC and dpm associated with ceramide were quantified using a scintillation counter. The data show an inverse relationship of *de-novo* sphingolipid synthesis and plasma HDL cholesterol levels (**FIG. 13a**) but no correlation with reverse sphingolipid synthesis (**FIG. 13b).** Recycling pathways do not correlate with plasma HDL levels.

# SEQUENCE LISTINGS

**SEQ ID NO. 1 Serine palmitoyltransferase Long Chain Base Subunit 1**

**Amino Acid Sequence Homo sapiens**

MATATEQWVLVEMVQALYEAPAYHLILEGILILWIIRLLFSKTYKLQERSDLTVKEKEEL
IEEWQPEPLVPPVPKDHPALNYNIVSGPPSHKTVVNGKECINFASFNFLGLLDNPRVKAA
ALASLKKYGVGTCGPRGFYGTFDVHLDLEDRLAKFMKTEEAIIYSYGFATIASAIPAYSK
RGDIVFVDRAACFAIQKGLQASRSDIKLFKHNDMADLERLLKEQEIEDQKNPRKARVTR
RFIVVEGLYMNTGTICPLPELVKLKYKYKARIFLEESLSFGVLGEHGRGVTEHYGINIDDI
DLISANMENALASIGGFCCGRSFVIDHQRLSGQGYCFSASLPPLLAAAAIEALNIMEENPG
IFAVLKEKCGQIHKALQGISGLKVVGESLSPAFHLQLEESTGSREQDVRLLQEIVDQCMN
RSIALTQARYLEKEEKCLPPPSIRVVVTVEQTEEELERAASTIKEVAQAVLL

**SEQ ID NO. 2 Serine palmitoyltransferase Long Chain Base Subunit 1**

**Nucleotide Sequence Homo sapiens**

atggcgaccgccacggagcagtgggttctggtggagatggtacaggcgctttacgaggct
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gcgtccaccatcaaggaggtagcccaggccgtcctgctctag

**SEQ ID NO. 3 Serine palmitoyltransferase Amino Acid Sequence Long Chain Base Subunit 2 Homo sapiens**

MRPEPGGCCCRRTVRANGCVANGEVRNGYVRSSAAAAAAAAAAGQIHHVTQNGGLYK
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QDFENFYTRNLYMRIRDNWNRPICSVPGARVDIMERQSHDYNWSFKYTGNIIKGVINMG
SYNYLGFARNTGSCQEAAAKVLEEYGAGVCSTRQEIGNLDKHEELEELVARFLGVEAA
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LGPTGRGVVEYFGLDPEDVDVMMGTFTKSFGASGGYIGGKKELIDYLRTHSHSAVYAT
SLSPPVVEQIITSMKCIMGQDGTSLGKECVQQLAENTRYFRRRLKEMGFIIYGNEDSPVV
PLMLYMPAKIGAFGREMLKRNIGVVVVGFPATPIIESRARFCLSAAHTKEILDTALKEIDE
VGDLLQLKYSRHRLVPLLDRPFDETTYEETED

**SEQ ID NO. 4 Serine palmitoyltransferase Amino Acid Sequence Long Chain Base Subunit 2 Homo sapiens**

Nucleic Acid sequence 1689 nucleotides
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cattctcatagtgcagtgtatgccacgtcattgtcacctcctgtagtggagcagatcatc
acctccatgaagtgcatcatggggcaggatggcaccagccttggtaaagagtgtgtacaa

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cgtcatcggttggtacctctactggacaggcctttgacgagacgacgtatgaagaaaca
gaagactga

**SEQ ID NO. 5 Sphingosine kinase 1 Homo sapiens**
**Amino Acid Sequence**

MDPVVGCGRGLFGFVFSAGGPRGVLPRPCRVLVLLNPRGGKGKALQLFRSHVQPLLAE
AEISFTLMLTERRNHARELVRSEELGRWDALVVMSGDGLMHEVVNGLMERPDWETAI
QKPLCSLPAGSGNALAASLNHYAGYEQVTNEDLLTNCTLLLCRRLLSPMNLLSLHTASG
LRLFSVLSLAWGFIADVDLESEKYRRLGEMRFTLGTFLRLAALRTYRGRLAYLPVGRVG
SKTPASPVVVQQGPVDAHLVPLEEPVPSHWTVVPDEDFVLVLALLHSHLGSEMFAAPM
GRCAAGVMHLFYVRAGVSRAMLLRLFLAMEKGRHMEYECPYLVYVPVVAFRLEPKD
GKGVFAVDGELMVSEAVQGQVHPNYFWMVSGCVEPPPSWKPQQMPPPEEPL

**SEQ ID NO. 6 Sphingosine kinase 1 Homo sapiens**
**Nucleic Acid Sequence**

atggatccagtggtcggttgcggacgtggcctctttggttttgttttctcagcgggcggc
ccccggggcgtgctcccgcggccctgccgcgtgctggtgctgctgaacccgcgcggcggc
aagggcaaggccttgcagctcttccggagtcacgtgcagccccttttggctgaggctgaa
atctccttcacgctgatgctcactgagcggcggaaccacgcgcgggagctggtgcggtcg
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gagcccccgcccagctggaagccccagcagatgccaccgccagaagagcccttatga

**SEQ ID NO. 7 Sphingosine kinase 2 Homo sapiens**
**Amino Acid Sequence**

MNGHLEAEEQQDQRPDQELTGSWGHGPRSTLVRAKAMAPPPPLAASTPLLHGEFGSY
PARGPRFALTLTSQALHIQRLRPKPEARPRGGLVPLAEVSGCCTLRSRSPSDSAAYFCIYT
YPRGRRGARRRATRTFRADGAATYEENRAEAQRWATALTCLLRGLPLPGDGEITPDLLP
RPPRLLLLVNPFGGRGLAWQWCKNHVLPMISEAGLSFNLIQTERQNHARELVQGLSLSE
WDGIVTVSGDGLLHEVLNGLLDRPDWEEAVKMPVGILPCGSGNALAGAVNQHGGFEP
ALGLDLLLNCSLLLCRGGGHPLDLLSVTLASGSRCFSFLSVAWGFVSDVDIQSERFRALG
SARFTLGTVLGLATLHTYRGRLSYLPATVEPASPTPAHSLPRAKSELTLTPDPAPPMAHS
PLHRSVSDLPLPLPQPALASPGSPEPLPILSLNGGGPELAGDWGGAGDAPLSPDPLLSSPP
GSPKAALHSPVSEGAPVIPPSSGLPLPTPDARVGASTCGPPDHLLPPLGTPLPPDWVTLEG
DFVLMLAISPSHLGADLVAAPHARFDDGLVHLCWVRSGISRAALLRLFLAMERGSHFSL
GCPQLGYAAARAFRLEPLTPRGVLTVDGEQVEYGPLQAQMHPGIGTLLTGPPGCPGREP

**SEQ ID NO. 8 Sphingosine kinase 2**
**Homo sapiens**
atgaatggacaccttgaagcagaggagcagcaggaccagaggccagaccaggagctgacc
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gtggacggggagcaggtggagtatgggccgctacaggcacagatgcaccctggcatcggt
acactgctcactgggcctcctggctgcccggggcgggagccctga

**SEQ ID NO. 9 UDP-glucose ceramide glucosyltransferase**
**Amino Acid Sequence Homo sapiens**

MALLDLALEGMAVFGFVLFLVLWLMHFMAIIYTRLHLNKKATDKQPYSKLPGVSLLKP
LKGVDPNLINNLETFFELDYPKYEVLLCVQDHDDPAIDVCKKLLGKYPNVDARLFIGGK
KVGINPKINNLMPGYEVAKYDLIWICDSGIRVIPDTLTDMVNQMTEKVGLVHGLPYVAD
RQGFAATLEQVYFGTSHPRYYISANVTGFKCVTGMSCLMRKDVLDQAGGLIAFAQYIA
EDYFMAKAIADRGWRFAMSTQVAMQNSGSYSISQFQSRMIRWTKLRINMLPATIICEPIS
ECFVASLIIGWAAHHVFRWDIMVFFMCHCLAWFIFDYIQLRGVQGGTLCFSKLDYAVA
WFIRESMTIYIFLSALWDPTISWRTGRYRLRCGGTAEEILDV

**SEQ ID NO. 10 UDP-glucose ceramide glucosyltransferase**
**Nucleotide Sequence Homo sapiens**
atggcgctgctggacctggccttggagggaatggccgtcttcgggttcgtcctcttcttg
gtgctgtggctgatgcatttcatggctatcatctacacccgattacacctcaacaagaag
gcaactgacaaacagccttatagcaagctcccaggtgtctctcttctgaaaccactgaaa
ggggtagatcctaacttaatcaacaacctggaaacattctttgaattggattatcccaaa
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atatacatttttttgtctgcattatgggacccaactataagctggagaactggtcgctac
agattacgctgtgggggtacagcagaggaaatcctagatgtataa

**SEQ ID NO. 11 Fatty acid desaturase (ceramide synthase) Homo sapiens**

**Gene Name DEGS2 Amino Acid Sequence**

MGNSASRSDFEWVYTDQPHTQRRKEILAKYPAIKALMRPDPRLKWAVLVLVLVQMLT
CWLVRGLAWRWLLFWAYAFGGCVNHSLTLAIHDISHNAAFGTGRAARNRWLAVFANL
PVGVPYAASFKKYHVDHHRYLGGDGLDVDVPTRLEGWFFCTPARKLLWLVLQPFFYSL
RPLCVHPKAVTRMEVLNTLVQLAADLAIFALWGLKPVVYLLASSFLGLGLHPISGHFVA
EHYMFLKGHETYSYYGPLNWITFNVGYHVEHHDFPSIPGYNLPLVRKIAPEYYDHLPQH
HSWVKVLWDFVFEDSLGPYARVKRVYRLAKDGL

**SEQ ID NO. 12 Fatty acid desaturase (ceramide synthase) Homo sapiens**
**Gene Name DEGS2 Nucleotide sequence**

atgggcaacagcgcgagccgcagcgacttcgagtgggtctacaccgaccagccgcacacg
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gatggtctgtga

**SEQ ID NO. 13 Fatty acid desaturase Gene Name DEGS1**
**Amino Acid Sequence Homo Sapiens**
MGSRVSREDFEWVYTDQPHADRRREILAKYPEIKSLMKPDPNLIWIIIMMVLTQLGAFYI
VKDLDWKWVIFGAYAFGSCINHSMTLAIHEIAHNAAFGNCKAMWNRWFGMFANLPIGI
PYSISFKRYHMDHHRYLGADGVDVDIPTDFEGWFFCTAFRKFIWVILQPLFYAFRPLFIN
PKPITYLEVINTVAQVTFDILIYYFLGIKSLVYMLAASLLGLGLHPISGHFIAEHYMFLKG
HETYSYYGPLNLLTFNVGYHNEHHDFPNIPGKSLPLVRKIAAEYYDNLPHYNSWIKVLY
DFVMDDTISPYSRMKRHQKGEMVLE

**SEQ ID NO. 14 Fatty acid desaturase Gene Name DEGS1**
**Nucleic Acid Sequence Homo Sapiens**

atggggagccgcgtctcgcgggaagacttcgagtgggtctacaccgaccagccgcacgcc
gaccggcgccgggagatcctggcaaagtatccagagataaagtccttgatgaaacctgat
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gtgctggagtaa

**Literature Cited**
**All of these references and those cited above are hereby incorporated by reference as if set forth fully herein.**


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## CLAIMS

**What is claimed is:**

1. A method for treating cystic fibrosis in an animal, comprising administering an agent that inhibits the synthesis of one or more sphingolipids through either *de novo* or recycling pathways, in cells from the animal in an amount that ameliorates one or more symptoms of cystic fibrosis.

2. The method of claim 1, wherein the agent specifically inhibits *de novo* sphingolipid synthesis.

3. The method of claim 1, wherein the agent inhibits the synthesis of one or more sphingolipids by its action on recycling pathways.

4. The method of claim 1, wherein the animal is a human.

5. The method of claim 1, wherein the agent specifically inhibits the activity of an enzyme that catalyzes part of the *de novo* ceramide pathway.

6. The method of claim 5, wherein the enzyme is serine-palmitoyl transferase, ceramide synthase, sphingosine kinase, UDP-glucose ceramide glucosyltransferase and glucosylceramide synthase.

7. The method of claim 5, wherein the enzyme is a member selected from the group comprising SPTLC1 serine palmitoyltransferase, long chain base subunit 1, Degs1 degenerative spermatocyte homolog 1, SGMS1 sphingomyelin synthase 1, Sphingomyelin synthase 2, ASAH2 N-acylsphingosine amidohydrolase (non-lysosomal ceramidase) 2, LASS6 LAG1 homolog, ceramide synthase 6, LASS5 LAG1 homolog, ceramide synthase 5, LASS4 LAG1 homolog, ceramide synthase 4, LASS3 LAG1 homolog, ceramide synthase 3, LASS2 LAG1 homolog, ceramide synthase 2, and LASS2 LAG1 homolog, ceramide synthase 1.

8. The method of claim 2, wherein the agent is selected from the group comprising of (a) myriocin; (b) cycloserine; (c) Fumonisin B1; (d) PPMP; (e) compound D609; (f) methylthiodihydroceramide; (g) propanolol; and (h) resvaratrol. (i) Dimethyl sphingosine, (j) dihydroceramide saturase (GT 11), (k) n-butyldeoxynojirimycin, and (l) N-oleyl-ethanolamine.

9. The method of claim 5, wherein the agent is a member of the group comprising ceramide, dihydroceramide, fenretinide, 4-oxo-HPR or a derivative, variant or fragment thereof.

10. The method of claim 5, wherein the agent is an unsaturated fatty acid that is a member of the group comprising linoleic acid, linolenic acid, cholesterol DHA, eicosapentaenoic acid, oleic acid, and arachidonic acid.

11. The method of claim 1, wherein the agent is an isolated antisense nucleic acid or small interfering RNA that is sufficiently complementary to the sense strand of the gene or to an mRNA encoding an enzyme in the sphingolipid synthesis pathway to permit hybridization to the sense strand of the gene or to the mRNA of the respective enzyme, and wherein the hybridization event prevents expression of the enzyme thereby decreasing the amount of sphingolipids produced in the cell.

12. The method of claim 1, wherein the enzyme is a member selected from the group comprising serine-palmitoyl transferase, ceramide synthase, sphingosine kinase, UDP-glucose ceramide glucosyltransferase and glucosylceramide synthase or a member selected from the group comprising SPTLC1 serine palmitoyltransferase, long chain base subunit 1, Degs1 degenerative spermatocyte homolog 1, SGMS1 sphingomyelin synthase 1, Sphingomyelin synthase 2, ASAH2 N-acylsphingosine amidohydrolase (non-lysosomal ceramidase) 2, LASS6 LAG1 homolog, ceramide synthase 6, LASS5 LAG1 homolog, ceramide synthase 5, LASS4 LAG1 homolog, ceramide synthase 4, LASS3 LAG1 homolog,

ceramide synthase 3, LASS2 LAG1 homolog, ceramide synthase 2, and LASS2 LAG1 homolog, ceramide synthase 1.

13. The method of claim 1, wherein the daily dose of the agent is between about 0.1 nanograms per kilogram body weight per day and about 20 milligrams per kilogram body weight per day, or between about 1 nanogram per kilogram body weight per day and about 10 milligrams per kilogram body weight per day.

14. The method as in claim 1, wherein the agent is administered to achieve a serum level of between about 1 nanogram per milliliter and about 10 micrograms per milliliter in the patient, or from between about 1 nanogram per milliliter and about 7 micrograms per milliliter.

15. The method as of claim 1, wherein the daily dose of the agent is between about 0.1 nanograms per kilogram body weight per day and about 20 milligrams per kilogram body weight per day, or between about 1 nanogram per kilogram body weight per day and about 10 milligrams per kilogram body weight per day.

16. A method for treating cystic fibrosis in an animal by administering a therapeutic amount of an agent that reduces sphingolipid synthesis in a biological sample taken from the animal, wherein the therapeutic amount is determined by:

a. taking a first biological sample from the animal before administering the agent,

b. determining a sphingolipid level in the first sample,

c. administering an amount of the agent,

d. taking a second biological sample from the animal after administering the agent,

e. determining a sphingolipid level in the second sample,

f. if the sphingolipid level in the second sample is significantly lower than in the first sample, then concluding that the amount of the agent is a therapeutic amount, and

g. if the sphingolipid level in the second sample is not lower than the level in the first sample, then repeating steps a-f

17. The method of claim 16, wherein the agent is a member of the group comprising (a) myriocin; (b) cycloserine; (c) Fumonisin B1; (d) PPMP; (e) compound D609; (f) methylthiodihydroceramide; (g) propanolol; and (h) resvaratrol (i) Dimethyl sphingosine, (j) dihydroceramide saturase (GT 11), (k) n-butyldeoxynojirimycin, and (l) N-oleyl-ethanolamine..

18. The method of claim 16, wherein the agent is a member of the group comprising ceramide, dihydroceramide, fenretinide, 4-oxo-HPR or a derivative, variant or fragment thereof.

19. The method of claim 16 wherein the agent is an unsaturated fatty acid.

20. The method of claim 16, wherein the unsaturated fatty acid is a member selected from the group comprising DHA, eicosapentaenoic acid, oleic acid, and arachidonic acid.

21. The method of claim 16, wherein the agent is an isolated antisense nucleic acid or small interfering RNA that is sufficiently complementary to the sense strand of the gene or to an mRNA encoding an enzyme in the sphingolipid synthesis pathway to permit hybridization to the sense strand of the gene or to the mRNA of the respective enzyme, and wherein the hybridization event prevents expression of the enzyme thereby decreasing the amount of sphingolipids produced in the cell.

22. The method of claim 16, wherein the enzyme is a member selected from the group comprising serine-palmitoyl transferase, ceramide synthase, sphingosine kinase, UDP-glucose ceramide glucosyltransferase and glucosylceramide synthase or a member selected from the group comprising SPTLC1 serine palmitoyltransferase, long chain base subunit 1, Degs1 degenerative spermatocyte homolog 1, SGMS1 sphingomyelin synthase 1, Sphingomyelin synthase 2, ASAH2 N-acylsphingosine amidohydrolase (non-lysosomal ceramidase) 2, LASS6 LAG1 homolog, ceramide synthase 6, LASS5 LAG1 homolog,

ceramide synthase 5, LASS4 LAG1 homolog, ceramide synthase 4, LASS3 LAG1 homolog, ceramide synthase 3, LASS2 LAG1 homolog, ceramide synthase 2, and LASS2 LAG1 homolog, ceramide synthase 1.

23. The method of claim 16, wherein the daily dose of the agent is between about 0.1 nanograms per kilogram body weight per day and about 20 milligrams per kilogram body weight per day, or between about 1 nanogram per kilogram body weight per day and about 10 milligrams per kilogram body weight per day.

24. The method as in claim 16, wherein the agent is administered to achieve a serum level of between about 1 nanogram per milliliter and about 10 micrograms per milliliter in the patient, or from between about 1 nanogram per milliliter and about 7 micrograms per milliliter.

25. The method as of claim 16, wherein the daily dose of the agent is between about 0.1 nanograms per kilogram body weight per day and about 20 milligrams per kilogram body weight per day, or between about 1 nanogram per kilogram body weight per day and about 10 milligrams per kilogram body weight per day.

26. The method of claim 16, wherein the level of sphingolipid synthesis in the biological sample taken from the animal is determined by measuring the level of sphingolipid mass or the amount of an individual sphingolipid that is a member of the group comprising sphinganine, C16, C18, C20, C22, C24, C 26, dihdyroceramide, sphingosine, sphingosine–s-phosphate, and sphingomyelin.

27. The method of claim 16, wherein the amount of sphingolipid mass is determined using mass spectrometry.

28. The method of claim 16, wherein the biological sample is peripheral blood mononuclear cells, bronchial lavage or lung epithelial cells.

29. The method of claim 16, wherein the agent is fenretinide or 4-oxo-HPR.

30. A method for treating a *P. aeruginosa* infection in an animal comprising administering an agent that inhibits the synthesis of one or more sphingolipids in cells from the animal in an amount that ameliorates one or more symptoms of cystic fibrosis.

31. The method of claim 30, wherein the agent specifically inhibits *de novo* sphingolipid synthesis.

32. The method of claim 30 wherein the agent inhibits the synthesis of one or more sphingolipids by its action on recycling pathways.

33. The method of claim 30, wherein the animal is a human.

34. The method of claim 30, wherein the agent specifically inhibits the activity of an enzyme that catalyzes part of the *de novo* sphingolipid pathway.

35. The method of claim 30, wherein the enzyme is serine-palmitoyl transferase or ceramide synthase.

36. The method of claim 30, wherein the enzyme is serine-palmitoyl transferase or ceramide synthase.

37. The method of claim 30, wherein the agent is selected from the group comprising of (a) myriocin; (b) cycloserine; (c) Fumonisin B1; (d) PPMP; (e) compound D609; (f) methylthiodihydroceramide; (g) propanolol; and (h) resvaratrol. (i) Dimethyl sphingosine, (j) dihydroceramide saturase (GT 11), (k) n-butyldeoxynojirimycin, and (l) N-oleyl-ethanolamine.

38. The method of claim 30, wherein the agent is a member of the group comprising ceramide, dihydroceramide, fenretinide, 4-oxo-HPR, or a derivative, variant or fragment thereof.

39. The method of claim 30, wherein the agent is an unsaturated and is a member selected from the group comprising DHA, eicosapentaenoic acid, oleic acid, and arachidonic acid.

40. The method of claim 30, wherein the agent is an isolated antisense nucleic acid or small interfering RNA that is sufficiently complementary to the sense strand of the gene or to an mRNA encoding an enzyme in the sphingolipid synthesis pathway to permit hybridization to the sense strand of the gene or to the mRNA of the respective enzyme, and wherein the hybridization event prevents expression of the enzyme thereby decreasing the amount of sphingolipids produced in the cell.

41. The method of claim 30, wherein the enzyme is a member selected from the group comprising serine-palmitoyl transferase, ceramide synthase, sphingosine kinase, UDP-glucose ceramide glucosyltransferase and glucosylceramide synthase or a member selected from the group comprising SPTLC1 serine palmitoyltransferase, long chain base subunit 1, Degs1 degenerative spermatocyte homolog 1, SGMS1 sphingomyelin synthase 1, Sphingomyelin synthase 2, ASAH2 N-acylsphingosine amidohydrolase (non-lysosomal ceramidase) 2, LASS6 LAG1 homolog, ceramide synthase 6, LASS5 LAG1 homolog, ceramide synthase 5, LASS4 LAG1 homolog, ceramide synthase 4, LASS3 LAG1 homolog, ceramide synthase 3, LASS2 LAG1 homolog, ceramide synthase 2, and LASS2 LAG1 homolog, ceramide synthase 1.

42. The method of claim 30, wherein the daily dose of the agent is between about 0.1 nanograms per kilogram body weight per day and about 20 milligrams per kilogram body weight per day, or between about 1 nanogram per kilogram body weight per day and about 10 milligrams per kilogram body weight per day.

43. The method as in claim 30, wherein the agent is administered to achieve a serum level of between about 1 nanogram per milliliter and about 10 micrograms per milliliter in the

patient, or from between about 1 nanogram per milliliter and about 7 micrograms per milliliter.

44. The method as of claim 30, wherein the daily dose of the agent is between about 0.1 nanograms per kilogram body weight per day and about 20 milligrams per kilogram body weight per day, or between about 1 nanogram per kilogram body weight per day and about 10 milligrams per kilogram body weight per day.

45. A method of identifying serine palmitoyl transferase inhibitors in an animal cell-based assay using cells expressing defective CFTR or no CFTR and overexpressing serine palmitoyl transferase, the method comprising:
a) providing test cells overexpressing serine palmitoyl transferase;
b) contacting the test cells with a test compound;
c) determining the level of ketosphinganine or sterol-regulatory element-binding protein produced by the test cells,
d) comparing the determined level in the test cells to a level of ketosphinganine or sterol-regulatory element-binding protein in control cells that are not exposed to the test compound, and

e) determining that the test compound is a serine palmitoyl transferase inhibitor if the level of ketosphinganine or sterol-regulatory element-binding protein is significantly lower in test cells compared to control cells.

46. The method of claim 45, wherein the expression of sterol-regulatory element-binding protein is indicated by a sterol-regulatory element reporter gene.

47. The method of claim 45, wherein the reporter gene is a member of the group comprising luciferase, green fluorescent protein and lacz.

48. A method of identifying a compound that reduces the production of one or more sphingolipids in an animal cell that expresses defective CFTR or no CFTR, the method comprising:

a) providing test cells and control cells expressing the one or more sphingolipids;

b) contacting the test cells with a test compound for a time and under conditions that permit the test compound to affect sphingolipid production;

c) determining the level of the one or more sphingolipids produced by the test cells and the control cells,

d) comparing the determined level in test cells to the level determined in control cells that were not exposed to the test compound, and

e) concluding that the test compound reduces the production of the one or more sphingolipids if the level in the test cells is lower than the level in control cells.

49. The method of claim 48, wherein the cell is a member selected from the group comprising an IB3 cell, an epithelial cell from an animal having cystic fibrosis, a cell transduced to express defective CFTR or no CFTR, an A549 cell.

50. A method for decreasing the amount of sphingolipid produced in an animal cell expressing defective CFTR or no CFTR, comprising contacting the cell with an agent that inhibits the synthesis of one or more sphingolipids in animal cells at a concentration that decreases the amount of the one or more sphingolipids produced in the cell.

51. The method of claim 50, wherein the agent is a member of the group comprising: (a) myriocin; (b) cycloserine; (c) Fumonisin B1; (d) PPMP; (e) compound D609; (f) methylthiodihydroceramide; (g) propanolol; and (h) resvaratro, (i) Dimethyl sphingosine, (j) dihydroceramide saturase (GT 11), (k) n-butyldeoxynojirimycin, and (l) N-oleyl-ethanolamine.

52. The method of claim 50, wherein the agent is an antisense nucleic acid that is sufficiently complementary to the sense strand of the gene or to an mRNA encoding an enzyme that is a member of the group comprising serine palmitoyl transferase, sphingosine kinase, ceramide synthase, and glucosylceramide synthase to permit hybridization to the sense strand of the gene or to the mRNA or the respective enzyme, and wherein the hybridization prevents expression of the enzyme thereby decreasing the amount of sphingolipids produced in the cell.

53. The method of claim 50, wherein the agent is a member of the group comprising ceramide, dihydroceramide, fenretinide, 4-oxo-HPR, and unsaturated fatty acids.

54. The method of claim 50, wherein the cell is a member selected from the group comprising an IB3 cell, an epithelial cell from an animal having cystic fibrosis, a cell transduced to express defective CFTR or no CFTR, an A549 cell.

55. The method as in one of claims 1, 16, 30 or 53, wherein the agent is ceramide and the amount of ceramide is from about 5-100 micromolar, preferably about 20 micromolar.

56. A pharmacological composition comprising ceramide and a compound that inhibits de novo sphingolipid synthesis.

57. A pharmacological composition comprising ceramide and a compound that is a member of the group comprising fenretinide, 4-oxo-HPR, unsaturated fatty acids including DHA and EPA.

58. A pharmacological composition comprising fenretinide and a compound that inhibits de novo sphingolipid synthesis.

59. The composition as in claim 56 or claim 58, wherein the compound is a member selected from the group comprising (a) myriocin; (b) cycloserine; (c) Fumonisin B1; (d) PPMP; (e) compound D609; (f) nethylthiodihydroceramide; (g) propanolol; and (h)

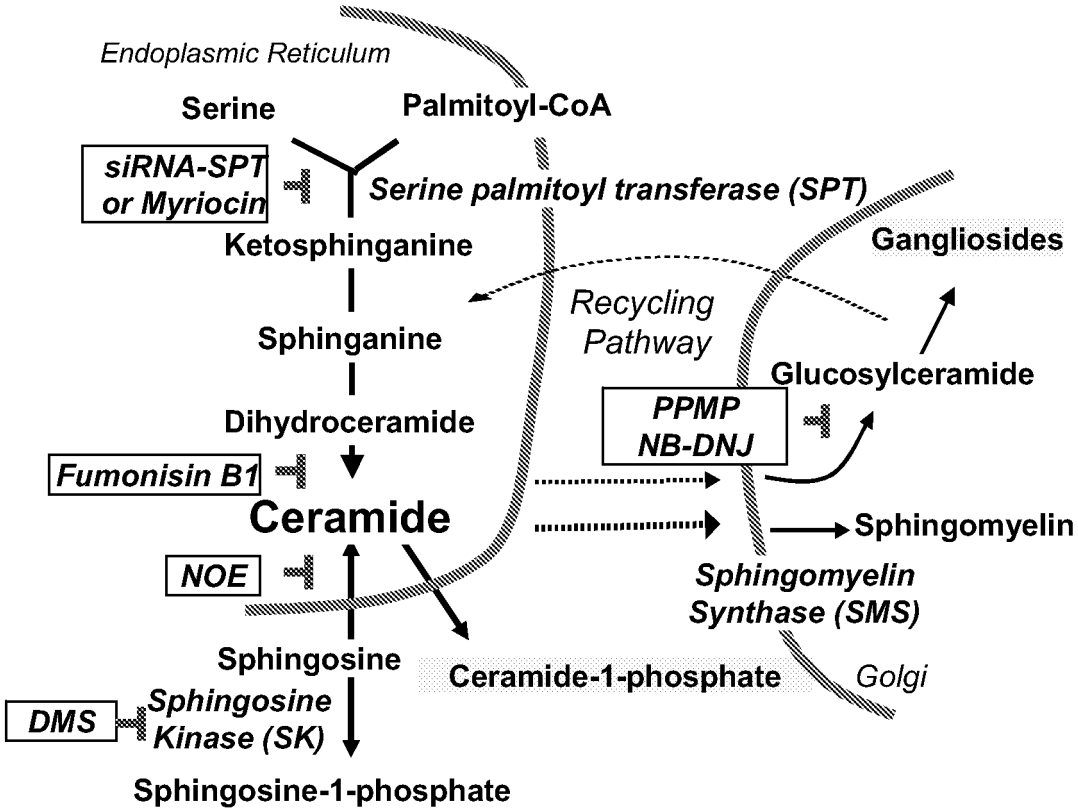
resvaratrol. (i) Dimethyl sphingosine, (j) dihydroceramide saturase (GT 11), (k) n-butyldeoxynojirimycin, and (l) N-oleyl-ethanolamine.

60. A pharmacological composition comprising an isolated nucleic acid that is a member of the group comprising an antisense DNA, antisense RNA, and small interfering RNA, which nucleic acid is sufficiently complementary to the gene or mRNA encoding an enzyme that is a member of the group comprising serine palmitoyl transferase, sphingosine kinase, ceramide synthase/fatty acid desaturase, UDP-glucose ceramide glucosyltransferase and glucosylceramide synthase to permit specific hybridization to the gene or mRNA, respectively.

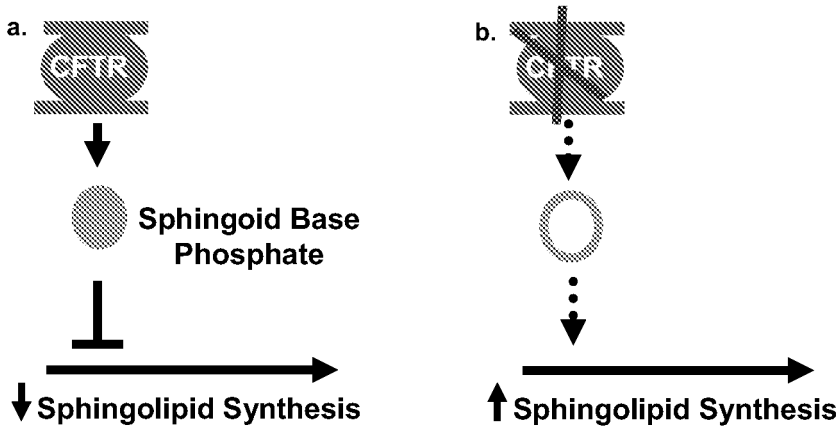
61. A method for treating cystic fibrosis, comprising inhibiting NF-kappa B activity by administering an amount of a pharmaceutical composition that is a member of the group comprising fenretinide, ceramide, ceramide plus fenretinide, ceramide plus fenretinide and 4-oxo-HPR, and fenretinide plus 4-oxo-HPR, ceramide plus parthenolide, ceramide plus parthenolide and fenretinide, ceramide plus parthenolide and fenretinide and 4-oxo-HPR, in an amount that inhibits NF-kappa B activity.

62. A method for treating cystic fibrosis, comprising inhibiting phospholipase A2 activity by administering a pharmaceutical composition that is a member of the group comprising fenretinide, ceramide, ceramide plus fenretinide, ceramide plus fenretinide and 4-oxo-HPR in an amount that inhibits phospholipase A2.

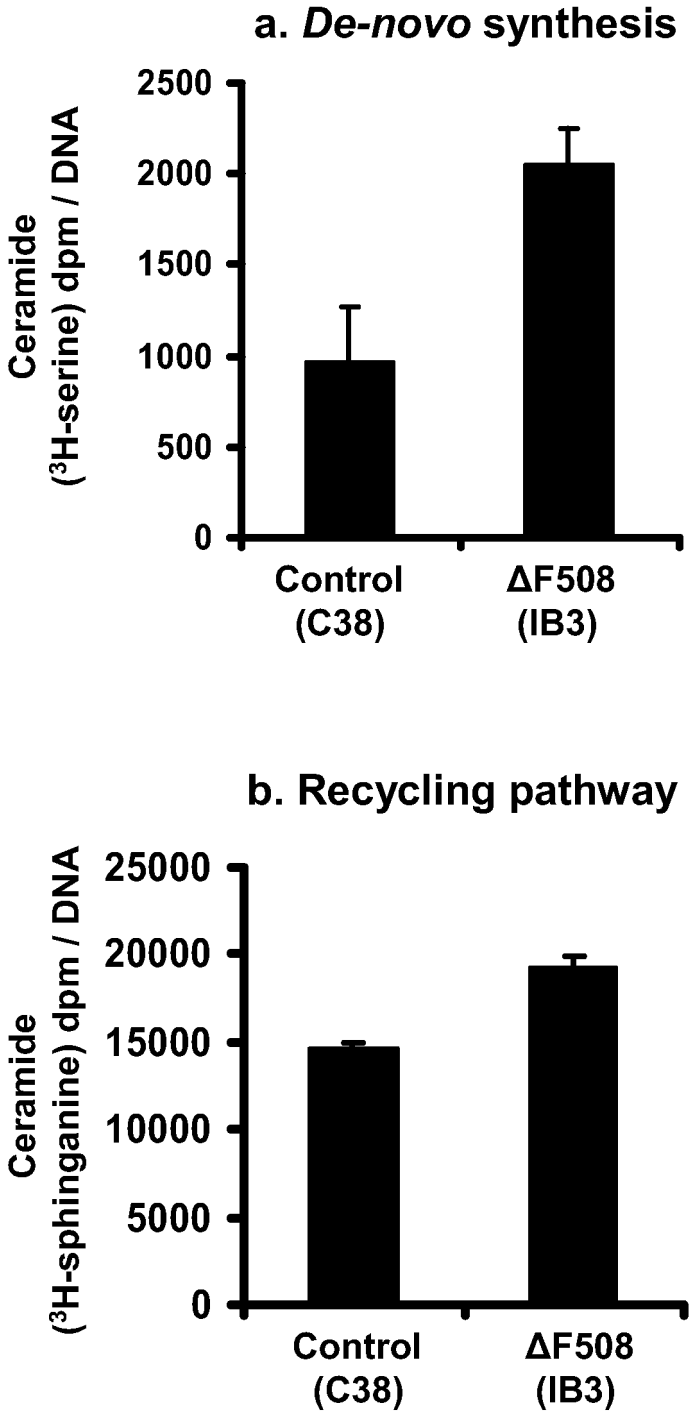
# FIG. 1


Endoplasmic Reticulum
Serine
Palmitoyl-CoA
siRNA-SPT
or Myriocin
Serine palmitoyl transferase (SPT)
Ketosphinganine
Gangliosides
Recycling
Pathway
Sphinganine
Glucosylceramide
PPMP
NB-DNJ
Dihydroceramide
Fumonisin B1
Ceramide
Sphingomyelin
NOE
Sphingomyelin
Synthase (SMS)
Sphingosine
Ceramide-1-phosphate
Golgi
DMS
Sphingosine
Kinase (SK)
Sphingosine-1-phosphate

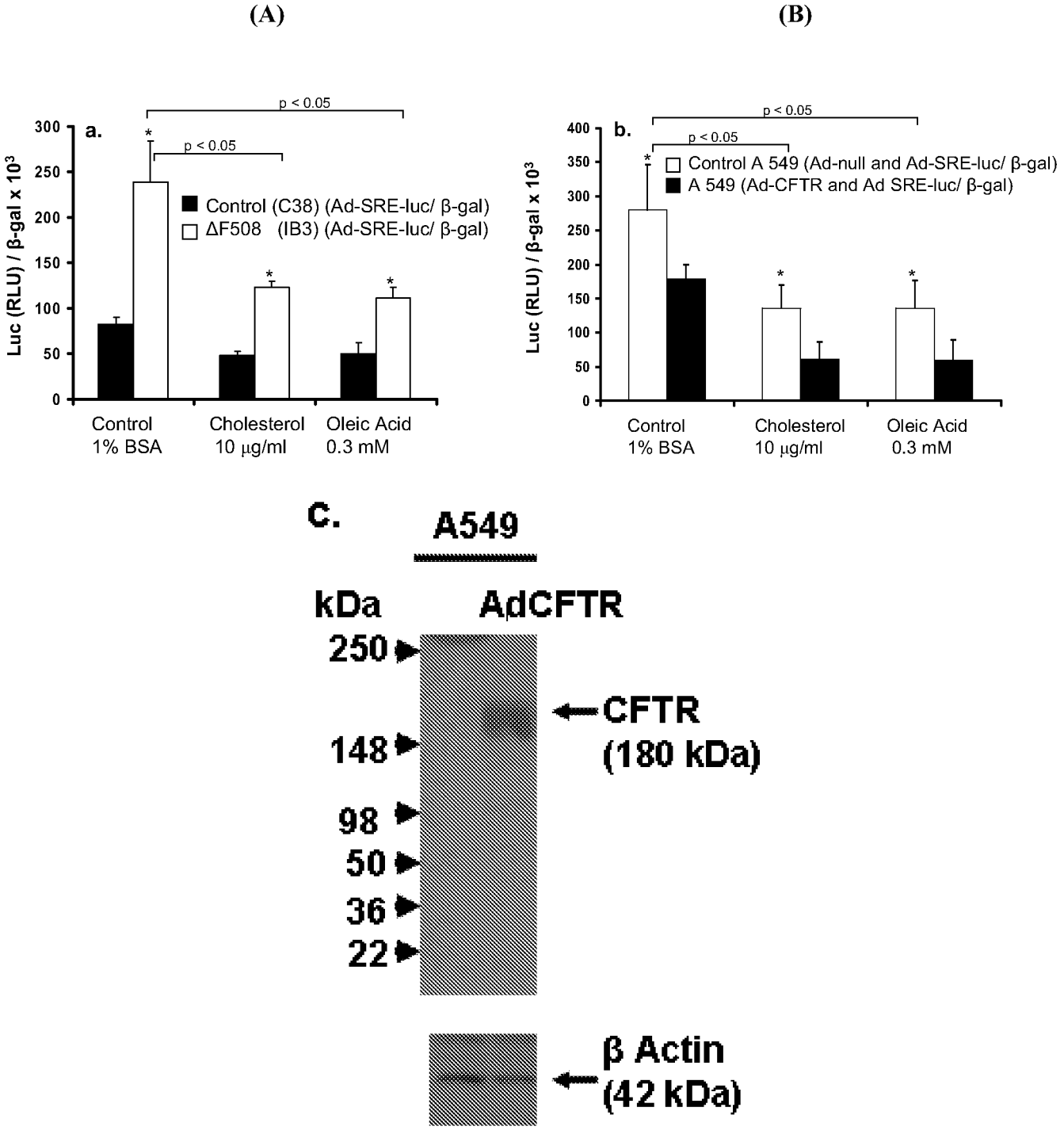
# FIG. 2


a.
CFTR
Sphingoid Base
Phosphate
Sphingolipid Synthesis
b.
CFTR
Sphingolipid Synthesis

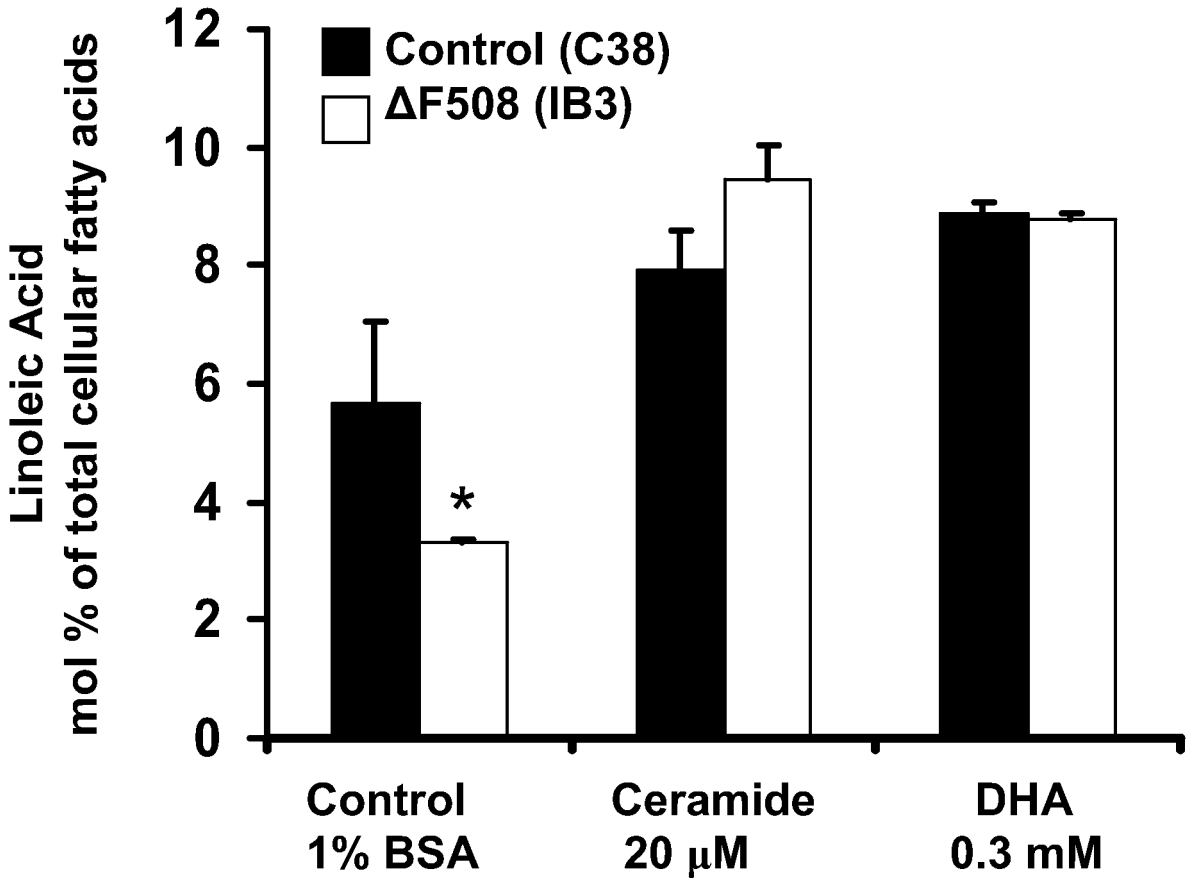
# FIG. 3


a. De-novo synthesis
Ceramide
(3H-serine) dpm / DNA
2500
2000
1500
1000
500
0
Control
(C38)
ΔF508
(IB3)
b. Recycling pathway
Ceramide
(3H-sphinganine) dpm / DNA
25000
20000
15000
10000
5000
0
Control
(C38)
ΔF508
(IB3)

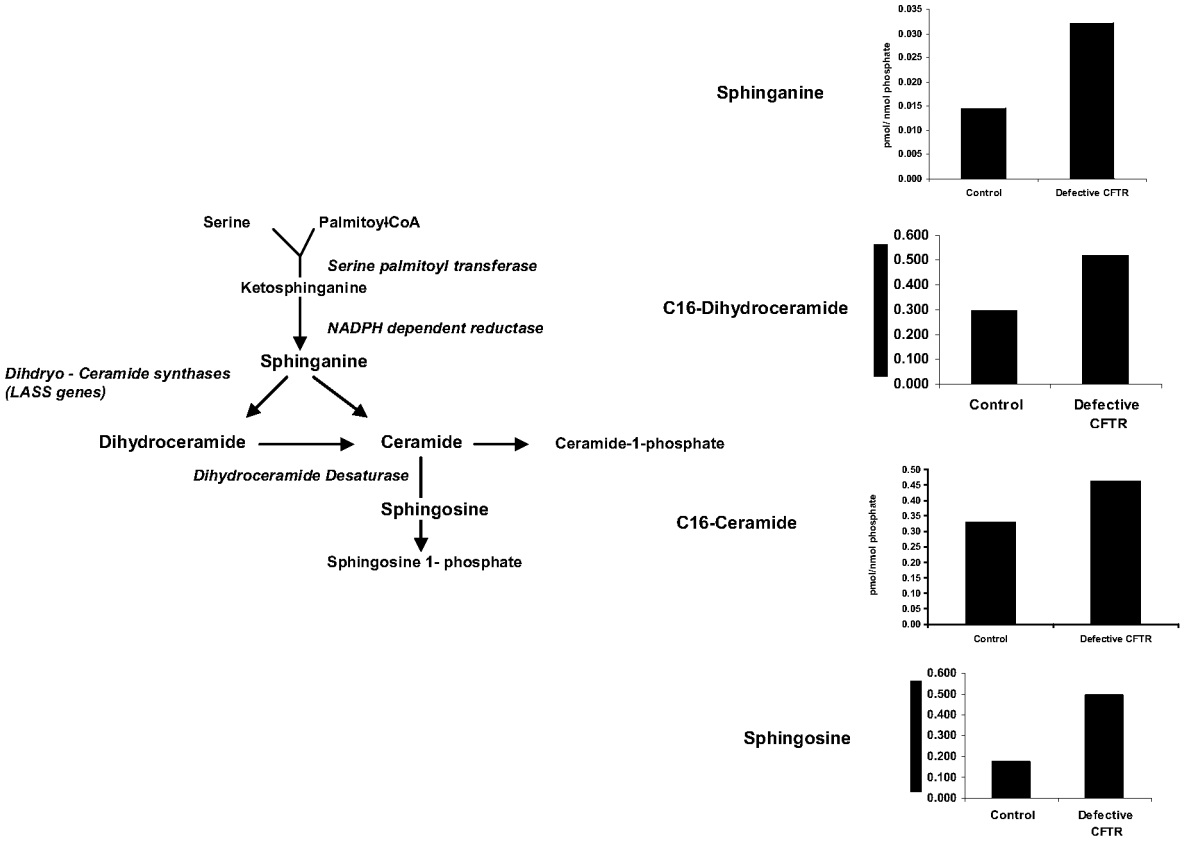
# FIG. 4


(A)
(B)
a.
b.
p < 0.05
p < 0.05
Luc (RLU) / β-gal x 10³
Control (C38) (Ad-SRE-luc/ β-gal)
ΔF508 (IB3) (Ad-SRE-luc/ β-gal)
Control A 549 (Ad-null and Ad-SRE-luc/ β-gal)
A 549 (Ad-CFTR and Ad SRE-luc/ β-gal)
Control 1% BSA
Cholesterol 10 μg/ml
Oleic Acid 0.3 mM
C.
A549
kDa
AdCFTR
250
148
98
50
36
22
CFTR (180 kDa)
β Actin (42 kDa)

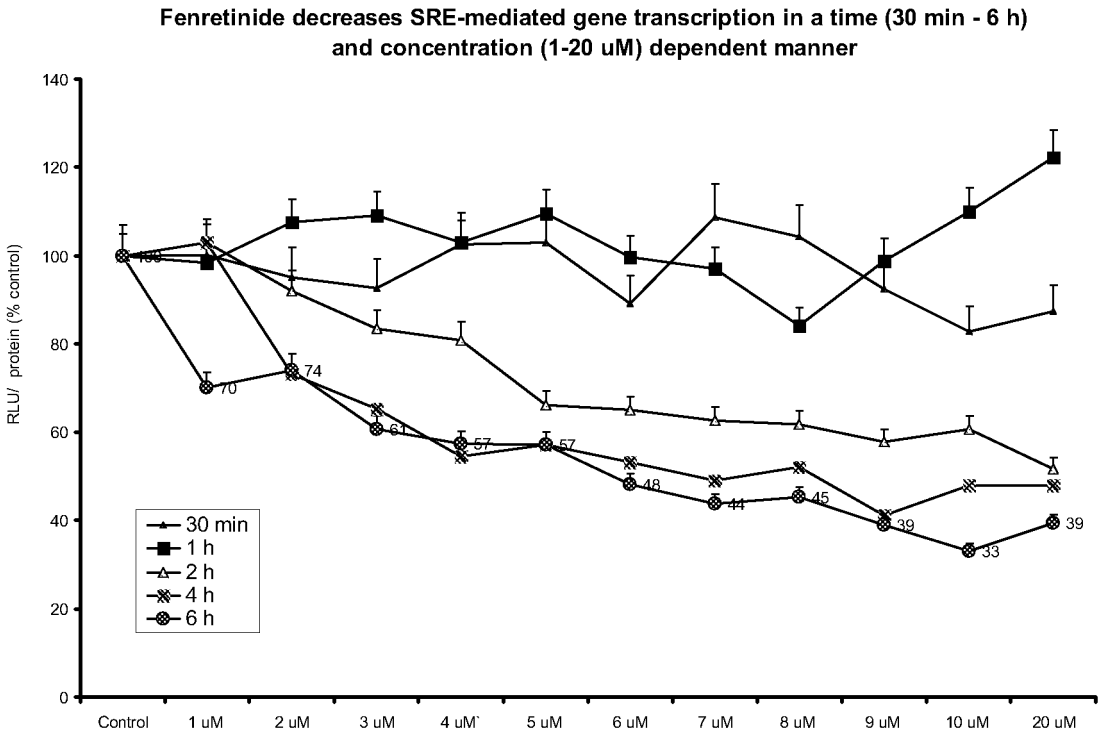
# FIG. 5


Linoleic Acid
mol % of total cellular fatty acids
12
10
8
6
4
2
0
Control (C38)
ΔF508 (IB3)
*
Control
1% BSA
Ceramide
20 μM
DHA
0.3 mM

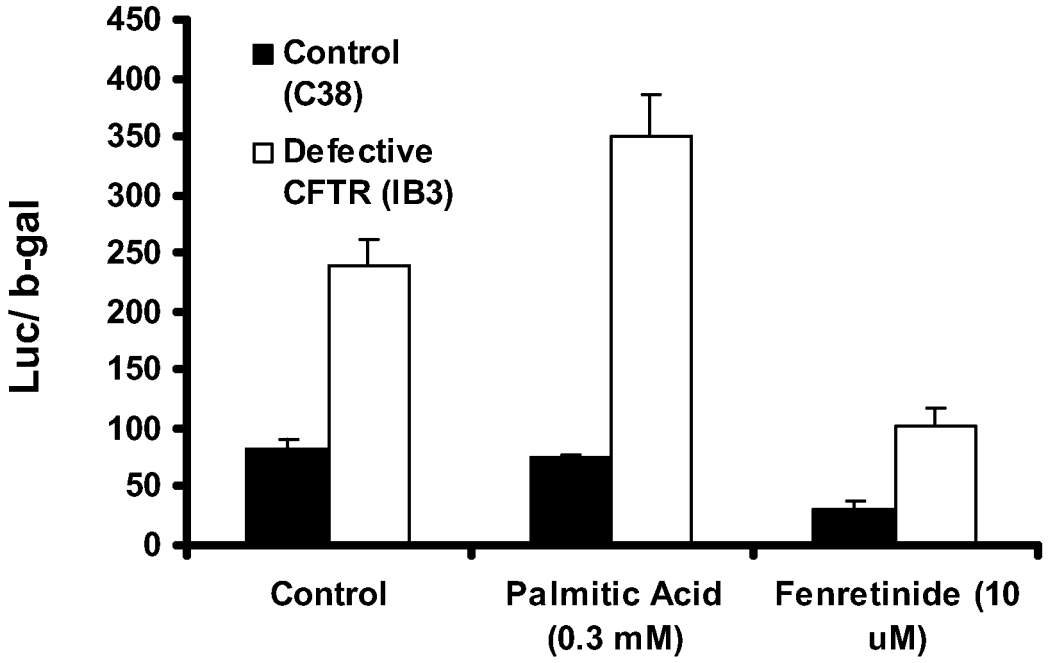
# FIG. 6


Serine
PalmitoylCoA
Serine palmitoyl transferase
Ketosphinganine
NADPH dependent reductase
Sphinganine
Dihdryo - Ceramide synthases
(LASS genes)
Dihydroceramide
Ceramide
Ceramide-1-phosphate
Dihydroceramide Desaturase
Sphingosine
Sphingosine 1- phosphate
Sphinganine
pmol/ nmol phosphate
0.035
0.030
0.025
0.020
0.015
0.010
0.005
0.000
Control
Defective CFTR
C16-Dihydroceramide
0.600
0.500
0.400
0.300
0.200
0.100
0.000
Control
Defective CFTR
C16-Ceramide
pmol/nmol phosphate
0.50
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
Control
Defective CFTR
Sphingosine
0.600
0.500
0.400
0.300
0.200
0.100
0.000
Control
Defective CFTR

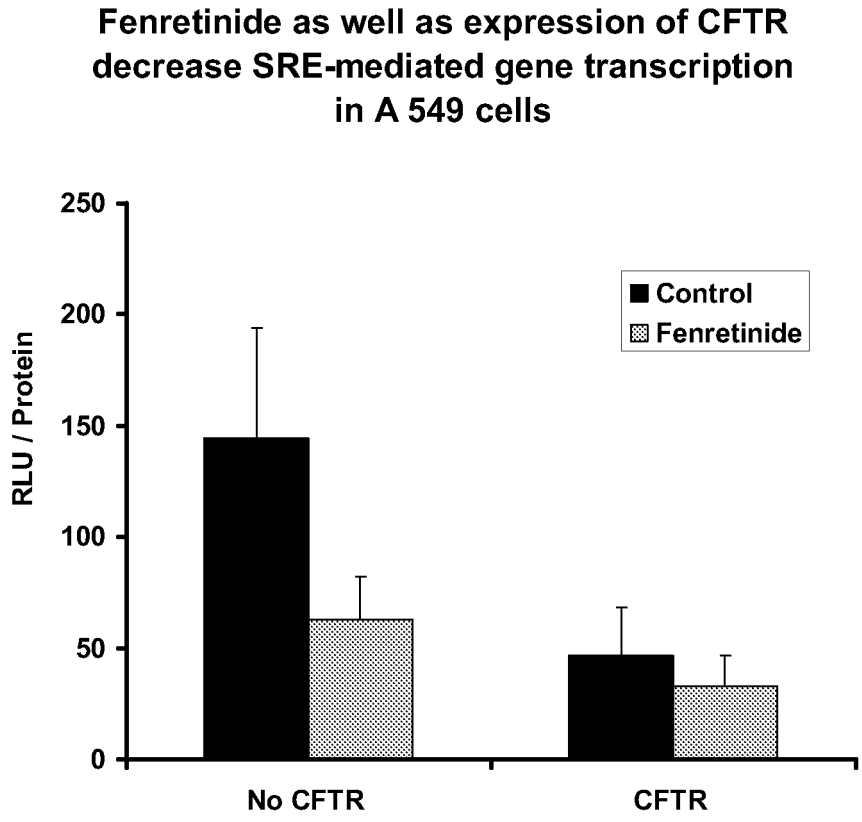
# FIG. 7


Fenretinide decreases SRE-mediated gene transcription in a time (30 min - 6 h) and concentration (1-20 uM) dependent manner
RLU/ protein (% control)
140
120
100
80
60
40
20
0
Control
1 uM
2 uM
3 uM
4 uM
5 uM
6 uM
7 uM
8 uM
9 uM
10 uM
20 uM
30 min
1 h
2 h
4 h
6 h
100
70
74
61
57
57
48
44
45
39
33
39

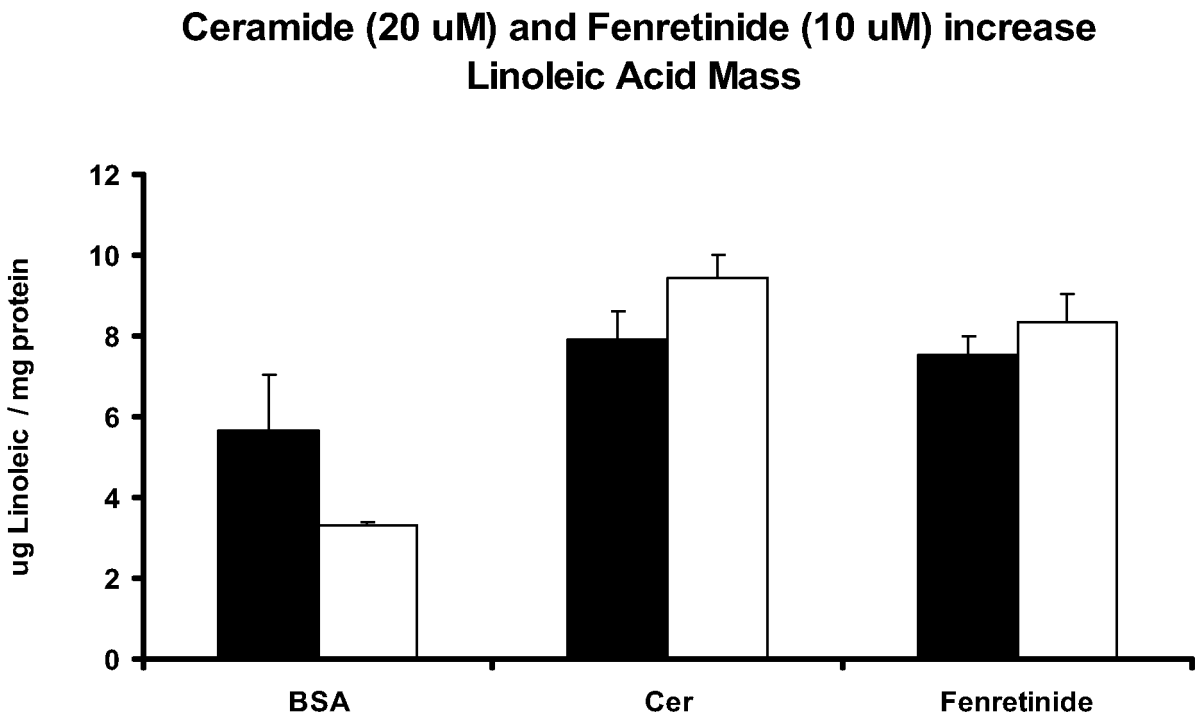
# FIG. 8


450
400
350
300
250
200
150
100
50
0
Luc/ b-gal
Control (C38)
Defective CFTR (IB3)
Control
Palmitic Acid (0.3 mM)
Fenretinide (10 uM)

# FIG. 9

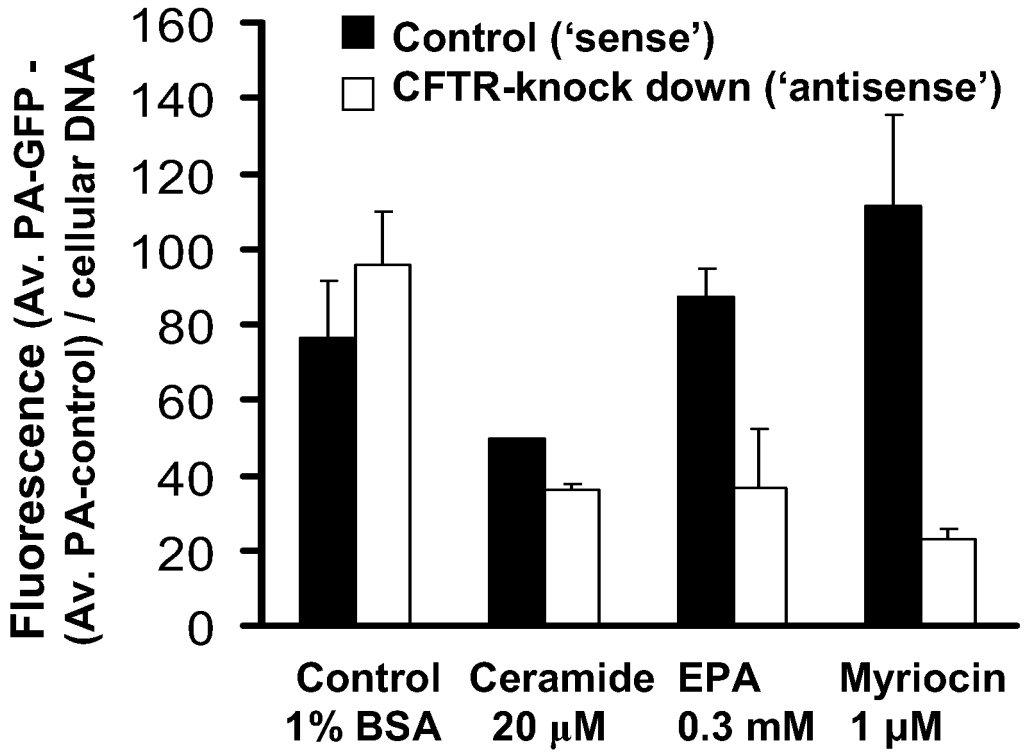

Fenretinide as well as expression of CFTR decrease SRE-mediated gene transcription in A 549 cells
RLU / Protein
250
200
150
100
50
0
Control
Fenretinide
No CFTR
CFTR

# FIG. 10

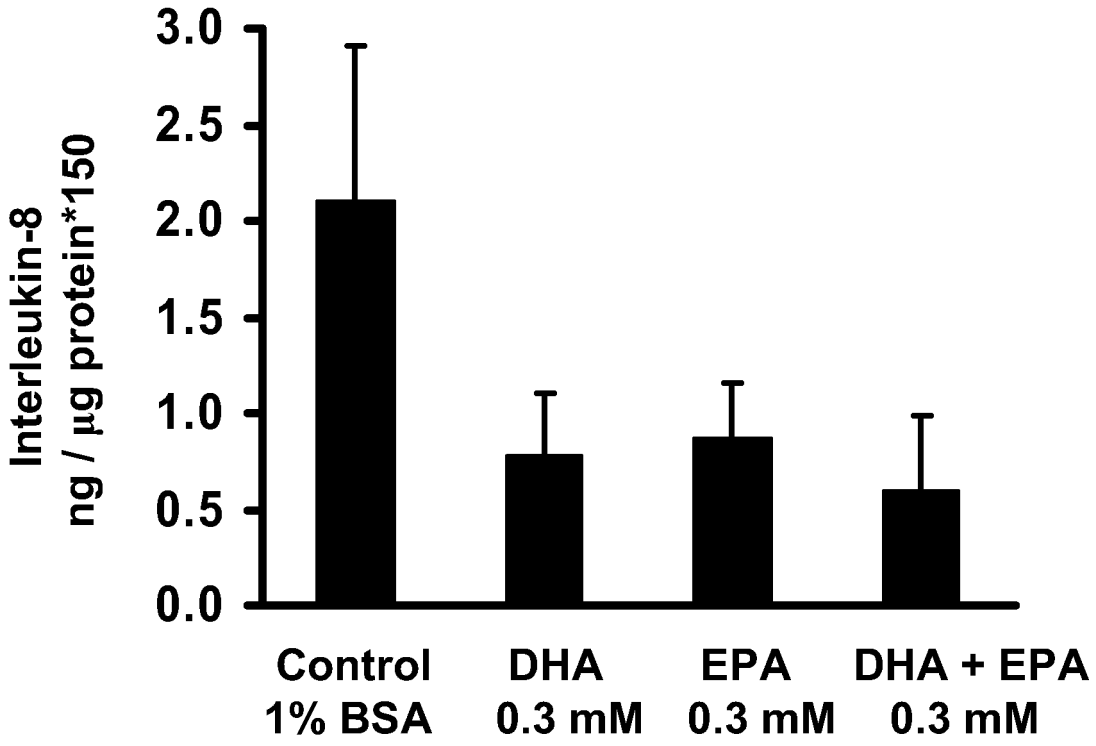

Ceramide (20 uM) and Fenretinide (10 uM) increase Linoleic Acid Mass
ug Linoleic / mg protein
12
10
8
6
4
2
0
BSA
Cer
Fenretinide

# FIG. 11

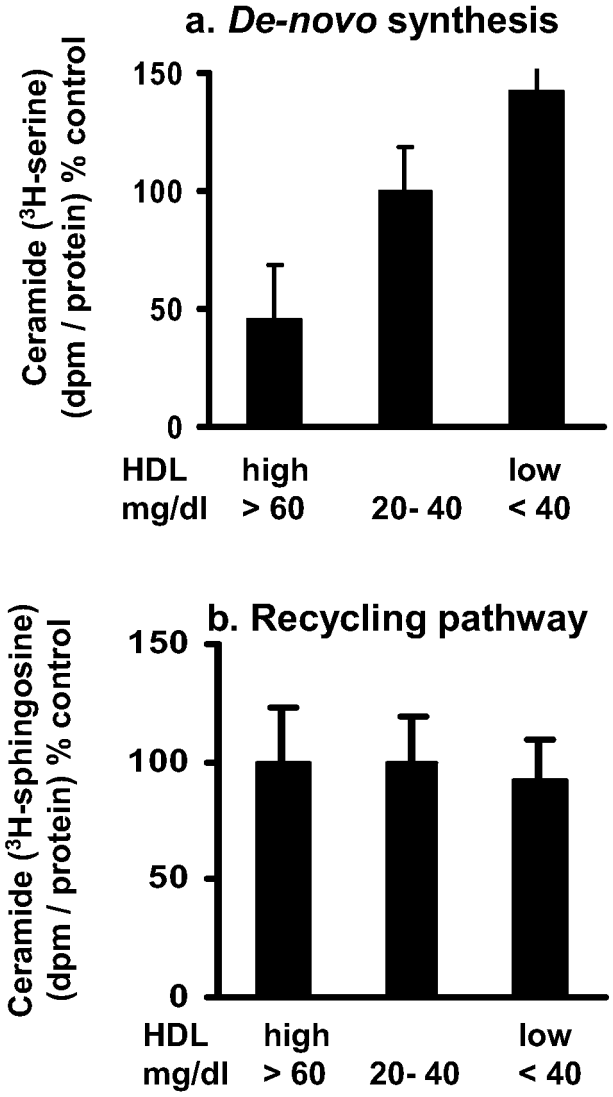
FIG. 11


Fluorescence (Av. PA-GFP - (Av. PA-control) / cellular DNA
160
140
120
100
80
60
40
20
0
Control ('sense')
CFTR-knock down ('antisense')
Control 1% BSA
Ceramide 20 µM
EPA 0.3 mM
Myriocin 1 µM

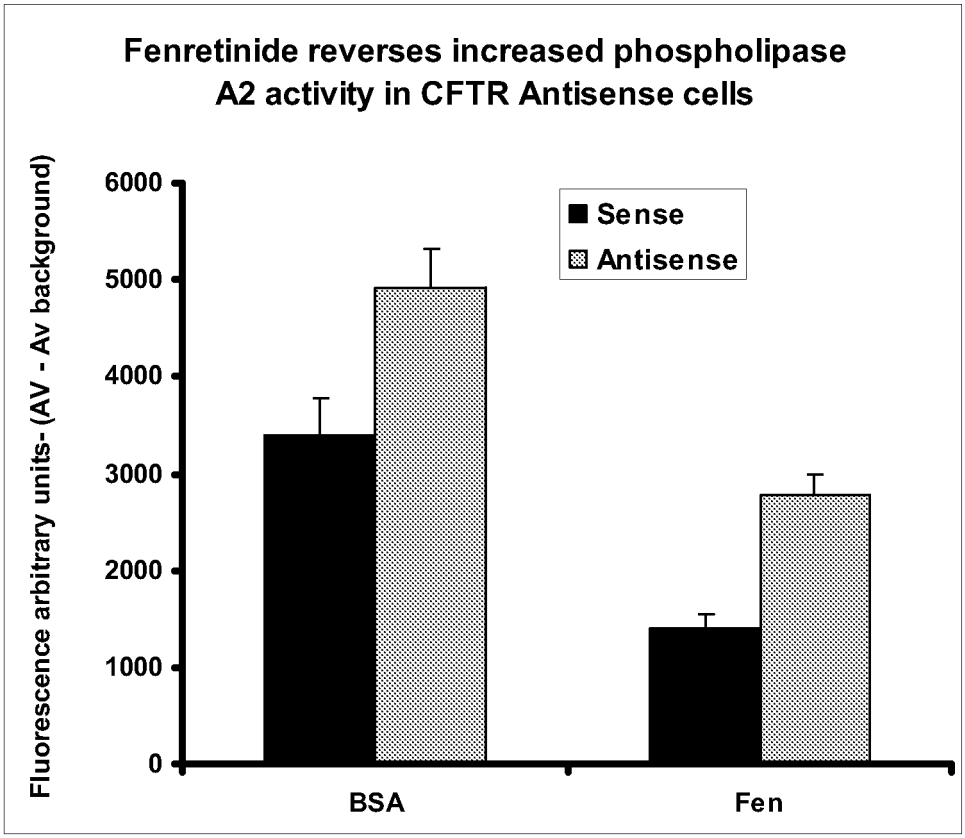
# FIG. 12


Interleukin-8
ng / µg protein*150
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Control
1% BSA
DHA
0.3 mM
EPA
0.3 mM
DHA + EPA
0.3 mM

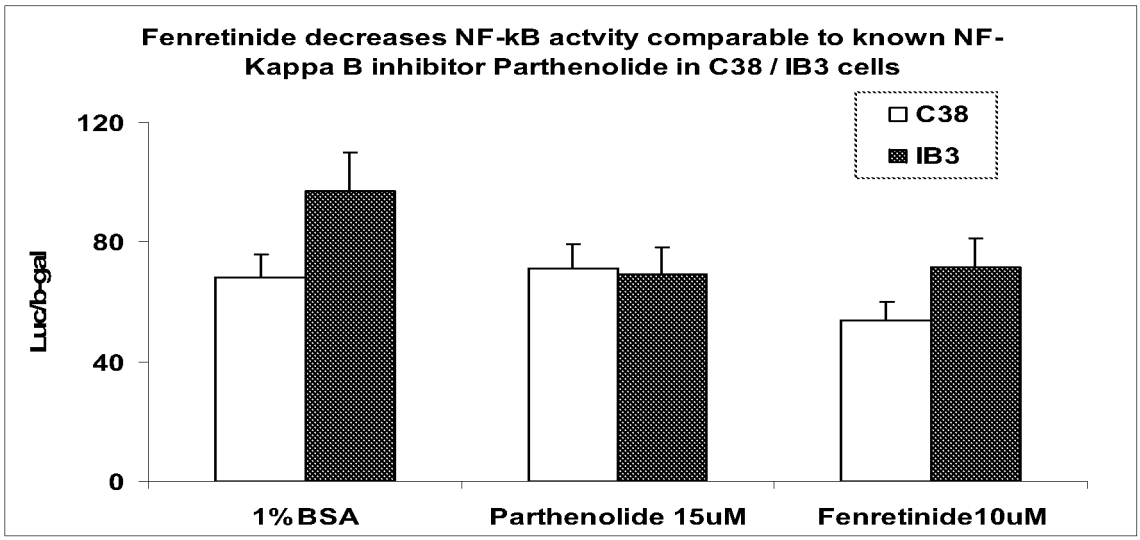
# FIG. 13


a. De-novo synthesis
Ceramide (3H-serine) (dpm / protein) % control
150
100
50
0
HDL high low
mg/dl > 60 20- 40 < 40
b. Recycling pathway
Ceramide (3H-sphingosine) (dpm / protein) % control
150
100
50
0
HDL high low
mg/dl > 60 20- 40 < 40

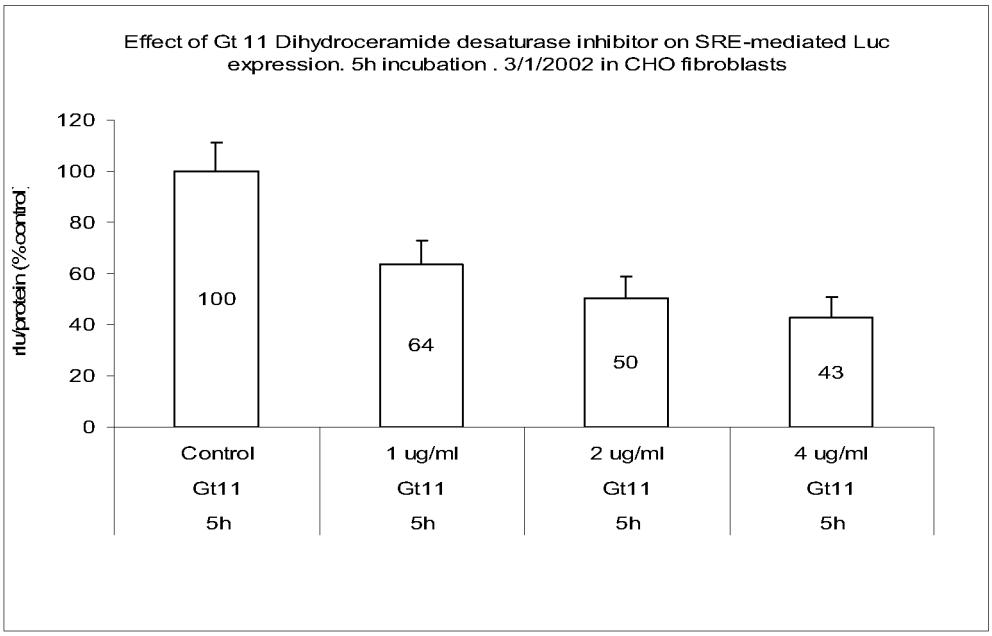
# FIG. 14


Fenretinide reverses increased phospholipase A2 activity in CFTR Antisense cells
Fluorescence arbitrary units- (AV - Av background)
6000
5000
4000
3000
2000
1000
0
Sense
Antisense
BSA
Fen

# FIG. 15


Fenretinide decreases NF-kB actvity comparable to known NF-Kappa B inhibitor Parthenolide in C38 / IB3 cells
C38
IB3
120
80
40
0
Luc/b-gal
1% BSA
Parthenolide 15uM
Fenretinide10uM

# FIG. 16


Effect of Gt 11 Dihydroceramide desaturase inhibitor on SRE-mediated Luc expression. 5h incubation . 3/1/2002 in CHO fibroblasts
rlu/protein (%control)
120
100
80
60
40
20
0
100
64
50
43
Control Gt11 5h
1 ug/ml Gt11 5h
2 ug/ml Gt11 5h
4 ug/ml Gt11 5h