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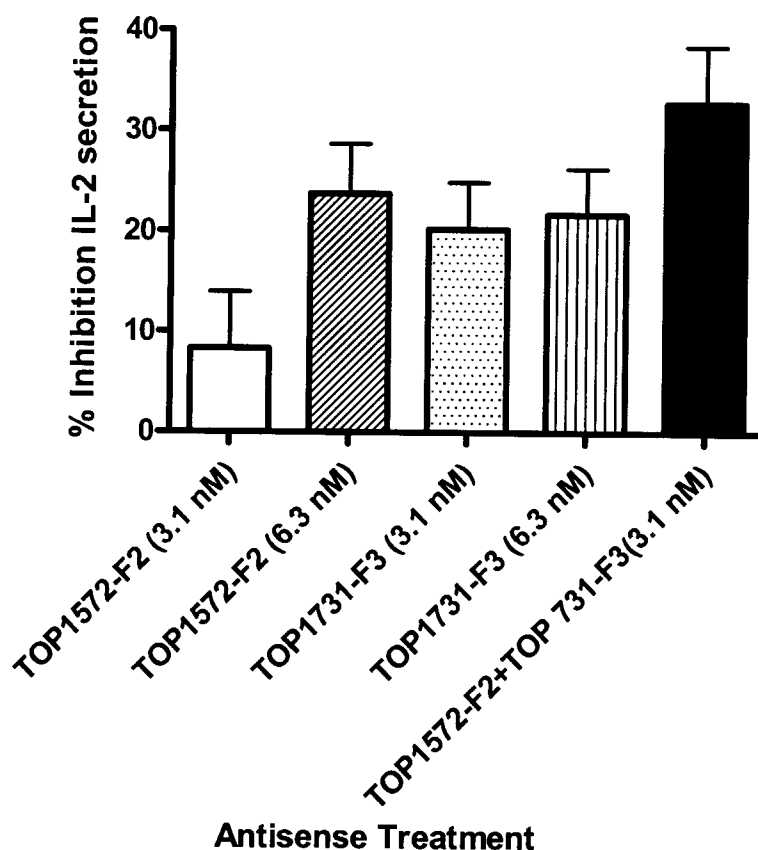
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(54) Title: OLIGONUCLEOTIDES AFFECTING EXPRESSION OF PHOSPHODIESTERASES



(57) Abstract: The invention relates to therapeutic antisense oligonucleotides directed against genes encoding phosphodiesterases (PDE) and the use of these antisense oligonucleotides in combination. These antisense oligonucleotides may be used as analytical tools and/or as therapeutic agents in the treatment of disease associated with reduced cellular cAMP in a patient, such as inflammatory diseases of the respiratory tract including, for example, asthma, chronic obstructive pulmonary disease (COPD), acute respiratory distress syndrome, bronchitis, chronic bronchitis, silicosis, pulmonary fibrosis, lung allograft rejection, allergic rhinitis and chronic sinusitis as well as other conditions in which an increase in cyclic AMP or a decrease in PDE levels is beneficial.

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OLIGONUCLEOTIDES AFFECTING EXPRESSION OF
PHOSPHODIESTERASES

Cross Reference to Related Applications

This application claims priority to U.S. Provisional Application No. 60/801,384 filed on May 19, 2006.

Field of the Invention

The invention relates to methods, reagents and compositions of use for antisense oligonucleotide-based therapy. In particular, the invention relates the application of antisense oligonucleotide-based therapy in the treatment of diseases associated with reduced cAMP in a patient including, for example, PDE-related disease such as inflammatory conditions and cancer. The invention also relates to gene therapy methods and methods for identifying novel antisense-based strategy wherein cyclic AMP phosphodiesterases are involved.

Background of the Invention

The alveolar and airway epithelium is recognized as a dynamic barrier that plays an important role in regulating inflammatory and metabolic responses to oxidative stress, sepsis, endotoxemia, and other critical illnesses in the lung. The respiratory epithelium, in particular, is a primary target of inflammatory conditions/infections at the epithelial-blood interface, and is itself capable of amplifying an inflammatory signal by recruiting inflammatory cells and producing inflammatory mediators.

Chronic Obstructive Pulmonary Disease (COPD) is one example of an inflammatory airway and alveolar disease where persistent upregulation of inflammation is thought to play a role. Inflammation in COPD is characterized

by increased infiltration of neutrophils, CD8 positive lymphocytes, and macrophages into the airways. Neutrophils and macrophages play an important role in the pathogenesis of airway inflammation in COPD because of their ability to release a number of mediators including elastase, metalloproteases, and oxygen radicals that promote tissue inflammation and damage. It has been suggested that inflammatory cell accumulation in the airways of patients with COPD is driven by increased release of pro-inflammatory cytokines and of chemokines that attract the inflammatory cells into the airways, activate them and maintain their presence. The cells that are present also release enzymes (like metalloproteases) and oxygen radicals which have a negative effect on tissue and perpetuate the disease. A vast array of pro-inflammatory cytokines and chemokines have been shown to be increased within the lungs of patients with COPD. Among them, an important role is played by tumor necrosis factor alpha (TNF-alpha), granulocyte-macrophage colony stimulating factor (GM-CSF) and interleukin 8 (IL-8), which are increased in the airways of patients with COPD.

Other examples of respiratory diseases where inflammation seems to play a role include: asthma, eosinophilic cough, bronchitis, acute and chronic rejection of lung allograft, sarcoidosis, pulmonary fibrosis, rhinitis and sinusitis. Asthma is defined by airway inflammation, reversible obstruction and airway hyperresponsiveness. In this disease the inflammatory cells that are involved are predominantly eosinophils, T lymphocytes and mast cells, although neutrophils and macrophages may also be important. A vast array of cytokines and chemokines have been shown to be increased in the airways and play a role in the pathophysiology of this disease by promoting inflammation, obstruction and hyperresponsiveness.

Eosinophilic cough is characterized by chronic cough and the presence of inflammatory cells, mostly eosinophils, within the airways of patients in the absence of airway obstruction or hyperresponsiveness. Several cytokines and chemokines are increased in this disease, although they are mostly eosinophil

directed. Eosinophils are recruited and activated within the airways and potentially release enzymes and oxygen radicals that play a role in the perpetuation of inflammation and cough.

Acute bronchitis is an acute disease that occurs during an infection or irritating event for example by pollution, dust, gas or chemicals, of the lower airways. Chronic bronchitis is defined by the presence of cough and phlegm production on most days for at least 3 months of the year, for 2 years. One can also find during acute or chronic bronchitis within the airways inflammatory cells, mostly neutrophils, with a broad array of chemokines and cytokines. These mediators are thought to play a role in the inflammation, symptoms and mucus production that occur during these diseases.

Lung transplantation is performed in patients with end stage lung disease. Acute and more importantly chronic allograft rejection occur when the inflammatory cells of our body, lymphocytes, do not recognize the donor organ as "self". Inflammatory cells are recruited by chemokines and cytokines and release a vast array of enzymes that lead to tissue destruction and in the case of chronic rejection a disease called bronchiolitis obliterans.

Sarcoidosis is a disease of unknown cause where chronic non-caseating granulomas occur within tissue. The lung is the organ most commonly affected. Lung bronchoalveolar lavage shows an increase in mostly lymphocytes, macrophages and sometimes neutrophils and eosinophils. These cells are also recruited and activated by cytokines and chemokines and are thought to be involved in the pathogenesis of the disease.

Pulmonary fibrosis is a disease of lung tissue characterized by progressive and chronic fibrosis (scarring) which will lead to chronic respiratory insufficiency. Different types and causes of pulmonary fibrosis exist but all are characterized by inflammatory cell influx and persistence, activation and proliferation of fibroblasts with collagen deposition in lung tissue. These

events seem related to the release of cytokines and chemokines within lung tissue.

Acute rhinitis is an acute disease that occurs during an infection or irritating event, for example, by pollution, dust, gas or chemicals, of the nose or upper airways. Chronic rhinitis is defined by the presence of a constant chronic runny nose, nasal congestion, sneezing and pruritis. One can also find within the upper airways during acute or chronic rhinitis inflammatory cells with a broad array of chemokines and cytokines. These mediators are thought to play a role in the inflammation, symptoms and mucus production that occur during these diseases.

Acute sinusitis is an acute, usually infectious disease of the sinuses characterized by nasal congestion, runny, purulent phlegm, headache or sinus pain, with or without fever. Chronic sinusitis is defined by the persistence for more than 6 months of the symptoms of acute sinusitis. One can also find during acute or chronic sinusitis within the upper airways and sinuses inflammatory cells with a broad array of chemokines and cytokines. These mediators are thought to play a role in the inflammation, symptoms and phlegm production that occur during these diseases.

There is a growing body of evidence suggesting an intimate link between inflammation and neoplastic diseases. The tumor microenvironment is shaped by cells entering it, and their functions reflect the local conditions. Successive changes occurring at the tumor site during tumor progression resemble chronic inflammation. This chronic inflammatory reaction seems to be largely orchestrated by the tumor, and it seems to promote tumor survival. It has become evident that early and persistent inflammatory responses observed in or around developing neoplasms regulates many aspects of tumour development (matrix remodelling, angiogenesis, malignant potential) by providing diverse mediators implicated in maintaining tissue homeostasis,

e.g., soluble growth and survival factors, matrix remodelling enzymes, reactive oxygen species and other bioactive molecules.

As described above, these inflammatory respiratory diseases or diseases in which inflammation plays a critical role are all characterized by the presence of mediators that recruit and activate different inflammatory cells which release enzymes or oxygen radicals causing symptoms, the persistence of inflammation and when chronic, destruction or disruption of normal tissue.

A logical therapeutic approach would be to downregulate cytokine and chemokine production and the inflammatory cell response. This has been performed in all the diseases described above by employing either topical or systemic corticosteroids with different levels of success. Corticosteroids are immune suppressive and have effects not only on inflammatory cells but also on other cells of the body that lead to toxicity when administered chronically.

Despite the availability of medications for COPD, asthma and other inflammatory respiratory diseases, the prevalence and morbidity of these diseases has remained stable or increased. It is obvious that there is an unmet medical need for the therapy of inflammatory respiratory diseases, and innovative therapeutic agents are urgently required. Antisense oligonucleotide-based therapy offers a new alternative approach to selectively decrease the expression of specific genes without the undesirable toxic effects of traditional therapeutic strategies. Antisense therapies are being investigated for the treatment of several diseases. It has been previously shown that antisense oligonucleotides directed against receptors for inflammatory mediators can be administered to the lungs and down-regulate their targets as described in WO9966037.

A therapeutic approach that would decrease pro-inflammatory cytokine and chemokine release by a vast array of cells while having a reduced effect on the release of anti-inflammatory mediators or enzymes may have an

advantage over current therapies for inflammatory respiratory diseases or any other systemic inflammatory disease.

The cyclic nucleotides cAMP and cGMP are ubiquitous second messengers participating in signaling transduction pathways and mechanisms. Mammalian cells have evolved a complex and highly conserved complement of enzymes that regulate the generation and inactivation of cyclic nucleotides through multiple and complex feedforward and feedback mechanisms. Both cAMP and cGMP are formed from their respective triphosphates (ATP and GTP) by the catalytic activity of adenylyl (adenylate) or guanylyl (guanylate) cyclase, respectively as described in Essayan D. M. *Cyclic nucleotide phosphodiesterase (PDE) inhibitors and immunomodulation*. Biochem. Pharmacol., 1999, 57, 965-973. Inactivation of cAMP/cGMP is achieved by hydrolytic cleavage of the 3'-phosphodiester bond catalyzed by the cyclic-nucleotide-dependent phosphodiesterases (PDEs), resulting in the formation of the corresponding, inactive 5'-monophosphate as described in Essayan, 1999 and Perry M.J. and Higgs G.A. *Chemotherapeutic potential of phosphodiesterase inhibitors*. 1998, Curr Opin Chem Biol.4:472-81.

It has been shown that the inflammatory response and its progression is exquisitely sensitive to modulations in the steady-state levels of cyclic nucleotides, where target cells for their effects extend beyond immune cells to include accessory cells, such as airway smooth muscle, epithelial and endothelial cells, and neurons as described in Perry and Higgs, 1998; Essayan, 1999. In this respect, the emerging concept that modulation of intracellular cyclic nucleotides plays a major role in regulating the inflammatory milieu has recently evolved into targeting and improving inflammatory/autoimmune responses. The cyclic nucleotide PDEs are a large, growing multigene family, comprising at least 11 families of PDE enzymes. The profile of selective and nonselective PDE inhibitors in vitro and in vivo, therefore, suggests a potential therapeutic utility as antidepressants,

antiproliferative, immunomodulatory, tocolytics, inotropes/chronotropes, and cytoprotective agents.

Intracellular cAMP seems to have a fundamental role, not only in smooth muscle relaxation, activation and proliferation but also in the modulation of the release of mediators by inflammatory cells. Decreased cAMP levels can lead to increased production of inflammatory mediators such as TNF-alpha, GM-CSF, and IL-8 in airway epithelial cells.

Insight into the molecular mechanisms of the regulatory role of cytokines in cellular homeostasis as well as inflammatory/autoimmune/infectious diseases has begun to provide new approaches to design therapeutic strategies for pharmacological interventions. One such novel approach is the chemotherapeutic potential of PDE enzyme blockade, which revealed a phenomenal diversity and complexity scheme for promising therapeutics across a broad spectrum of disease states. One mechanistic understanding of PDE inhibition is centered on the immunomodulatory properties of cyclic nucleotides (cAMP/cGMP), thereby paving a channel through which anti-inflammatory, therapeutic applications could be clearly demonstrated.

The inflammatory response and its progression is exquisitely sensitive to modulations in the steady-state levels of cyclic nucleotides, where target cells for their effects extend beyond immune cells to include structural cells, such as epithelial, smooth muscle and endothelial cells, and neurons (Perry and Higgs, 1998; Essayan, 1999). Modulation of intracellular cyclic nucleotides plays a major role in regulating the inflammatory response. The cyclic nucleotide PDEs are a large, growing multigene family, comprising at least 11 families of PDE isoenzymes. PDEs differ in their tissue and cellular distribution as well as in their molecular and physicochemical characteristics including nucleotides and protein sequences, substrate specificity, inhibitor sensitivity, and cofactor requirements. Within different families, tissue specific

isoforms are generated from the same gene by alternative mRNA splicing and differential promoter usage.

The cAMP-specific PDE4 enzyme family is one of the most extensively studied PDEs. Enzymes within this family are found in most pro-inflammatory and immune cells, where they play a key role in the regulation of cAMP metabolism. PDE4 enzymes are expressed in macrophages, neutrophils, cytotoxic CD8⁺ T cells, bronchial epithelial cells, and airway smooth muscle cells. Moreover, PDE4 inhibitors modulate inflammation in animal models of respiratory diseases, suggesting that PDE4 may represent a suitable target in a therapeutic based strategy for intervention with small molecule inhibitors. Anti-PDE4 drugs inhibit the hydrolysis of intracellular cAMP, which in turn provides bronchodilation and suppression of the inflammatory response. Selective PDE4 inhibitors such as cilomilast and roflumilast are active in animal models of neutrophil inflammation (Bundschuh DS et al. J. Pharmacol. Exp. Ther. 2001, 297: 280-290). Although the use of PDE4 inhibitors for the treatment of airway inflammation is under intensive clinical investigations, several inhibitors have been dropped because of their toxicity, dose-limiting side effects, of which nausea and vomiting are the most common physiological manifestations. Consequently, improving the therapeutic ratio of PDE4 inhibitors raised a major challenge that is still an important field of investigation.

Considering the distribution of enzymes in target tissues, with high activity of PDE3 and PDE4 in airway smooth muscle and inflammatory cells, selective inhibitors of these enzymes may add to the therapy of chronic airflow obstruction. Generally small molecule inhibitors have been focused on one or several PDE without assessing the potential detrimental effects of inhibiting all the isoenzymes of a PDE. For example it has been suggested that most of the toxicity attributed to the PDE4 antagonists will occur through inhibition of the PDE4 isotype D. In addition, as shown herein, inhibition of certain isoenzymes of PDEs does not decrease all pro-inflammatory mediators however, a

combination of isotype specific oligonucleotides can lead to an effect that is much broader when the right combination of antisense oligonucleotides is employed.

The PDE3 family contains two different genes, PDE3A and PDE3B, which are cGMP-inhibited and display a high affinity towards cAMP. Each PDE3 gene codes for at least two splice isoforms. The PDE3A has been identified in smooth muscle, platelets and cardiac tissues. The PDE3B is most abundant in adipocytes and liver cells. However, initial data from clinical trials with selective PDE3 inhibitors or a combination with PDE4 inhibitors have been somewhat disappointing and have tempered the expectations considerably since these drugs had limited efficacy and their use was clinically limited through side effects.

PDE7 was first isolated from a human glioblastoma. PDE7A codes for a cAMP-specific PDE that is insensitive to cGMP and inhibitors of PDE3 and PDE4 and has an amino acid sequence distinct from other cAMP PDEs. In humans two genes (PDE7A and PDE7B) have been characterized. The PDE7A gene codes for three isoenzymes (PDE7A1, PDE7A2, and PDE7A3) derived from the same gene by alternative mRNA splicing. In humans, PDE7A2 mRNA is expressed abundantly in skeletal muscle, heart, and kidney, whereas the testis, lung, and immune system (thymus, spleen, lymph node, blood leukocytes) are rich sources of PDE7A1. In addition, activated, but not naïve, human T lymphocytes express the splice variant PDE7A3. In contrast, PDE7B exists as a single isoenzyme in humans, it shares ~70% sequence similarity to PDE7A, but distinct kinetic properties. PDE7B is expressed predominantly in the brain and in a number of other tissues including liver, heart, thyroid glands, and skeletal muscle. Stimulation of human naïve T cells with anti-CD3 and anti-CD28 antibodies has been shown to promote IL-2 production and clonal amplification. These effects were attributed to PDE7A and down regulation of this enzyme prevents lymphocyte proliferation (Li L et al., Science, 1999, 283, 848-851).

The PDE4 family contains 4 different genes (PDE4 A-D). Due to alternative splicing of the genes, multiple splice variants are reported and classified into two main groups, the long and the short forms. PDE4A, B and D gene products are found in most immune and inflammatory cells. These are present either constitutively or after activation as described in Burnouf C. and Pruniaux M.P. Current Pharmaceutical Design 2002; 8:1255-1296. Inhibition of all or certain isotypes of PDE4 is associated with downregulation of several inflammatory mediators; however, an increase in certain pro-inflammatory cytokines (e.g. IL-6) has been described (Giembycz M.A. Expert Opin Invest Drugs 2001, 10:1361-1379).

It would therefore be desirable to inhibit all three PDE enzymes; however this approach may be plagued by the toxicity that has been described with administration of inhibitors of each of these enzymes. The topical application of antisense oligonucleotides could circumvent the systemic toxicity of enzyme inhibition but there appear to be too many isoenzymes of these PDE for this approach to be practical. Inhibition of one or several isotypes of PDE enzymes may not be as effective as full PDE inhibition since other isotypes would be present to have their pro-inflammatory effects.

It would therefore appear desirable to seek a way to down-regulate pro-inflammatory mediators and their cells while affecting less anti-inflammatory mediators or inhibitory enzymes for the therapy of inflammatory respiratory diseases. The therapeutic application of a combination of antisense oligonucleotides directed against selected isotypes of PDE enzymes is therefore herein proposed as a therapy for inflammatory respiratory diseases or any disease where increased cyclic AMP plays a role.

SUMMARY OF THE INVENTION

The present invention provides antisense oligonucleotide compounds that are effective at down-regulating PDE isotype genes that they are directed against

as well as selected antisense oligonucleotide compounds that are effective at down-regulating not only the PDE isotype genes they are directed against but also other related genes including other PDE isotype genes, inflammatory and mitogenic genes.

In another aspect, the present invention provides a composition comprising at least 2 antisense oligonucleotide compounds, each antisense oligonucleotide compound being capable of downregulating a different gene. In some embodiments of this aspect, the combination of the at least 2 antisense oligonucleotide compounds leads to a significant downregulation of each the genes that is more than the additive ability of each antisense to downregulate each gene individually. In further embodiments, the present invention also provides a composition comprising at least 2 antisense oligonucleotide compounds, each antisense oligonucleotide compound being capable of downregulating a different gene, each antisense oligonucleotide compound being present at a concentration at which the antisense oligonucleotide compound is practically ineffective on its own to downregulate the gene it is directed against, the combination of the at least 2 antisense oligonucleotide compounds leading to a significant downregulation of each of the genes that the antisense oligonucleotide compounds are directed against and optionally other related genes. In still further embodiments of this aspect of the present invention, there is provided a composition comprising a combination of at least two antisense oligonucleotide compounds each directed against a different PDE target gene and each being effective to downregulate or inhibit a PDE target gene, each oligonucleotide compound being present in the combination at a concentration at which it exhibits less than 20% inhibition of its target gene, the combination of the oligonucleotide compounds exhibiting more than 20% inhibition and at least doubling the inhibition of at least one of the target genes.

The present invention further provides an antisense oligonucleotide effective in the compositions and methods of the present invention having a sequence selected from the group consisting of Seq. ID No. 1 to 92 and 113 to 126.

The present invention further provides pharmaceutical compositions comprising a pharmaceutically acceptable carrier and an oligonucleotide of the present invention or a combination of at least two antisense oligonucleotides as described above. The present invention also provides for the use of such compositions in the treatment and/or prevention of inflammatory respiratory diseases and other inflammation-related diseases.

The combinations of the present invention appear to exert an inhibitory action by an approach that has been termed multiple gene knock down. Multiple gene knock down encompasses situations in which:

- 1) an antisense oligonucleotide downregulates not only the gene that it is directed against but also downregulates other related genes; and/or
- 2) a combination of at least 2 antisense oligonucleotides, each present at a concentration at which the antisense oligonucleotide is practically ineffective to downregulate the gene it is directed against on its own, the combination leading to significant downregulation of both genes that the antisense oligonucleotides are directed against and optionally other related genes.

The present invention uses the above approach to provide methods, compositions and kits for treating and/or preventing disease associated with reduced cAMP in a patient, including PDE-related disease, inflammatory disease including respiratory diseases and more particularly COPD and asthma, and other diseases in which inflammation plays an important role including cancer.

The invention also provides methods, reagents and compositions for reducing the expression and consequently the activity of cell cyclic-AMP phosphodiesterases and thereby maintaining the correct balance of intracellular c-AMP and cytokine/chemokine production.

The invention further provides methods and tools for identifying/screening *in vitro*, *in vivo* and *ex vivo*, combinations of novel antisense oligonucleotide compounds, drugs and vaccines that are capable of interfering with PDE expression and elevating the intracellular c-AMP level.

The present invention also provides gene-based therapy and transfection methods in which one or more antisense oligonucleotide compounds are used for cell transfection or delivered to humans and animals for interfering with PDE isotype gene expression.

The present invention further provides antisense oligonucleotides effective against PDE3 isotypes, such as PDE3A and PDE3B; PDE4 isotypes such as PDE4A, PDE4B and PDE4D and PDE7 isotypes such as PDE7A, among other PDEs.

According to another broad aspect of the invention, a method is provided for increasing at least one of nuclease stability and target gene inhibition activity of an antisense oligonucleotide comprising replacing at least one nucleotide of the oligonucleotide with an arabinose modified nucleotide, preferably 2'-deoxy-2'-fluoroarabinonucleotide (FANA).

Other objects and advantages of the present invention will be apparent upon reading the following non-restrictive description of several preferred embodiments made with reference to the accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

The present invention will now be described in greater detail having regard to the appended drawings in which:

Figure 1 shows the efficacy of different AON at inhibiting their specific target knockdown. (A) A549 cells were transfected with specific AON targeting PDE3A, PDE3B, PDE7A, PDE4A, PDE4B or PDE4D for 24h using Lipofectamine™ 2000 (Invitrogen, ratio 1ug oligonucleotide:1uL Lipofectamine™ 2000) and 200ng to 400ng of AON (AS) or the corresponding mismatch sequence (Mi/Sense). PDE3B, PDE4A and PDE4B mRNA expression levels were quantified using the Quantigene® assay (Panomics). Real-time PCR was used to quantify PDE3A, PDE7A and PDE4D expression. PDE expression was normalized to the expression of a control gene (β 2M, Ppib or HPRT). Data expressed as mean % inhibition relative to expression level in untreated cells. (**p<0.01, ***p<0.001, n=3). (B) 293 cells transfected overnight with AON that targets more than one PDE4 isoform. PDE4 isoform mRNA expression level was quantified using real-time PCR and normalized to the expression of the control gene β 2M. Data expressed as mean % inhibition relative to expression level of cells transfected with relevant sense or mismatch AON.

Figure 2 shows the biological effect on AON on lung epithelial cells. A549 cells transfected overnight with AON as described above were stimulated with IL-1 β (10 ng/mL) for 4 h to induce secretion of chemokines (IL-8, MCP-1) and cytokine (GM-SCF). Cell supernatants were harvested and levels of chemokines/cytokines determined using ELISA. Data expressed mean % inhibition of specific secretion which compares levels of secretion between PDE-AON targeted cells with mismatch/sense AON targeted cells.

Figure 3 shows the biological effect of AON on immune cells. Human PBMCs transfected with AON for 20h using lipidic reagent Escort IV (1ug AON:0.5uL

Lipid) were then stimulated with PHA (10 ug/mL) for 6 hrs. Cell supernatants were harvested and levels of cytokines TNF- α and IL-2 secreted by PBMCs in response to PHA were assessed using ELISA (BD Biosciences). Decrease of cytokine was compared to levels in PHA stimulated cells in the absence of AON delivery. Data expressed as the % inhibition of AON relative to the levels of specific cytokine inhibition observed with mismatch or sense control sequence AON (left hand axis). Cell lysates were harvested for cAMP determination using cAMP EIA (Cayman Chemical) as a measure of PDE activity. Increase in cAMP compared levels in PHA stimulated cells in the absence of AON delivery. Data expressed as % specific increase of cAMP relative to levels achieved with mismatch or sense control sequence AON (right hand axis). Data expressed as mean % change $\pm$ SE of between 9-15 donors.

Figure 4 shows the potency of 2' F-ANA-containing AON at inhibiting target gene expression. A549 cells transfected for 24 h with PDE4D-specific AON composed of PS-DNA or composed with PS-FANA modifications or corresponding mismatch sequences using Lipofectamine™ 2000 (Invitrogen, 1ug AON:1uL Lipofectamine™ 2000) at indicated doses (A). PDE4D mRNA expression level of target genes was quantified using the Quantigene® assay (Panomics) and was normalized to β 2M gene expression. Data expressed as specific % inhibition relative to expression level in cells treated with the sense control sequence. A549 cells transfected with for 24 h with PDE4D-specific AON composed of PS-DNA or composed with PS-FANA modifications or corresponding mismatch sequences using Lipofectamine™ 2000 (Invitrogen, 1ug AON:1uL Lipofectamine™ 2000) with a single dose for different time points (8, 24 or 48 h) (B). PDE4D mRNA expression level of target genes was quantified using the Quantigene® assay (Panomics) and was normalized to β 2M gene expression. Data expressed as specific % inhibition relative to expression level in cells treated with the sense control sequence. Legend below figure demonstrates FANA modifications in the TOP 1497 (PDE4-specific) AON.

Figure 5 shows the effect of 2'-F-ANA-containing AON on biological effects of lung epithelial cells. A549 cells transfected with PDE4B/4D-dual specific AON composed of PS-DNA (TOP 1545) or modified with FANA (PS-FANA, TOP 1545-F3) overnight and were then stimulated with IL-1 β (10 ng/mL) for 4 h. Supernatants were analyzed for IL-8 and MCP-1 secretion by ELISA (BD Biosciences) with levels normalized to cell viability determined by the alamarBlue™ assay. Data expressed as mean % inhibition relative to expression level in cells treated with corresponding sense control sequence. (**p<0.01, n=3).

Figure 6 shows the effect of 2'-F-ANA-containing AON in immune cells. (A) Human PBMCs transfected with FANA modified dual specific PDE4B/4D AON (TOP 1545) or FANA modified PDE7A specific AON (TOP 1731) or both for 20h and were then stimulated with PHA (10 ug/mL) for 6 hrs. mRNA expression of target genes were assessed using Quantigene® assay (Panomics) with all PDE gene expression levels normalized to β 2M gene expression. Data expressed as % specific inhibition of mRNA expression for PDE4B (n=8), PDE4D (n=6) and PDE7A (n=8) relative to levels in cells transfected with sense AON. P values determined for unpaired t-test comparing indicated columns. (B) Cell supernatants were harvested and levels of cytokines TNF- α and IL-2 secreted by PBMCs in response to PHA were assessed using ELISA (Medicorp). Decrease of cytokine was compared to levels in PHA stimulated cells in the absence of AON delivery. Data expressed as the % inhibition of AON relative to the levels of inhibition observed with same dose of mismatch or sense control oligonucleotide. Data expressed as mean % change $\pm$ SE of between 8 donors.

Figure 7 shows the inhibition of thymidylate synthase (TYMS) gene expression in 293 cells transfected with PDE4B/4D specific AON (TOP-1545-F3) or TYMS specific AON (TOP-1549) using Lipofectamine™ 2000 (Invitrogen, ratio 1ug oligonucleotide:1uL Lipofectamine™ 2000) and 200ng, 400ng and 800 ng of either TOP-1545-F3 or TOP-1549 AON. Real-time PCR

was used to quantify TYMS expression which was normalized to the expression of a control gene (Ppib). Data expressed as mean % inhibition relative to expression level of cells transfected with relevant sense or mismatch AON.

Figure 8 shows the efficacy of (A) TOP1572-F2 at reducing PDE4D and PDE4B mRNA expression and (B) TOP1731-F3 at reducing PDE7A mRNA expression in the CYNOM-K1 monkey cell line. Cells were transfected for 24h with 250nM of AON or corresponding control complexed to LipofectamineTM 2000 at ratio 1ug oligo: 2uL lipid in antibiotic free media. PDE4D, PDE4B and PDE7A mRNA expression levels were measured by RT-PCR with relative quantification to the expression of PPIB control gene. Percentage of PDE mRNA inhibition was determined relative to the gene level in non-transfected cells. Statistical analysis was performed using one way Anova followed by Dunnett comparison, **p<0.01.

Figure 9 shows the efficacy of TOP1572-F2 and TOP1545 at reducing (A) PDE4D mRNA expression and (B) MCP-1 protein secretion in normal human bronchial epithelial cells (NHBE). Cells were transfected for 24h with 250nM of AON or corresponding control complexed to LipofectamineTM 2000 at ratio 1ug oligo: 1uL lipid in antibiotic free media. Cells were stimulated for 4h with cytomix (500U/mL TNF- α + 10ng/mL IL1- β + 10ng/mL IFN γ) at the end of the transfection period. MCP-1 protein secretion was measured in supernatant by ELISA, with normalization to the cell viability as measured by AlamarBlue assay. PDE4D, mRNA expression level was measured by RT-PCR with relative quantification to the expression of B2M control gene. Percentage of inhibition was determined relative to cells treated with the corresponding control. Statistical analysis was performed using t test, *p<0.05, ***p<0.001.

Figure 10 shows the efficacy of the antisense oligonucleotide (AON) TOP 1572 at inhibiting cytokine secretion by PHA-stimulated human PBMCs. PBMCs were transfected with either TOP 1572 (6.2 nM) or a relevant control AON (TOP 1572s, 6.3 nM) overnight and then stimulated the next day with the mitogen PHA (10 ug/ml) for 6 h. Levels of cytokines, IL-2 (A) and TNF- α

(B) in cell supernatants were quantified using ELISA. Data represent mean values of triplicate wells $\pm$ SE of IL-2 (pg/ml) or TNF- α (ng/ml). Statistical analysis was performed using one way ANOVA followed by a Bonferroni Multiple comparison test comparing cytokine levels following AON transfection to PHA-stimulated cells, or to relevant control AON treated cells, **p<0.01 and ***p<0.001. % inhibition of cytokine secretion is relative to levels obtained in response to PHA.

Figure 11 shows the efficacy of the AON TOP 1731 at inhibiting cytokine secretion by mitogen-stimulated human PBMCs. PBMCs were transfected with either TOP1731 (3.1 nM) or relevant control AON (TOP 1731s, 3.1 nM) complexed with Escort IV overnight and then stimulated the following day with mitogen PHA (10 ug/ml) for 6 h. Levels of cytokines IL-2 (A) and TNF- α (B) in cell supernatants were quantified by ELISA. Data represents mean values of triplicate wells $\pm$ SE of IL-2 (pg/ml) or TNF- α (ng/ml). Statistical analysis was performed using one way ANOVA followed by a Bonferroni multiple comparison test comparing cytokine levels following AON-treatment to PHA-stimulated cells or to relevant control AON treated cells, *** p<0.001. %inhibition of cytokine secretion is relative to levels obtained in response to PHA.

Figure 12 shows the enhanced efficacy when F'ANA modified TOP 1572 and TOP 1731 are combined in reducing the cytokine secretion by PHA-stimulated PBMCs. Human PBMCs were transfected overnight with either TOP 1572-F2 (3.1 and 6.3 nM), TOP1731-F3 (3.1 and 6.3 nM) or both AON (3.1nM each, total 6.3 nM). The following day PBMCs were stimulated with PHA (10 ug/ml) for 6 h and then levels of cytokine IL-2 in cell supernatants were quantified by ELISA. % inhibition of IL-2 secretion was determined compared to levels produced by untransfected cells following PHA stimulation. Values represent mean $\pm$ S.E.

Figure 13 compares the efficacy of TOP1572 (PS-DNA) to the efficacy of TOP1572-F1, TOP1572-F2 and TOP1572-F3 (PS-FANA versions) at reducing (A) PDE4D and (B) PDE4B mRNA expression in the 293 cell line.

Cells were transfected for 24h, 48h or 72h with 250nM of AON or corresponding control complexed to Lipofectamine™ 2000 at ratio 1ug oligo: 1uL lipid in antibiotic free media. PDE4D and PDE4B mRNA expression levels were measured by RT-PCR with relative quantification to the expression of PPIB control gene. Percentage of specific PDE mRNA inhibition was determined relative to the gene level in cells transfected with the corresponding control.

Figure 14 compares the efficacy of TOP1731 (PS-DNA) to the efficacy of TOP1731-F3, (PS-FANA) at reducing PDE7A mRNA expression in the A549 cell line. Cells were transfected for 48h with 250nM, 125nM or 75nM of AON or corresponding control oligo complexed to Lipofectamine™ 2000 at ratio 0.8ug oligo: 1uL lipid in antibiotic free media. PDE7A mRNA expression level was measured by Quantigene® assay with relative quantification to the expression of B2M control gene. Percentage of specific PDE mRNA inhibition was determined relative to the gene level in cells transfected with the corresponding control.

BRIEF DESCRIPTION OF THE TABLES

Table 1a identifies human PDE7A antisense oligonucleotides in accordance with the present invention.

Table 1b identifies human PDE3A antisense oligonucleotides in accordance with the present invention.

Table 1c identifies antisense oligonucleotides with dual specificity against more than one PDE4 isoform (either PDE4A/4D or PDE4B/4D) in accordance with the present invention. Base pair mismatches between PDE4 isoforms in AON sequences are indicated in underline. Replacement of base pairs with inosine in AON indicated by letter I.

Table 1d identifies antisense oligonucleotides with specificity against PDE 3B, PDE4D and PDE7A and dual specificity against PDE4A and 4B.

Table 2a identifies human PDE7A antisense oligonucleotides containing FANA bases in accordance with the present invention. FANA modified bases indicated by bold lettering.

Table 2b identifies human PDE3A antisense oligonucleotides containing FANA bases in accordance with the present invention. FANA modified bases indicated by bold lettering.

Table 2c identifies human PDE3B antisense oligonucleotides containing FANA bases in accordance with the present invention. FANA modified bases indicated by bold lettering.

Table 2d identifies human PDE4A antisense oligonucleotides containing FANA bases in accordance with the present invention. FANA modified bases indicated by bold lettering.

Table 2e identifies human PDE4D antisense oligonucleotides containing FANA bases in accordance with the present invention. FANA modified bases indicated by bold lettering.

Table 2f identifies human PDE4B antisense oligonucleotides containing FANA bases in accordance with the present invention. FANA modified bases indicated by bold lettering.

Table 2g identifies human antisense oligonucleotides with dual specificity against more than one PDE4 isoform and containing FANA bases in accordance with the present invention. FANA modified bases indicated by bold lettering.

Table 3 shows human oligonucleotide primers used in real-time PCR.

DETAILED DESCRIPTION

The invention herein relates to antisense oligonucleotide-based compounds, therapeutic compositions and methods for the treatment of disease associated with reduced cellular cAMP and/or disease associated with elevated levels of at least one PDE. The invention is aimed at increasing the level of cAMP in cells, while decreasing the level of pro-inflammatory mediators as well as enzymes that are released by inflammatory cells.

To obtain these effects, the present invention utilizes, in one of its aspects, what is herein referred to as "multiple gene knock down". Multiple gene knock down refers to the inhibition or downregulation of multiple genes by either a single antisense oligonucleotide compound in accordance with the present invention which downregulates not only the isoenzyme gene that it is directed against but also a related gene(s), or by a combination of at least two antisense oligonucleotide compounds which each downregulate a different isoenzyme gene.

Diseases associated with reduced levels of cellular cAMP include diseases in which there is increased levels of at least one cAMP-specific PDE (i.e. a PDE-related disease).

The term "downregulate" is used herein to refer to at least partial inhibition of the expression of a gene. In accordance with the invention, an antisense oligonucleotide compound downregulates or inhibits a gene that it is directed against, i.e. a gene to which the antisense oligonucleotide compound exhibits sequence complementarity sufficient to cause inhibition.

The term "related genes" refers to other genes that may also play a role in the pathophysiology of disease associated with reduced cellular cAMP and/or

increased levels of at least one PDE but to which the oligonucleotide antisense compound is not complementary, either completely or partially. Such genes include, but are not limited to, genes that encode PDE enzymes or isotypes, mediators, for example, cytokines and chemokines and genes that encode enzymes. Examples of mediators include IL-2, IL-6, IL-7, IL-8, IL-15 and TNF-alpha. Examples of appropriate enzymes include, but are not limited to, matrix metalloproteinases (MMPs), such as MMP-1, MMP-2, MMP-3, MMP-9 and MMP-12.

In accordance with the present invention, antisense oligonucleotide (AON) compounds are herein defined as oligonucleotides, naturally occurring or modified, preferably nuclease resistant, that exhibit a complementarity to DNA or mRNA coding for a particular target protein such that they are capable of interfering with the transcription or translation of the mRNA and/or induce RNase or RNase-like activity and thereby function to reduce expression of the target protein. Expression of the target protein is reduced when the oligonucleotide compound hybridizes to the target DNA or mRNA, thereby interfering/preventing its transcription/translation. The present oligonucleotide compounds, thus, must be sufficiently complementary to the nucleic acid to which it is directed in order to hybridize thereto. Thus, although in a most preferred embodiment, the oligonucleotide compounds are 100% complementary to the nucleic to which they are directed, 100% complementarity is not necessary for hybridization to occur between an oligonucleotide compound and the nucleic acid to which it is directed, as one of skill will appreciate. As such, in some embodiments the oligonucleotide compounds may be about 95%, 90%, 85%, 80%, 75%, 70%, 60% and 50% complementary to the nucleic to which they are directed.

The terms "nucleic acid" and "nucleic acid molecule" as used interchangeably herein, refer to a molecule comprised of nucleotides, i.e., ribonucleotides, deoxyribonucleotides, or both. The term includes monomers and polymers of ribonucleotides and deoxyribonucleotides, with the ribonucleotide and/or

deoxyribonucleotides being connected together, in the case of the polymers, via 5' to 3' linkages. However, linkages may include any of the linkages known in the nucleic acid synthesis art including, for example, nucleic acids comprising 5' to 2' linkages. The nucleotides used in the nucleic acid molecule may be naturally occurring or may be synthetically produced analogues that are capable of forming base-pair relationships with naturally occurring base pairs. Examples of non-naturally occurring bases that are capable of forming base-pairing relationships include, but are not limited to, aza and deaza pyrimidine analogues, aza and deaza purine analogues, and other heterocyclic base analogues, wherein one or more of the carbon and nitrogen atoms of the purine and pyrimidine rings have been substituted by heteroatoms, e.g., oxygen, sulfur, selenium, phosphorus, and the like.

The invention herein also relates to modifications to an antisense oligonucleotide(s) that do not significantly adversely effect their activity to reduce or inhibit expression of a target protein, but which may enhance this activity.

The present antisense oligonucleotide compounds may also be modified by insertion or deletion of 1 or more bases without significant adverse effect to their activity. In particular, the addition or deletion of bases at the terminal ends of the oligonucleotides that exhibit 100% complementation to the gene they are directed against can generally be made without significant loss of inhibitory activity. Such modifications may be made in order to increase activity or to provide enhanced stability of the oligonucleotide. In addition, substitution of 1 or more bases in the present antisense oligonucleotide compounds may also be made without adverse effect to activity, for example, substitution of purine with another purine (adenine, guanine) and pyrimidine with pyrimidine (cytosine, thymine, uracil).

Antisense oligonucleotide compounds in accordance with the present invention also include siRNAs (small interfering RNAs) and the RISCs (RNA-

induced silencing complexes) containing them that result from the RNAi (RNA interference) approach. The RNA interference (RNAi) approach, which has been described recently, is considered as a new tool for the inhibition of target gene expression. As already known some years ago, RNAi is based on an ancient anti-viral defence mechanism in lower eukaryotes. It is induced by double-stranded RNA and its processing to 21-23 nt small interfering RNAs (siRNAs), which cause the degradation of homologous endogenous mRNA after hybridizing to the target mRNA in a single stranded fashion with the assistance of the RISC complex. The way RNAi works is still to be fully elucidated, but it already serves as a first-choice approach to generate loss-of-function phenotypes among a broad variety of eukaryotic species, such as nematodes, flies, plants, fungi and mammals.

Antisense oligonucleotide compounds in accordance with the present invention also include ribozymes and short nucleotide sequences, single or double stranded, RNA or DNA, which may incorporate chemical modifications as described above, capable of inhibiting gene transcription and/or translation in vitro and/or in vivo.

The compositions and methods of the present invention, in one aspect, while not to be bound by a particular mode of action, appear to act by a mechanism that has been termed "multiple gene knock down". Multiple gene knock down in the context of the present invention refers to:

- 1) an antisense oligonucleotide compound that downregulates not only the gene that it is directed against but also downregulates other related genes; and
- 2) a combination of at least 2 antisense oligonucleotide compounds each capable of downregulating a gene, each being present at a concentration at which the antisense oligonucleotide compound is practically ineffective on its own to downregulate a gene it is directed against, the combination leading to

significant downregulation of both genes that the antisense oligonucleotides are directed against and may also downregulate other related genes. This effect was initially disclosed in applicant's PCT Published Application No: WO05/030787.

In one aspect, the present invention provides a composition comprising at least 2 antisense oligonucleotide compounds, each antisense oligonucleotide compound being capable of downregulating a different gene. In some embodiments of this aspect, the combination of the at least 2 antisense oligonucleotide compounds leads to a significant downregulation of each the genes that is more than the additive ability of each antisense to downregulate each gene individually.

In one aspect, the present invention provides a pharmaceutical composition for treating and/or preventing disease associated with reduced cellular cAMP and/or elevated levels of at least one PDE, said composition comprising a pharmaceutically acceptable carrier and at least two antisense oligonucleotide compounds, each oligonucleotide compound being directed against at least a portion of a different target PDE-encoding gene, each oligonucleotide compound being capable of downregulating the target gene it is directed against, each oligonucleotide compound being present at a concentration at which it exhibits less than 20% inhibition of its target PDE gene, the combination exhibiting greater than 20% inhibition and at least doubling the inhibition of at least one of the target genes and optionally other related genes.

As one of skill in the art will appreciate, the combination of oligonucleotide compounds in accordance with the present invention at very low concentrations, for example, concentrations with exhibit less than 1% inhibition of a target PDE gene, may not combine to exhibit an inhibition of at least 20%. The lowest concentration at which each oligonucleotide compound can be used to form a combination in accordance with the present

invention will vary, and this concentration may be that at which 0.5% inhibition is achieved, or that concentration at which 1% to 5% inhibition is achieved.

The present invention provides a pharmaceutical composition for treating and/or preventing a disease associated with reduced cellular cAMP and/or elevated levels of at least one PDE, said composition comprising a pharmaceutically acceptable carrier and at least two antisense oligonucleotides that are each capable of downregulating a different target gene, at least one of the oligonucleotides being present at a concentration at which it is practically ineffective on its own, e.g. a concentration exhibiting less than 20% inhibition of the target gene, the combination of the oligonucleotides exhibiting more than 20% inhibition and at least doubling the inhibition of at least one of the target genes. and optionally other related genes.

The present invention further provides the use of a combination of at least two antisense oligonucleotide compounds for treating and/or preventing disease associated with reduced cellular cAMP, such as respiratory inflammatory disease, each oligonucleotide compound being directed against a gene coding for a different PDE isotype, each oligonucleotide compound being capable of downregulating the gene it is directed against, the oligonucleotide compounds being present at a concentration at which each oligonucleotide is practically ineffective on its own to downregulate the gene it is directed against, the combination of the at least two oligonucleotide compounds being effective to downregulate at least one of the genes the oligonucleotide compounds are directed against and optionally other related genes.

The present invention further provides a method of treating and/or preventing disease associated with reduced cellular cAMP, such as inflammatory respiratory disease, the method comprising administering to a subject at least two antisense oligonucleotide compounds each being capable of downregulating a different target PDE gene, the oligonucleotide compounds being administered at a concentration at which each oligonucleotide compound is practically ineffective on its own to downregulate its target gene,

for example, a concentration exhibiting less than 20% inhibition of the target gene, the combination of the oligonucleotide compounds being effective to downregulate at least one of the genes the oligonucleotide compounds are directed against and optionally other related genes.

The present invention further provides the use of a pharmaceutical composition as described above for the manufacture of a medicament for the treatment and/or prevention of a disease associated with reduced cellular cAMP or increased cAMP-specific PDE levels (PDE-related disease) such as inflammatory disease and cancer. Inflammatory disease to which the present methods and compositions are directed is generally defined as diseases in which inflammatory cells and/or mediators are present. Examples of inflammatory diseases include, but are not limited to, multiple sclerosis, contact dermatitis, allergic and non-allergic eye diseases, rheumatoid arthritis, septic shock, osteoporosis and cognitive disorders. Inflammatory respiratory disease to which the present methods and compositions are directed is generally defined as diseases of the respiratory tract and lungs in which inflammatory cells and mediators are present. Examples of inflammatory respiratory disease include, but are not limited to, COPD, asthma, eosinophilic cough, bronchitis, acute and chronic rejection of lung allograft, sarcoidosis, pulmonary fibrosis, rhinitis and sinusitis.

The present invention further provides the use of a pharmaceutical composition as described above for the manufacture of a medicament for the treatment and/or prevention of a disease where decreased cyclic AMP is involved in the physiopathology.

The present invention further provides methods for modifying antisense oligonucleotides so that it may reach the target nucleotide and/or be more effective against the target gene for the treatment and/or prevention of inflammatory respiratory diseases.

The present invention further provides a formulation, including the composition described above, the formulation being systemic and/or topical.

The present invention further provides an *in vivo* method of delivering a pharmaceutical composition to a target polynucleotide, comprising administering to a subject the composition as described above in combination with a surfactant that permits the composition to reach the target gene(s).

Preferably, the subjects or patients that may be treated using the antisense oligonucleotide compounds of the present invention include, but are not limited to, invertebrates, vertebrates, birds, mammals such as pigs, goats, sheep, cows, dogs, cats, and particularly humans. Oligonucleotide compounds in accordance with the present invention are designed to be appropriate to the particular animal to be treated. In particular, the sequence of the antisense oligonucleotide compounds will vary with the subject being treated given inter-species genome differences.

The present invention further provides antisense oligonucleotides effective against, e.g. capable of inhibiting the expression of, the PDE family of enzymes, including, but not limited to, PDE3, PDE4 and PDE7. As will be appreciated by one of skill in the art, each PDE subtype may also includes isotypes. For example, PDE3 subtype include the PDE3A and PDE3B isotypes; the PDE4 subtype includes isotypes PDE4A, PDE4B, PDE4C and PDE4D; and the PDE7 subtype includes isotypes PDE7A1, PDE7A2, PDE7A3 and PDE7B.

When used herein the term "concentration at which the antisense oligonucleotide compound is practically ineffective" refers to the use of each oligonucleotide compound, when taken alone, at a concentration at which practically no downregulation is observed for the gene against which the oligonucleotide compound is directed (at a concentration at which less than 20% inhibition of the target gene is exhibited). This is in contrast to the effect

of the combination of at least two oligonucleotide compounds in accordance with the present invention, each present at a concentration at which they are ineffective alone to downregulate the gene they are directed against (at a concentration at which less than 20% inhibition is exhibited), this combination exhibiting greater than 20% inhibition and being effective to at least double the inhibition of at least one of the genes against which the oligonucleotide compounds are directed.

The term "oligonucleotide" as used herein refers to a nucleic acid molecule comprising from about 1 to about 100 nucleotides, more preferably from 1 to 80 nucleotides, and even more preferably from about 4 to about 35 nucleotides. The term "oligonucleotide" also includes modified oligonucleotides and oligonucleotide compounds as set out above.

The terms "modified oligonucleotide" and "modified nucleic acid molecule" as used herein refer to nucleic acids, including oligonucleotides, with one or more chemical modifications at the molecular level of the natural molecular structures of all or any of the nucleic acid bases, sugar moieties, internucleoside phosphate linkages, as well as molecules having added substituents, such as diamines, cholesteryl or other lipophilic groups, or a combination of modifications at these sites. The internucleoside phosphate linkages can be phosphodiester, phosphotriester, phosphoramidate, siloxane, carbonate, carboxymethylester, acetamidate, carbamate, thioether, bridged phosphoranidate, bridged methylene phosphonate, phosphorothioate, methylphosphonate, phosphorodithioate, bridged phosphorothioate and/or sulfone internucleotide linkages, or 3'-3', 2'-5' or 5'-5' linkages, and combinations of such similar linkages (to produce mixed backbone modified oligonucleotides). The modifications can be internal (single or repeated) or at the end(s) of the oligonucleotide molecule and can include additions to the molecule of the internucleoside phosphate linkages, such as cholesteryl, diamine compounds with varying numbers of carbon residues between amino groups and terminal ribose, deoxyribose and phosphate modifications which

cleave or cross-link to the opposite chains or to associated enzymes or other proteins. Electrophilic groups such as ribose-dialdehyde may be covalently linked with an epsilon amino group of the lysyl-residue of such a protein. A nucleophilic group such as n-ethylmaleimide tethered to an oligomer could covalently attach to the 5' end of an mRNA or to another electrophilic site. The term modified oligonucleotides also includes oligonucleotides comprising modifications to the sugar moieties such as 2'-substituted ribonucleotides, or deoxyribonucleotide monomers, any of which are connected together via 5' to 3' linkages. Modified oligonucleotides may also be comprised of PNA or morpholino modified backbones where target specificity of the sequence is maintained.

Optionally, the presently described oligonucleotides may be formulated with a variety of physiological carrier molecules. The presently described oligonucleotides may also be complexed with molecules that enhance their ability to enter the target cells. Examples of such molecules include, but are not limited to, carbohydrates, polyamines, amino acids, peptides, lipids, and molecules vital to cell growth. For example, the oligonucleotides may be combined with a lipid, the resulting oligonucleotide/lipid emulsion, or liposomal suspension may, inter alia, effectively increase the in vivo half-life of the oligonucleotide.

Alternatively, oligonucleotides directed at PDE targets may also be protonated/acidified to function in a dual role as phosphodiesterase inhibitors and antibacterial agents. Accordingly, another embodiment of the presently described invention is the use of a PDE modulating therapeutic oligonucleotide that is additionally protonated/acidified to increase cellular uptake, improve encapsulation in liposomes, so it can also serve as an antibiotic. Additionally, the oligonucleotide may be complexed with a variety of well established compounds or structures that, for instance, further enhance the in vivo stability of the oligonucleotide, or otherwise enhance its

pharmacological properties (e.g., increase *in vivo* half-life, reduce toxicity, etc.).

The term "nucleic acid backbone" as used herein refers to the structure of the chemical moiety linking nucleotides in a molecule. This may include structures formed from any and all means of chemically linking nucleotides. A modified backbone as used herein includes modifications to the chemical linkage between nucleotides, as well as other modifications that may be used to enhance stability and affinity, such as modifications to the sugar structure. For example an [alpha]-anomer of deoxyribose may be used, where the base is inverted with respect to the natural [beta]-anomer. In a preferred embodiment, the 2'-OH of the sugar group may be altered to 2'-O-alkyl or 2'-O-alkyl-n(O-alkyl), which provides resistance to degradation without comprising affinity.

The term "acidification" and "protonation/acidification" as used interchangeably herein refers to the process by which protons (or positive hydrogen ions) are added to proton acceptor sites on a nucleic acid. The proton acceptor sites include the amine groups on the base structures of the nucleic acid and the phosphate of the phosphodiester linkages. As the pH is decreased, the number of these acceptor sites which are protonated increases, resulting in a more highly protonated/acidified nucleic acid.

The term "protonated/acidified nucleic acid" refers to a nucleic acid that, when dissolved in water at a concentration of approximately 16 A260 per ml, has a pH lower than physiological pH, i.e., lower than approximately pH 7. Modified nucleic acids, nuclease-resistant nucleic acids, and antisense nucleic acids are meant to be encompassed by this definition. Generally, nucleic acids are protonated/acidified by adding protons to the reactive sites on a nucleic acid, although other modifications that will decrease the pH of the nucleic acid can also be used and are intended to be encompassed by this term.

The term "end-blocked" as used herein refers to a nucleic acid with a chemical modification at the molecular level that prevents the degradation of selected nucleotides, e.g., by nuclease action. This chemical modification is positioned such that it protects the integral portion of the nucleic acid, for example the coding region of an antisense oligonucleotide. An end block may be a 3' end block or a 5' end block. For example, a 3' end block may be at the 3'-most position of the molecule, or it may be internal to the 3' ends, provided it is 3' of the integral sequences of the nucleic acid.

The term "substantially nuclease resistant" refers to nucleic acids that are resistant to nuclease degradation, as compared to naturally occurring or unmodified nucleic acids. Modified nucleic acids of the invention are at least 1.25 times more resistant to nuclease degradation than their unmodified counterpart, more preferably at least 2 times more resistant, even more preferably at least 5 times more resistant, and most preferably at least 10 times more resistant than their unmodified counterpart. Such substantially nuclease resistant nucleic acids include, but are not limited to, nucleic acids with modified backbones such as phosphorothioates, methylphosphonates, ethylphosphotriesters, 2'-O-methylphosphorothioates, 2'-O-methyl-p-ethoxy ribonucleotides, 2'-O-alkyls, 2'-O-alkyl-n(O-alkyl), 3'-O-alkyls, 3'-O-alkyl-n(O-alkyl), 2'-fluoros, 2'-deoxy-erythropentofuranosyls, 2'-O-methyl ribonucleosides, methyl carbamates, methyl carbonates, inverted bases (e.g., inverted T's), or chimeric versions of these backbones.

The term "substantially acid resistant" as used herein refers to nucleic acids that are resistant to acid degradation as compared to unmodified nucleic acids. Typically, the relative acid resistance of a nucleic acid will be measured by comparing the percent degradation of a resistant nucleic acid with the percent degradation of its unmodified counterpart (i.e., a corresponding nucleic acid with "normal" backbone, bases, and phosphodiester linkages). A nucleic acid that is acid resistant is preferably at least 1.5 times more resistant to acid degradation, at least 2 times more resistant, even more preferably at

least 5 times more resistant, and most preferably at least 10 times more resistant than their unmodified counterpart.

The terms "PDE3A, PDE3B, PDE4A, PDE4B, PDE4C, PDE4D, PDE7A, PDE7B oligonucleotide" as used herein each refer to an oligonucleotide that is targeted, respectively, to sequences that affect PDE3A, PDE3B, PDE4A, PDE4B, PDE4C, PDE4D, PDE7A, PDE7B expression or activity. These include, but are not limited to, PDE3A, PDE3B, PDE4A, PDE4B, PDE4C, PDE4D, PDE7A, PDE7B DNA coding sequences, PDE3A, PDE3B, PDE4A, PDE4B, PDE4C, PDE4D, PDE7A, PDE7B DNA promoter sequences, PDE3A, PDE3B, PDE4A, PDE4B, PDE4C, PDE4D, PDE7A, PDE7B DNA enhancer sequences, mRNA encoding PDE3A, PDE3B, PDE4A, PDE4B, PDE4C, PDE4D, PDE7A, PDE7B, and the like.

As discussed above, one embodiment of the present invention provides antisense oligonucleotides targeted to sequences that affect PDE3A, PDE3B, PDE4A, PDE4B, PDE4D, PDE7A, expression or activity. In one embodiment the antisense oligonucleotides may have one of the sequences identified in Tables 1a-d or Tables 2a-g. In another embodiment, the antisense oligonucleotide may comprise fragments or variants of these sequences, as will be understood by a person skilled in the art, that may alter the oligonucleotide make-up and/or length, but which maintains or increases the activity of the oligonucleotide to downregulate gene expression. In another embodiment the present invention provides for combinations of at least two antisense oligonucleotides having the sequences identified in Tables 1a-d or Tables 2a-g.

The comparison of effectiveness of a single oligonucleotide is illustrated in, but not limited to, Figure 1a, Figure 1b and Figure 5. For example in Figure 1a TOP 1512 (Seq ID No 91) is directed against human PDE4A and PDE4B inhibits both of these targets *in vitro*. Similarly Figure 1b shows TOP 1545 (Seq ID No 14) and TOP 1556 (Seq ID No 24) which recognize sequences

shared by PDE4B and PDE4D, demonstrate inhibition of both target sequences. TOP 1507 (Seq ID No 10) and TOP 1508 (Seq ID No 11) which recognize sequences shared by PDE4A and PDE4D function to inhibit both target genes.

The comparison of effectiveness of combinations of at least 2 oligonucleotide compounds is illustrated in, but not limited to, Figure 6a and 12. For example in Figure 6 the combination of TOP 1545-F3 (Seq ID No. 85) and TOP-1731-F2 (Seq. ID No. 53) show a greater effect at decreasing expression of both PDE4B, PDE4D and PDE7A than either of the oligonucleotides had on their respective targets when used alone. In Figure 6b the combination of TOP-1545-F3 (seq. ID No. 85) and TOP-1731-F2 (Seq. ID No. 53) show a greater effect at decreasing IL-2-release in human PBMC than either of the oligonucleotides had on IL-2 when used alone at a the same total dose (6.3 nM). Referring to Figure 12, the combination of TOP 1572-F2 (Seq ID No.122) and TOP 17314-F3 (Seq. ID No. 54) show a greater effect of inhibiting IL-2 secretion than when used alone.

Many combinations of antisense oligonucleotide compounds may be used in accordance with the present invention, the combinations leading to multiple gene knock down as described above. Examples of combinations of antisense oligonucleotides may include, but are not limited to, at least one oligonucleotide compound directed against PDE3 with at least one oligonucleotide compound directed against PDE7. An alternative example of a combination may include at least one oligonucleotide directed against PDE4 and at least one oligonucleotide directed against PDE7. An alternative example of a combination may include at least one oligonucleotide directed against PDE3, at least one oligonucleotide directed against PDE4 and at least one oligonucleotide directed against PDE7. Such antisense oligonucleotide compounds may be chosen from, but are not limited to, the oligonucleotides provided in Tables 1a-d and Tables 2a-2g.

The terms "treatment", "treating", "therapy" and the like are used herein to generally mean obtaining a desired pharmacologic and/or physiologic effect. The effect may be prophylactic in terms of completely or partially preventing a disease or symptom thereof and/or may be therapeutic in terms of a partial or complete cure for a disease and/or amelioration of an adverse effect attributable to the disease. "Treatment" as used herein covers any treatment of a disease in a subject as previously defined, particularly a human, and includes:

- (a) preventing a disease from occurring in a subject which may be predisposed to the disease but has not yet been diagnosed as having it;
- (b) inhibiting a disease, i.e., arresting its development; or
- (c) relieving a disease, i.e., causing regression of the disease.

The term "pharmaceutically acceptable" as it is used herein with respect to carriers, surfactants and compositions refers to substances which are acceptable for use in the treatment of a subject patient that are not toxic or otherwise unacceptable for administration by any of the routes herein described.

The invention is generally directed toward the treatment of subjects by the administration of therapeutically effective amounts of antisense oligonucleotide compounds in accordance with the present invention, including siRNA, ribozymes, short nucleotide sequences as single or double stranded including RNA and/or DNA that may be complementary to a target nucleic acid, or may optionally be modified as described above, an RNA oligonucleotide having at least a portion of said RNA oligonucleotide capable of hybridizing with RNA to form an oligonucleotide-RNA duplex, or a chimeric oligonucleotide, that will downregulate or inhibit the expression of an endogenous gene *in vivo*.

By "therapeutically effective" amount is meant a nontoxic but sufficient amount of an antisense oligonucleotide compound to provide the desired therapeutic effect. In the present case, that dose of antisense oligonucleotide compound effective to relieve, ameliorate, or prevent symptoms of the condition or disease being treated, e.g. disease associated with reduced cellular cAMP, PDE-related disease, inflammatory disease such as inflammatory respiratory disease.

The pharmaceutical compositions provided herein comprise antisense oligonucleotide compounds described above and one or more pharmaceutically acceptable surfactants. Suitable surfactants or surfactant components for enhancing the uptake of the antisense oligonucleotides of the invention have been previously described in U.S. Application Publication No. 2003/0087845, the contents of which are incorporated with respect to surfactants. The application states that suitable surfactants "...include synthetic and natural as well as full and truncated forms of surfactant protein A, surfactant protein B, surfactant protein C, surfactant protein D and surfactant protein E, di-saturated phosphatidylcholine (other than dipalmitoyl), dipalmitoylphosphatidylcholine, phosphatidylcholine, phosphatidylglycerol, phosphatidylinositol, phosphatidylethanolamine, phosphatidylserine; phosphatidic acid, ubiquinones, lysophosphatidylethanolamine, lysophosphatidylcholine, palmitoyl-lysophosphatidylcholine, dehydroepiandrosterone, dolichols, sulfatidic acid, glycerol-3-phosphate, dihydroxyacetone phosphate, glycerol, glycerol-3-phosphocholine, dihydroxyacetone, palmitate, cytidine diphosphate (CDP) diacylglycerol, CDP choline, choline, choline phosphate; as well as natural and artificial lamellar bodies which are the natural carrier vehicles for the components of surfactant, omega-3 fatty acids, polyenic acid, polyenoic acid, lecithin, palmitinic acid, non-ionic block copolymers of ethylene or propylene oxides, polyoxypropylene, monomeric and polymeric, polyoxyethylene, monomeric and polymeric, poly (vinyl amine) with dextran and/or alkanoyl side chains, Brij 35, Triton X-100 and synthetic surfactants ALEC, Exosurf, Survan and

Atovaquone, among others. These surfactants may be used either as single or part of a multiple component surfactant in a formulation, or as covalently bound additions to the 5' and/or 3' ends of the antisense oligonucleotides (oligos)."

The antisense component of the present compositions, which includes the combination of at least two antisense oligonucleotide compounds, may be contained in a pharmaceutical formulation within a lipid particle or vesicle, such as a liposome or microcrystal. As described in U.S. Patent No. 6,025,339, the lipid particles may be of any suitable structure, such as unilamellar or plurilamellar, so long as the antisense oligonucleotide is contained therein. Positively charged lipids such as N-[1-(2, 3-dioleoyloxy) propyl]-N, N, N-trimethyl-ammoniummethylsulfate, or "DOTAP," are particularly preferred for such particles and vesicles. The preparation of such lipid particles is well known. See, e.g., U.S. Pat. Nos. 4,880,635 to Janoff *et al.*; 4,906,477 to Kurono *et al.*; 4,911,928 to Wallach; 4,917,951 to Wallach; 4,920,016 to Allen *et al.*; 4,921,757 to Wheatley *et al.*; etc.

The composition of the invention may be administered by any means that transports the antisense oligonucleotide compound to the desired site, such as the lung. The antisense compounds disclosed herein may be administered to the lungs of a patient by any suitable means, but are preferably administered by inhalation of an aerosol comprised of respirable particles that comprise the antisense compound.

The composition of the present invention may be administered into the respiratory system as a formulation including particles of respirable size, e.g. particles of a size sufficiently small to pass through the nose, mouth and larynx upon inhalation and through the bronchi and alveoli of the lungs. In general, respirable particles range from about 0.5 to 10 microns in size. Particles of non-respirable size that are included in the aerosol tend to deposit in the throat and be swallowed, and the quantity of non-respirable particles in

the aerosol is preferably thus minimized. For nasal administration, a particle size in the range of 10-500 μM (micro-meter)) is preferred to ensure retention in the nasal cavity:

Liquid pharmaceutical compositions of active compound (the antisense oligonucleotide compound(s)) for producing an aerosol may be prepared by combining the antisense compound with a suitable vehicle, such as sterile pyrogen free water or phosphate buffered saline.

A solid particulate composition comprising the antisense compound may optionally contain a dispersant that serves to facilitate the formation of an aerosol as well as other therapeutic compounds. A suitable dispersant is lactose, which may be blended with the antisense compound in any suitable ratio, e.g., a 1 to 1 ratio by weight.

The antisense compositions may be administered in an anti-bronchoconstriction, anti-allergy(ies) and/or anti-inflammatory effective amount, which amount depends upon the degree of disease being treated, the condition of the subject patient, the particular formulation, the route of administration, the timing of administration to a subject, etc. In general, intracellular concentrations of the oligonucleotide of from 0.05 to 50 μM , or more particularly 0.2 to 5 μM , are desirable. For administration to a mammalian patient such as a human, a dosage of about 0.001, 0.01, 0.1, or 1 mg/Kg up to about 50, or 100 mg/Kg or more is typically employed. However, other doses are also contemplated. Depending on the solubility of the active compound in any particular formulation, the daily dose may be divided among one or several unit dose administrations.

The aerosols of liquid particles comprising the antisense compound may be produced by any suitable means, such as with a nebulizer. Nebulizers are commercially available devices that transform solutions or suspensions of the active ingredient into a therapeutic aerosol mist either by means of

acceleration of a compressed gas, typically air or oxygen, through a narrow venturi orifice or by means of ultrasonic agitation. Suitable formulations for use in nebulizers comprise the active antisense oligonucleotide ingredient in a liquid carrier in an amount of up to 40% w/w preferably less than 20% w/w of the formulation. The carrier is typically water or a dilute aqueous alcoholic solution, preferably made isotonic with body fluids by the addition of, for example, sodium chloride. Optional additives include preservatives if the formulation is not prepared sterile, for example, methyl hydroxybenzoate, anti-oxidants, anti-bacterials, flavorings, volatile oils, buffering agents and emulsifiers and other formulation surfactants.

The aerosols of solid particles comprising the active oligonucleotide compound(s) and a pharmaceutically acceptable surfactant may likewise be produced with any solid particulate medicament aerosol generator. Aerosol generators for administering solid particulate medicaments to a subject produce particles that are respirable, as explained above, and generate a volume of aerosol containing a predetermined metered dose of a medicament at a rate suitable for human administration. The active oligonucleotide ingredient typically comprises from 0.1 to 100 w/w of the formulation. A second type of illustrative aerosol generator comprises a metered dose inhaler. Metered dose inhalers are pressurized aerosol dispensers, typically containing a suspension or solution formulation of the active ingredient in a liquified propellant. During use these devices discharge the formulation through a valve adapted to deliver a metered volume, typically from 10 to 150 μ l, to produce a fine particle spray containing the active ingredient. Suitable propellants include certain chlorofluorocarbon compounds, for example, dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane or hydrofluoroalkanes and mixtures thereof. The formulation may additionally contain one or more co-solvents, for example, ethanol, emulsifiers and other formulation surfactants, such as oleic acid or sorbitan trioleate, anti-oxidants and suitable flavoring agents.

The aerosol, whether formed from solid or liquid particles, may be produced by the aerosol generator at a rate of from about 1 to 150 liters per minute.

The formulations of the present invention may be oral, intrabuccal, intrapulmonary, rectal, intrauterine, intratumor, intracranial, nasal, intramuscular, subcutaneous, intravascular, intrathecal, inhalable, transdermal, intradermal, intracavitary, implantable, iontophoretic, ocular, vaginal, intraarticular, otical, intravenous, intramuscular, intraglandular, intraorgan, intralymphatic, implantable, slow release or enteric coating formulations. The carriers used in the formulations may be, for example, solid and/or liquid carriers.

Reference may be made to "Remington's Pharmaceutical Sciences", 17th Ed., Mack Publishing Company, Easton, Pa., 1985, for other carriers that would be suitable for combination with the present oligonucleotide compounds to render compositions/formulations suitable for administration to treat respiratory disease.

In a further aspect of the present invention, an article of manufacture is provided which includes packaging material contained within which is a pharmaceutically acceptable antisense oligonucleotide composition that is therapeutically effective to treat conditions associated with reduced cellular cAMP, or elevated levels of PDE, including inflammatory disease. In one embodiment, the composition comprises an antisense oligonucleotide compound that is effective to inhibit a PDE gene, said oligonucleotide compound being at least 50% complementary to the gene. In another aspect, the composition comprises at least 2 antisense oligonucleotide compounds, each antisense oligonucleotide compound being capable of downregulating a different PDE gene, each antisense oligonucleotide compound being present at a concentration at which the antisense oligonucleotide compound is practically ineffective on its own to downregulate the gene it is directed against, the combination of the antisense oligonucleotide compounds being

effective to downregulate at least one of the genes that the antisense oligonucleotides are directed against.

In one embodiment, the packaging material of the article comprises a label which indicates that the composition can be used to treat inflammatory disease and may additionally include an indication that the disease is one of multiple sclerosis, contact dermatitis, allergic and non-allergic eye diseases, rheumatoid arthritis, septic shock, osteoporosis and cognitive disorders.

In another embodiment, the packaging material of the article comprises a label which indicates that the composition can be used to treat inflammatory respiratory disease, and may additionally include an indication that the disease is one of COPD, asthma, eosinophilic cough, bronchitis, acute and chronic rejection of lung allograft, sarcoidosis, pulmonary fibrosis, rhinitis or sinusitis.

For the purposes of the present invention, the packaging material may be any suitable material for packaging a nucleotide-containing composition in accordance with the present invention, including a bottle or other container (either plastic or glass), a carton, a tube, or other protective wrapping. As will be appreciated, the packaging may vary with the nature of the oligonucleotide composition, for example, a liquid formulation may be packaged differently than an aerosol formulation.

The present invention is illustrated in further detail by the following non-limiting examples.

EXAMPLES

Materials and Reagents

AIM-V media (Invitrogen, Cat#31035-025); Opti MEM I (Invitrogen, cat#31985-07, lot#1244206); FBS (Fetal Bovine Serum, Wisent, cat# 80150);

Penicillin, Streptomycin (GIBCO, cat# 15140-122); HEPES (Wisent, cat# 26060CI) and L-glutamine (Gibco, cat# 25030-081); DMEM/F12 (Wisent, cat# 10090CV); Sodium Pyruvate (Wisent, cat# 25000-Ci); PBS Sterile (GIBCO, cat# 25030-081); Trypsin 0,25% and EDTA 0,01% (Wisent, cat# 25-052-Ci); HBSS (Hank's balanced salt solution, Wisent, Cat# 21-022CV); PHA (Phytohemagglutinin, Sigma, cat#L-9132, lot#: 015K88913); Ficoll paque (Amersham, Cat# 17-1440-03); Trizol (Invitrogen, cat#15596-018); Dnase I (Fermentas, cat#EN0521); Superscript First-Strand Synthesis System for RT-PCR kit (Invitrogen, cat#11904-018); dNTPs (Invitrogen, cat# 10297-018); oligo (dT)₁₂₋₁₈ (Invitrogen, cat#11904-018); Qiagen RNAeasy Mini Kit (Qiagen, Cat#74106); QiaVac 24 Manifold (Qiagen, Cat#19403); Disposable Vacconnectors (Qiagen, Cat#19407); RiboGreen Quantification Reagent (Invitrogen-Molecular probes, Cat # R-11490); Light-Cycler Instrument version 1.5 (Roche, Cat# 3531414); LC Capillaries for 20ml reactions (Roche, cat# 1909339); LC FastStart DNA Master SYBR Green 1 PLUS (100ml) (Roche, Cat# 03752186001); K3EDTA tubes (Greiner Bio-one, cat#: 455036B110306); Cyclic AMP EIA Kit (Cayman, cat.# 581001) Human TNF- α ELISA kit, (BioSource Cat#CHC-1754, lot# 041703); Human IL-8/CXCL8 ELISA kit (Biosource, cat# CHC1303); Human MCP-1/CCL2 ELISA kit (Biosource, cat# KHC1012); cAMP EIA kit (Cayman, cat# 581001); Human GMCSF ELISA (Medicorp, cat#CHC0904); Human IL-2 ELISA (BD Biosciences, cat#555190); ELISA plate reader (filter 450 nm, reference filter 650nm, Biorad, model680); Escort IV transfection agent (Sigma-Aldrich, cat#3287, lot#094K0565); hIL-1 β (Peprotech, cat.#11195D195); Forskolin (Sigma-Aldrich cat#F3917); Rolipram (Sigma-Aldrich Cat # R6520, lot # 022K4604); Alamar Blue, (Biosource cat#DAL1100); 12-well plate high binding (Costar, cat #665 102); TMB chromogen reagent for ELISA (MediCorp, cat # SB01); LipofectamineTM2000 transfection reagent (Invitrogen, cat#11668-019); Quantigene® Screen Kit (Panomics, cat# QG-000-050); 20XSSC (3M NaCl, 0,3M sodium citrate dihydrate, pH 7,0); Lithium dodecyl sulfate 10% w/v (Sigma, cat# L9781); Incubator set at 53°C, 0% CO₂; MW96 Plate Washer (Beckman Coulter); Thermo Luminoskan (LabSystem);

EMEM (HyClone, cat#SH30024.01); non-essential amino acids (HyClone, cat#30238.01); BEBM (Clonetics, cat#CC-3171); BEBM kit (Clonetics, cat#3170); Human TNF- α (Peprotech, cat.#300-01A); Human IFN γ (Peprotech, cat#315-05).

Cell culture

293 cells (human transformed embryonic kidney cell line; ATCC cat#: CRL-1573) were cultivated in DMEM containing 2mM L-glutamine, 0.1 mM non-essential amino acids, 1.5g/l sodium bicarbonate, 1mM sodium pyruvate, 10% FBS, penicillin 100 U/mL and streptomycin 100 microg/mL. A549 (Human lung carcinoma cell lines; ATCC) were cultivated in Ham's F12 media containing 2 mM L-glutamine; 1.5 g/L sodium bicarbonate; 10% FBS, Penicillin 100 U/mL, Streptomycin 100 microg/mL. Human peripheral blood mononuclear cells (PBMC) (peripheral blood mononuclear cells) were obtained from healthy volunteers. PBMC were isolated by Ficoll-Hypaque density gradient centrifugation of EDTA K3 blood from normal donors. PBMC were plated at 2×10^6 cells/mL/well in 12 well plates in AIM-V culture media supplemented with Penicillin 100 U/mL, Streptomycin 100 microg/mL. CYNOM-K1 cells were cultivated in EMEM containing 2mM L-glutamine, 1% non-essential amino acids, 10% FBS, penicillin 100U/mL and streptomycin 100microg/mL. NHBE cells were cultivated in BEBM (500mL supplemented with BPE, 2 ml; Hydrocortisone, 0.5 ml; hEGF, 0.5 ml; Epinephrine, 0.5 ml; Transferrin, 0.5 ml; Insulin, 0.5 ml; Retinoic Acid, 0.5 ml; Triiodothyronine, 0.05 ml).

Cell viability

Cell viability was systematically assayed using Alamar Blue test following the manufacturer's guidelines.

Preparation of Antisense

Phosphorothioate-DNA antisenses (Sigma Genosys) and phosphothiorate-FANA antisenses (Topigen, Montreal or UCDN, Calgary) were resuspended in sterile water and diluted in Opti-MEM for transfection. References to DNA (non-FANA) oligonucleotides in the examples are phosphorothioate-DNA oligonucleotides.

Antisense Treatment of Cells:*A549 cell line:*

Cells were trypsinized and re-suspended in HAM-F12 medium (0.5×10^5 cells/well in 48 well plates) supplemented with 10% serum in antibiotic starvation conditions then incubated overnight at 37°C. The next day, adherent cells were transfected with antisense/Lipofectamine complexes (ratio of 1 ug oligonucleotide: 1 uL Lipofectamine) at concentrations indicated in figure descriptions, and incubated at 37°C for a determined period of time.

293 cell line:

Cells were trypsinized and resuspended (1×10^5 cells/well in 48 well-plates) in DMEM medium supplemented with 10% serum in antibiotic deprivation conditions then incubated overnight at 37°C. The next day, adherent cells were transfected with antisense/Lipofectamine complexes (ratio of 1ug oligonucleotide: 1uL Lipofectamine) at concentrations indicated in figure legends and incubated at 37°C for a determined period of time.

PBMC

The day of isolation PBMCs were plated (2.0×10^6 /ml in 12 well plates) in AIM-V media. Antisenses were complexed with Escort IV reagent (Sigma) (ratio of 1 ug oligonucleotide: 0.5 uL Escort IV) and were added at concentrations indicated in figures. Cells were cultured for 18-24 hours at 37°C, 5%CO₂, humidity.

CYNOM-K1 cell line:

Cells were trypsinized and resuspended in EMEM medium supplemented with 10% serum in antibiotic starvation conditions (0.5×10^5 cells/well in 200uL in 48 well plates) then incubated overnight at 37°C. The next day, adherent cells were transfected with antisense/Lipofectamine 2000 complexes (ratio of 1ug oligonucleotide: 2uL Lipofectamine) at concentrations indicated in figure descriptions, and incubated at 37° for a determined period of time.

NHBE cells:

Cells were trypsinized and resuspended in BEBM complete medium in antibiotic starvation conditions (1×10^5 cells/well in 200uL in 48 well plates) then incubated overnight at 37°C. The next day, adherent cells were transfected with antisense/Lipofectamine 2000 complexes (ratio of 1ug oligonucleotide: 1uL Lipofectamine) at concentrations indicated in figure descriptions, and incubated at 37° for a determined period of time.

RNA Extraction

RNA was extracted from cell pellets according to RNAeasy mini kit protocol using the QiaVac 24 manifold from Qiagen and treated with DNase-I according to Fermentas procedure. RNA was quantified using the RiboGreen reagent according to the manufacturer protocol (Invitrogen-Molecular probes).

Reverse Transcription (RT)

Preparation of first-strand cDNA was performed using the Superscript First-Strand Synthesis System for RT-PCR kit, in a total reaction volume of 20 µL. 1µg of RNA was first denatured at 65°C for 5 minutes, with 0.5mM of each dNTPs, 0.5 µg of oligo (dT)₁₂₋₁₈ and chilled on ice for at least 1 min .The mixture was incubated at 42°C for 2 minutes and a second pre-mix containing 1X First-Strand Buffer, 10mM DTT, and 40 units of SuperScript II RT was added. Reactions were incubated at 42°C for 10 minutes, at 50°C for 1 hour and inactivated by heating at 70°C for 15 minutes.

Real-time PCR

PCR reaction mixtures were performed with 3 μ L of cDNA reaction in a total volume of 20 μ L in presence of 0.4 mM of each PCR primer and 4 μ L of LC FastStart DNA Master SYBR Green 1 PLUS. Step 1 (Denaturation): 95°C, 10 min (slope 20°C/sec); Step 2 (Cycles x 40): 95°C, (slope 20°C/sec); 57°C or 59°C, 5 sec. (slope 20°C/sec) 72°C, 10 sec. (slope 20°C/sec); Step 3 (Melting curve): 95°C, (slope 20°C/sec); 70°C, 30 sec. (slope 20°C/sec); 95°C, 0 sec. (slope 0,1°C/sec); Step 4 (Cool): 40°C, 30 sec (slope 20°C/sec)

PCR primer sequences used for each gene are described in Table 3. Quantification of PCR products was performed using the RelQuant program (Roche).

Quantigene Assay

Cells were harvested and lysed for 30 minutes at 53°C in 1X lysis mixture from the Quantigene® kit. Cell lysates were hybridized overnight at 53°C in Quantigene® capture plates in the presence of specific probe sets and mRNA expression was linearly quantified by luminescence signal using Luminoskan.

Cellular Assays: (ELISAs, EIA)*A549 cells*

At the end of the transfection period, A549 cells were incubated for 4 h with IL-1 β (10 ng/ml). Supernatants were harvested and analysed using ELISA for human IL-8, human MCP-1 and human GM-CSF.

PBMCs

After overnight transfection, PHA (10 μ g/mL) was added to cells for 6 hrs. Some cells received treatment with Rolipram (10 μ g/mL) or Forskolin (2 μ M) for 30 minutes prior to cell harvesting. Supernatant were collected and frozen

until time to perform IL-2 and TNF- α ELISA following manufacturer's guidelines. Determination of intracellular cAMP levels was performed on cell pellets that were lysed and lysates was frozen until time to perform cAMP EIA according to manufacturer's protocols.

NHBE cells:

NHBE cells were stimulated for 4h with cytomix (500U/mL TNF- α +10ng/mL IL1- β + 10ng/mL IFN γ) at the end of the transfection period. MCP-1 protein secretion was measured in supernatant by ELISA, with normalization to the cell viability as measured by AlamarBlue assay.

Example 1 : Composition of Antisense and Potency in vitro

The sequence and composition of the antisense oligonucleotides prepared in this study are shown in Tables 1a, 1b, 1c and 1d. All oligonucleotides were purified by anion exchange HPLC or gel electrophoresis and desalted via a size-exclusion chromatography using Sephadex G-25 beads. The potency of some selected sequences listed in Tables 1a-1d are demonstrated in Figures 1a and 1b which show the reduction in gene expression of specific PDE isoforms in cells following transfection with indicated AON. Figure 1A shows cell lines, either A549 for PDE3A, PDE3B and PDE7A or 293 cells for PDE4A, PDE4B and PDE4D had decreased levels of target isoforms following overnight transfection with specific AON. PDE mRNA expression level was quantified using the Quantigene® assay (Panomics) for PDE3A/B, PDE4D and PDE7A and real-time PCR was used to quantify expression of PDE4A/4B. PDE isoform levels were normalized to the expression of a control gene (β 2M, Ppib or HPRT) and these levels were compared to expression levels of untreated cells.

Although the majority of antisense oligonucleotides designed were specific for PDE isoforms, some AON, in particular for PDE4 isoforms, demonstrated dual specificity for target knockdown of more than one PDE4 isoform. Sequences

listed in Table 1c describe AON which have demonstrated more than one target gene knockdown with the single AON. Figure 1b demonstrates the efficacy of these AON that were designed for dual target specificity. AON TOP 1545 (Seq ID No 14) and TOP 1556 (Seq ID No 24) each recognize gene sequences shared between PDE4B isoform and PDE4D isoform (Table 1c), and demonstrate target gene knockdown for PDE4B and PDE4D while AON. TOP 1507 (Seq ID No 10) and 1508 (Seq ID No 11) are effective in decreasing mRNA levels of PDE4A and PDE4D in transfected cells and both AON recognize sequences shared between PDE4A and PDE4D isoforms (Table 1c). Data is expressed as % specific inhibition determined by comparing the levels of inhibition obtained with the control mismatch or sense AON sequences with the % inhibition obtained with their respective PDE-specific AON sequences.

Example 2 Effect of AON delivery on biological functions of cells

This example relates to efficacy of AON on the biological function of different cell types, specifically the secretion of cytokines and chemokines. Cytokines and chemokines are important mediators of cell activation and recruitment. Lung epithelial cells can play a role in the pathophysiology of inflammatory respiratory diseases through the secretion of chemokines that lead to the recruitment of immune cells, such as neutrophils and monocytes/macrophages. Interleukin-8 (IL-8/CXCL8) and monocyte chemoattractant protein-1 (MCP-1/CCL2) levels are increased in COPD patients (Szilasi M *et al.* Pathology Oncology Research. 2006 12:52-60). The levels of IL-8 and MCP-1 may be involved in neutrophil and macrophage recruitment respectively. Both IL-8 and MCP-1 are secreted by the lung epithelial cell line, A549 in response to stimulation with IL-1 β . Stimulated A549 cells also secrete granulocyte-macrophage cell stimulating factor (GM-CSF), a cytokine known to activate neutrophils and macrophages for enhanced function. Figure 2 demonstrates the efficacy of AON TOP 1497 (Seq ID No 90) which is specific for PDE4D isoform, to limit the secretion of

chemokines IL-8/CXCL-8 and MCP-1/CCL2 and cytokine GM-CSF in A549 cells following overnight transfection of AON then stimulated with IL-1 β for 4 h. The use of this assay allows the verification of PDE isoforms involved in the release of inflammatory mediators by lung epithelial cells. In Figure 2, the contribution of PDE4D, through its inhibition by TOP 1497 (Seq ID No 90), in the A549 cells is clearly demonstrated whereas there is no contribution of PDE7A in these cells for the release of chemokines and cytokines although transfection with AON TOP 1716 (Seq ID No 92) was successful in reducing mRNA levels of this gene. Figure 2 demonstrates functional changes, specifically the reduction of inflammatory potential through the decrease in secretion of inflammatory mediators that can occur in lung epithelial cells following transfection with AON.

Figure 3 demonstrates the efficacy of AON to limit biologic function of different cell types, specifically human peripheral blood mononuclear cells (PBMCs) PBMCs refer to a mixed population of immune cells that secrete cytokines, specifically TNF α which is known to activate macrophages and IL-2 which is known to activate and stimulate T cells and NK cells. Human PBMCs transfected overnight with AON targeting PDE isoforms then stimulated the following day with phytohemagglutinin (PHA) secreted less TNF α and IL-2 than PBMCs transfected with mismatched or sense AON sequences (Figure 3). Transfection of PBMC with the described AON led to a decrease in the inflammatory potential of these cell types. In COPD release of proinflammatory mediators and chemoattractants by inflammatory cells and activated epithelial cells contribute to further increased levels of inflammation (Szilasi M *et al.* Pathology Oncology Research. 2006 12:52-60).

It is expected that inhibition of PDE gene expression, would lead to reduced PDE activity, that would in turn result in a build up of cAMP inside cells. Figure 3 demonstrates that transfection with AON targeting specific PDE isoforms led to increased intracellular cAMP levels in the PBMCs (right hand axis). Data represent the changes in levels of either cytokine or cAMP in PBMCs

following transfection with PDE-specific AON compared to levels following transfection with mismatch or sense sequences.

Cytokines play a critical role in the orchestration of chronic inflammation in all diseases, including asthma and COPD. TNF- α is one of the most important pro-inflammatory cytokines that is expressed in several inflammatory diseases. TNF α levels are markedly increased in induced sputum of patients with COPD (Keatings *et al.*, Am. J. Respir. Crit. Care Med. 1996; 153; 530–534.). Furthermore, there is evidence that COPD patients with weight loss show increased release of TNF α from circulating cells and that TNF α may induce apoptosis of skeletal muscle cells, resulting in the characteristic muscle wasting and cachexia seen in some patients with severe COPD (De Godoy *et al.*, Am. J. Respir. Crit. Care Med. 1996, 153, 633–637.). TNF- α induces the gene expression of many pro-inflammatory cytokines including itself and IL-8. Cytokines, specifically TNF α are amongst the mediators that have been shown to be increased and/or play a role in the pathophysiology of inflammatory respiratory diseases and involved in tissue destruction.

PDE4 inhibitors caused a decrease in the release of cytokines and chemokines from inflammatory cells via an increase in intracellular cyclic AMP (Torphy, Am. J. Respir. Crit. Care Med. 1998, 157, 351–370). In contrast to corticosteroids, PDE4 inhibitors have a potent inhibitory effect on neutrophils (Au *et al.*, Br. J. Pharmacol. 1998, 123, 1260–1266.) indicating that they may be useful anti-inflammatory treatments for COPD. There is preliminary evidence that a PDE4 inhibitor Cilomilast improves lung function and symptoms in patients with COPD and this may be due to cytokine inhibition (Compton *et al.*, Lancet. 2001, 358, 265–270).

As TNF α , is considered as a common denominator in inflammatory and chronic respiratory diseases, this invention provides a broad therapeutic application for treating several inflammatory diseases.

Example 3 AON modified with 2'-F-ANA chemistry demonstrate increased efficacy and prolonged action for target gene knockdown

This example relates to the enhanced efficacy of PDE-specific AON when FANA modifications are made into the chemistry of the AON. Tables 2a-2g describe the compositions of AON modified with F-ANA residues. In Figure 4, A549 cells were transfected for 24h with PDE4D-specific AON TOP 1497 (Seq. ID No. 90) either in phosphothiorate composition (PS-DNA) or containing FANA base modifications (PS-FANA; TOP 1497-F1 (Seq ID No. 72), TOP-1497-F5 (Seq ID No.76), TOP 1497-F6 (Seq ID No. 77)) as indicated in the legend provided in Figure 4. PDE4D mRNA knockdown was determined using the Quantigene® assay (Panomics) with PDE levels normalized to β 2M gene expression. Modification of TOP 1497 sequence with FANA monomers, (TOP 1497-F1 (Seq ID No. 72)) enhanced the efficacy of the AON to inhibit target gene expression at doses between 100-400 ng, clearly showing an advantage of this modification for AON activity (Figure 4a). 2'-F-ANA modifications are expected to enhance the stability of the AON, rendering it more resistant to nucleosidase digestion, which would prolong its activity. In Figure 4b, comparison of different formulations of PDE4D-specific AON TOP 1497 at a single dose over time clearly shows the 2'-F-ANA containing AON have sustained inhibitory activity up to 48 hp compared to PS-DNA AON. At 48 h after transfection, no inhibition is observed with the PS-DNA formulation of AON, however, even by modifying only 5 of the 19 bases AON TOP 1497-F6 (Seq ID No 77) with 2'-F-ANA, was sufficient to sustain an inhibitory effect for this time point. The sustained inhibition was increased by incorporating more 2'-F-ANA residues into the original sequence as shown following transfection with TOP 1497-F5 (Seq ID No 76) and TOP 1497-F1 (Seq ID No 72). The greater amount of 2'-F-ANA composition in AON yielded the most prolonged effect, although by overnight transfection (24h), PS-FANA containing 7 residues yielded a similar level of inhibition as PS-FANA containing 11 residues.

Example 4 AON modified with 2'-F-ANA chemistry retains biological function in cells

This example refers to the tolerance of 2'-F-ANA chemistry modification of PDE-specific AON in reducing the inflammatory responses of cells. In Figure 2, the efficacy of PDE-specific AON to reduce chemokine secretion by lung epithelial cells was demonstrated. In Figure 5 the effect of 2'-F-ANA containing AON on biological function of A549 was studied. A549 cells transfected with PDE4B/4D dual specific AON TOP 1545 (Seq ID No 14) as PS-DNA or with 2'-F-ANA modified TOP 1545-F3 (Seq ID No 85) demonstrate higher levels of inhibition of chemokine secretion when 2'-F-ANA containing AON were transfected in the A549 cells compared to the unmodified AON.

This retention of biological efficacy of 2'-F-ANA containing AON was demonstrated in primary cells, specifically human PBMCs. Combination of PDE4B/4D specific AON TOP 1545-F3 (Seq ID No 85) with PDE7A specific AON TOP 1731-F2 (Seq ID No 53) increased inhibition of their respective gene targets when the second antisense was present, although the antisense sequence has no homology with the other target gene (Figure 6a). In Figure 6a the solid bars represent the % specific inhibition of target gene mRNA of either PDE4B, PDE4D or PDE7A when both AON are used in combination with each at a low dose (3.2 nM) compared to the AON used alone, at low and high doses (6.3 nM), demonstrating that 2'-F-ANA containing AON retain the function of target gene knockdown in primary immune cells. For biological function transfection of PBMCs with combination of PDE4B/4D dual specific TOP 1545-F3 (Seq ID No 85) with PDE7A specific AON TOP 1731-F2 (Seq ID No 53) led to inhibition of both TNF- α and IL-2 secretion. 2'-F-ANA containing AON retain their functionality to reduce the inflammatory potential of immune cells.

The results in Figure 6a also show the enhanced effect that occurs when combinations of AON specific for two different target genes are administered

to cells at the same time. Although AON were administered at a concentration that had little to no effect on their target genes when employed alone, when combined, the AON induced significant inhibition on the gene expression of their respective targets (PDE4B and PDE4D for TOP 1545-F3 (Seq ID No 85), and PDE7A for TOP 1731-F2 (Seq ID No. 53)).

Example 5: Effect of PDE4B/4D AON on Mitogenic Regulatory Genes

Table 1c describes the sequence of TOP 1545 AON which has dual specificity for both PDE4B and PDE4D. Sequence of TOP 1549 (Seq ID No 17) AON has 100% homology with thymidylate synthase (TYMS) gene sequence and 5 base pair mismatches with PDE4B and PDE4D. TOP 1549 (Seq ID No 17) AON inhibits its target gene expression (TYMS) in a dose response manner (200, 400 and 800 ng) following 6 h transfection. Despite having 5 base pair mismatches with the TYMS gene sequence, TOP 1545 (Seq ID No 14) AON containing 2'-F-ANA is also efficient to inhibit TYMS gene expression in 293 cells following 6 h transfection. TYMS is a key rate-limiting enzyme of DNA synthesis and is upregulated in some cancers. TOP 1545 (Seq ID No. 14) AON has the potential to not only inhibit PDE function through the decrease of PDE4B and PDE4D mRNA levels, but could also function to inhibit proliferation of cells through its effects on TYMS gene expression. This figure demonstrates that TOP1545 (Seq ID No 14) is a novel antisense molecule that has a dual specificity for phosphodiesterase 4 (PDE4) and thymidylate synthase (TS).

Inhibitors of PDE4 are also described in the literature as cAMP elevating agents; the inhibition of PDE4 blocks the breakdown of cAMP into AMP, resulting in an accumulation of cAMP. It is well known that high intracellular levels of cAMP can effectively kill cancer cells *in vitro*.

Thymidylate synthase (TYMS) is the source of thymidine monophosphate for most organisms, which is necessary for DNA synthesis and cell division, and is therefore an obvious anticancer target. TYMS is not structurally related to

PDEs, except for a small stretch of 17 consecutive nucleotides in its coding region. TOP1545 (Seq ID No. 14) targets this region of homology between TYMS and PDE4B and PDE4D, making TOP1545 (Seq ID No 14) a unique anti-cancer compound.

Example 6: Potency of AON in vitro

This example shows the reduction in gene expression in CYNOM-K1 monkey cells transfected with the PDE4D/4B specific AON TOP1572-F2 (Seq ID No. 122; Figure 8 A) or the PDE7A specific AON TOP1731-F3 (Seq ID No. 54, Figure 8 B). PDE4D and PDE4B mRNA levels were respectively reduced by 53% and 66% (Figure 8 A) and PDE7A gene expression was reduced by 70% (Figure 8 B) as compared to non transfected cells.

Example 7: Effect of AON delivery on biological functions of cells

This example relates the efficacy of the PDE4D/4B specific AON TOP1572-F2 (Seq ID No. 122) and TOP1545 (Seq ID No. 14) at reducing the PDE4D mRNA expression level (Figure 9 A) and the secretion of MCP-1 cytokine stimulated in NHBE cells in response to a 4 hours stimulation with a cytomix (500U/mL TNF- α + 10ng/mL IL1- β + 10ng/mL IFN γ) (Figure 9 B). TOP1572-F2 (Seq ID No. 122) reduces the PDE4D mRNA expression level by 71% correlated with 34% inhibition of MCP-1 secretion. Similarly, TOP1545 (Seq ID No. 14) reduces the PDE4D mRNA expression levels by 36% correlated with 46% inhibition of MCP-1 secretion relative to cells transfected with corresponding controls.

Example 8: Effect of AON delivery on biological effect in cells

This example refers to the efficacy of PDE-specific AON to inhibit the secretion of pro-inflammatory cytokines by PHA-stimulated human peripheral blood mononuclear cells (PBMCs). PBMCs are a mixed population of

leukocytes including T lymphocytes, B lymphocytes and monocytes/macrophages, all of which can be stimulated to release pro-inflammatory cytokines. Human PBMCs transfected overnight with the PDE4B/4D specific TOP 1572 (Figure 10) and then stimulated the following day with the mitogen phytohemagglutinin (PHA, 10 ug/ml) for 6 h, secreted 51% less pro-inflammatory cytokine IL-2 (Figure 10 A) and 42% less TNF- α (Figure 10 B) as compared to those cells stimulated with PHA but not transfected. Similarly, PBMCs transfected with the PDE7A specific AON TOP 1731 (Figure 11) secreted 48% less IL-2 and 60% less TNF- α than those PBMCs which were not transfected but stimulated with PHA.

Example 9: Enhanced efficacy in inhibiting immune responses when TOP 1572 and TOP 1731 are combined

This example refers to the effect in reducing IL-2 secretion by PHA-stimulated PBMCs when AON are combined. PBMCs transfected with 3.1 nM each of TOP 1572-F2 (Seq ID No. 122) and TOP 1731-F3 (Seq ID No. 54) resulted in a level of IL-2 inhibition (33%) which was greater than the inhibition levels observed following transfection with each AON alone at either 3.1 nM or 6.3 nM doses (Figure 12). In this example, a low dose of TOP 1572-F2 yielded less than 10% inhibition of IL-2 whereas a higher dose (6.3 nM) yielded 24% inhibition of IL-2 (Figure 12). With regard to TOP 1731-F3, a low dose (3.1 nM) yielded 20% inhibition of IL-2 and this level was not statistically increased when the dose was increased to 6.3 nM (22% inhibition) (Figure 12). The combination of both TOP 1572-F2 with TOP 1731-F3 to result in a 33% inhibition of IL-2 secretion may have been due to targeting the different PDE isoforms of PDE4B, PDE4D and PDE7A simultaneously (Figure 12).

Example 10: Comparison of PS-DNA and PS-FANA chemistries in cell lines

This example compares the efficacy of PS-DNA AON to the efficacy of PS-FANA AON at reducing target mRNA expression. TOP1572-F2 version (Seq

ID No. 122) presents a longer duration of action than the standard PS-DNA version TOP1572 (Seq ID No. 40) at reducing the mRNA expression of both PDE4D (Figure 13A) and PDE4B (Figure 13B). After 72h transfection in 293 cells, TOP1572-F3 still decreases 72% of PDE4B and 55% of PDE4D mRNA expression whereas TOP1572 efficacy is reduced at 18% for PDE4B and 29% for PDE4D. Increase of FANA AON activity is also observed at lower doses. TOP1731-F3 FANA version (Seq ID No. 54) is better than the PS-DNA version TOP1731 (Seq ID No. 2) at blocking the PDE7A mRNA expression in A549 cells transfected for 48h at 125nM and 75nM doses (Figure 14).

Although preferred embodiments of the invention have been described herein, it will be understood by those skilled in the art that variations may be made thereto without departing from the spirit of the invention or the scope of the appended claims. All documents cited in this specification are hereby incorporated by reference.

Table 1a.

Target gene	Accession number	Antisense ID	Antisense sequence (5'-3')	Seq ID No.
PDE7A	NM_002603	TOP1730	TCATGAGTGGCAGCTGCAA	1
		TOP1731	TCATGAGTGGCAGCTGCAATT	2
		TOP1732	CATGAGTGGCAGCTGCAATTAA	3
		TOP1733	GAGTGGCAGCTGCAATTAAGC	4
		TOP1734	ATGAGTGGCAGCTGCAATTAA	5
		TOP1735	AGATCATGAGTGGCAGCTGCA	6
		TOP1736	GAGTGGCAGCTGCAATTAA	7
		TOP1737	GATCATGAGTGGCAGCTGCAA	8

Table 1b.

Target gene	Accession number	Antisense ID	Antisense sequence (5'-3')	Seq ID No.
PDE3A	NM_000921	TOP1313	CCTCAGCACCAGCCATGTCTG	9

Table 1c.

Target genes	Accession numbers	Antisense ID	Antisense sequence (5'-3')	Seq ID No.
PDE4A+4D	NM_006202	TOP1507	GTGCTTGTCACACATGG	10
	NM_006203	TOP1508	GTCCTCCAAAGTGTCC	11
PDE4B+4D	NM_002600 NM_006203	TOP1511	GGTCTCTAGCTGGTC	12
		TOP1544	ATGGTAATGGTCTTC	13
		TOP1545	TCTGCCCATGTCTCCCA	14
		TOP1546	AAGACCCCATTTGTTCA	15
		TOP1547	TCTGCCCAGGTCTCCCA	16
		TOP1549	GAGGCCCATGTCTCCCG	17
		TOP1550	TCTGCCCATGTCTCCCAGA	18
		TOP1551	TCTGCCCATGTCTCCCACA	19
		TOP1552	TCTGCCCATGTCTCCCAGAG	20
		TOP1553	TCTGCCCATGTCTCCCACAA	21
		TOP1554	GGTTGCTCAGGTCTGCACAGT	22
		TOP1555	GGTTGCTCAGATCTGCACAGT	23
		TOP1556	GGTTGCTCAGTCTGCACAGT	24
		TOP1557	TCAGCATGGTAATGGTCTT	25
		TOP1558	GCCACITCAGCATGGTAAT	26
		TOP1559	TACATCAAIGCAAGTTC	27
		TOP1560	ATGTCACAGTITTCTTC	28
		TOP1561	TGTCAATACCATTTTCCT	29
		TOP1562	GTTTCIACCATIGTCTTCA	30
		TOP1563	CTTTCTTAGTTTCIACCAT	31
		TOP1564	TCTGCACAGTGCACCAT	32
		TOP1565	GTCTGTAIGGTCTCTAGCT	33
		TOP1566	TGTAIGGTCTCTAGCTGGT	34
		TOP1567	TTCTTGACTCCACTIATCT	35
		TOP1568	AATCAAGTCATCICCGTGT	36
		TOP1569	GTTGTGATAIGCCACITCA	37
		TOP1570	ATTITACATCAAIGCAAGT	38
		TOP1571	TGTTTIGACATATCIGTT	39
		TOP1572	GGTTGCTCAGITCTGCACA	40
		TOP1573	GGTTGCTCAGATCTGCACA	113
		TOP1574	TTGCTCAGATCTGCA	114

Table 1d.

Target gene	Accession number	Antisense ID	Antisense sequence (5'-3')	Seq ID No.
PDE3B		TOP1360	TCAGCAGCGTCCGCAGCCAG	89
PDE4D	NM_006203	TOP1497	CTGCCTCCTCTTCAACCTG	90
PDE4A+4B	NM_006202	TOP1512	CCATGATGCGGTCTGTCCA	91
	NM_002600			
PDE7A	NM_002603	TOP1716	TCATGAGTGGCAGCTGC	92

Table 2a.

Target gene	Antisense ID	FANA-Antisense ID	Antisense sequence (5'-3')	Seq ID NO
PDE7A	TOP1716	TOP1716-F1	PS-TCA TGA gtg gca GCT GC	41
		TOP1716-F2	PS-TCA tgA GTg gCA gcT GC	42
		TOP1716-F3	PS-tCa TgA gTg GcA gCt gC	43
		TOP1716-F4	PS-TCA tGA gtg gca GCT GC	44
		TOP1716-F5	PS-TCA tga gtg gca GCT GC	45
	TOP1714	TOP1714-F1	PS-GCT TAg gtt cct TTA AGT	46
	TOP1729	TOP1729-F1	PS-CAG ATc atg agt ccC AGC TG	47
	TOP1730	TOP1730-F1	PS-TCA TGa gtg gca gCT GCA A	48
		TOP1730-F2	PS-TCA Tga gtg gca gct GCA A	49
		TOP1730-F3	PS-TCa tga gtg gca gct GCA A	50
		TOP1730-F4	PS-TCa tga gtg gcA GCT GCA A	51
	TOIP1731	TOP1731-F1	PS-TCA TGa gtg gca gct GCA ATT	52
		TOP1731-F2	PS-TCA Tga gtg gca gct gcA ATT	53
		TOP1731-F3	PS-TCa tga gtg gca gct gcA ATT	54
		TOP1731-F4	PS-TCa tga gtg gcA GCT GCA ATT	55
		TOP1731-F5	PS-TCA Tga gtg GCa gct gcA ATT	115
		TOP1731-F6	PS-TCa tGA gtg GCA gct gcA ATT	116
	TOP1733	TOP1733-F1	PS-GAG TGg cag ctg caa TTA AGC	56
	TOP1734	TOP1734-F1	PS-ATG AGt ggc agc tgc AAT TAA	57
	TOP1736	TOP1736-F1	PS-GAG TGg cag ctg cAA TTA A	58
	TOP1737	TOP1737-F1	PS-GAT CAt gag tgg cag CTG CAA	59

Table 2 b.

Target gene	Antisense ID	FANA-Antisense ID	Antisense sequence (5'-3')	Seq ID No.
PDE3A	TOP1311	TOP1311-F1	PS- CGG CCA AGC agc tga g CA CC	60
		TOP1311-F2	PS- CGG CCA Agc agc tg A GCA CC	61
		TOP1311-F3	PS- CGG CCA agc agc TGA GCA CC	62

Table 2c.

Target gene	Antisense ID	FANA-Antisense ID	Antisense sequence (5'-3')	Seq ID No.
PDE3B	TOP1360	TOP1360-F1	PS- TCA GCA Gcg tcc g CA GCC AG	63
		TOP1360-F2	PS- TCA gca gCG Tcc g CA gcc AG	64
		TOP1360-F3	PS- TCA gCa gCg TCC gCa gCC ag	65

Table 2d.

Target gene	Antisense ID	FANA-Antisense ID	Antisense sequence (5'-3')	Seq ID No.
PDE4A	TOP1413	TOP1413-F1	PS- GCC ACG ctc gcg CTC TC	66
		TOP1413-F2	PS- GCC acg CTC gcg ctc TC	67
		TOP1413-F3	PS-gcc ACG ctc GCG CTc tc	68

Table 2e.

Target gene	Antisense ID	FANA-Antisense ID	Antisense sequence (5'-3')	Seq ID No.
PDE4D	TOP1498	TOP1498-F1	PS- CTT CAg gct gg C TTT CCT C	69
		TOP1498-F2	PS-ct T Cag gCT gg C Ttt CCT c	70
		TOP1498-F3	PS-ctt cag gct g GC TTT CCT C	71
	TOP1497	TOP1497-F1	PS- CTG CCT cct ctt ca A CCT G	72
		TOP1497-F2	PS-ctg c CT CCT ct T CAA cct g	73
		TOP1497-F3	PS-ctg c CT CCT Bt T CAA cct g	74
		TOP1497-F4	PS- CTg cCT cct ctt ca A CCT G	75
		TOP1497-F5	PS- CTg cct cct ctt ca A CCT G	76
		TOP1497-F6	PS-ctg cct cct ctt ca A CCT G	77

Table 2f.

Target gene	Antisense ID	FANA-Antisense ID	Antisense sequence (5'-3')	Seq ID No.
PDE4B	TOP1432	TOP1432-F1	PS-GAC CGG Tag gtc tgT ATG GT	78
		TOP1432-F2	PS-GAC CGG Tag gtc tgT BTG GT	79
		TOP1432-F3	PS-GAC Cgg tag gtC TGT BTG GT	80

Table 2g.

Target gene	Antisense ID	FANA-Antisense ID	Antisense sequence (5'-3')	Seq ID No/
PDE4A+ 4D	TOP1505	TOP1505-F1	PS-GTC CAC tgg cgG TAC AG	81
PDE4A+ 4B	TOP1512	TOP1512-F1	PS-CCA TGA tgc ggt cTG TCC A	82
PDE4B+ 4D	TOP1545	TOP1545-F1	PS-TCT GCC cat gtC TCC CA	83
		TOP1545-F2	PS-TCt gcc cat gtc TCC CA	84
		TOP1545-F3	PS-TCt gcc cat gtc tCC CA	85
		TOP1545-F4	PS-TCt gcc cat gTC tcC CA	86
		TOP1545-F5	PS-TcT gCc CaT gTc TcC cA	87
		TOP1545-F6	PS-TCt gcc CAT gtc tCC CA	88
	TOP1556	TOP1556-F1	PS-GGT TGc tca git ctg CAC AGT	117
		TOP1556-F2	PS-GGt tgc tca git ctg cAC AGT	118
	TOP1570	TOP1570-F1	PS-ATt ita cat caa igC AAG T	119
		TOP1570-F2	PS-ATt ita CAT caa igC AAG T	120
	TOP1572	TOP1572-F1	PS-GGT TGc tca git cTG CAC A	121
		TOP1572-F2	PS-GGt tgc tca git ctG CAC A	122
		TOP1572-F3	PS-GGt tgc TCA git ctG CAC A	123
		TOP1572-F4	PS-GGt tgc tca git ctg CAC A	124
	TOP1573	TOP1573-F2	PS-GGt tgc tca gat ctg CAC A	125
	TOP1574	TOP1574-F2	PS-TTG ctc aga tct GCA	126

Table 3.

Target gene	Sense primer (5'-3')	SEQ ID No.	Product size (bp)
PDE7A	TCAGGCCATGCACTGTTACT	93	229
PDE3A	CAACAGTGACAGCAGTGACATT	95	166
PDE3B	GATTCTTTGGGATTGGGACT	97	212
PDE4A	ATCAACACCAATTCGGAGCT	99	199
PDE4B	TGGCAGACCTGAAGACAATG	101	181
PDE4D	CAGAATATGGTGCACTGTGC	103	192
HPRT	ATCAGACTGAAGAGCTTTGTAATGACCA	105	230
B2M	CAAGGACTGGTCTTTCTATCTCTTGT	107	338
PPIB	AGAGCATCTACGGTGAGCG	109	196
TYMS	AAGAATCATCATGTGCGCTT	111	226

Target gene	Antisense primer (5'-3')	SEQ ID No.	Product size (bp)
PDE7A	CCTGATTCTCTCAATAAGCCC	94	229
PDE3A	TTGAGTCCAGGTTATCCATGAC	96	166
PDE3B	ATCTTTGGCCTACAGGAACC	98	212
PDE4A	CCAGCACCATGTCGATGAC	100	199
PDE4B	AAATTCCTCCATGATGCGG	102	181
PDE4D	AGTCTATGAAGCCCACCTGTG	104	192
HPRT	TGGCTTATATCCAACACTTCGTG	106	230
B2M	GTGGAGCAACCTGCTCAGATAC	108	338
PPIB	CTTCCGCACCACCTCCA	110	196
TYMS	GTGTGTATAAAGTCACCTGGCTT	112	226

We Claim:

1. An oligonucleotide having a base sequence selected from the group consisting of (i) SEQ ID NOS. 1-92 and 113-126 and (ii) SEQ ID NOS. 1-92 and 113-126 containing a one base insertion, deletion or substitution.
2. The oligonucleotide of claim 1 having a base sequence selected from the group consisting of SEQ ID NOS. 1-92 and 113-126.
3. The oligonucleotide of claim 1 having a phosphodiester backbone.
4. The oligonucleotide of claim 1 having a phosphorothioate backbone.
5. The oligonucleotide of claim 4 wherein at least one nucleotide of the oligonucleotide is a 2'-deoxy-2'-fluoroarabinonucleotide.
6. The oligonucleotide of claim 5 selected from the group consisting of SEQ ID NOS. 41-88 and 115-126.
7. A pharmaceutical composition comprising at least one oligonucleotide of any one of claims 1-6 in combination with a pharmaceutically acceptable carrier.
8. A pharmaceutical composition comprising at least two oligonucleotides of any one of claims 1-6 in combination with a pharmaceutically acceptable carrier.
9. The pharmaceutical composition of claim 8, wherein a first oligonucleotide of the at least two oligonucleotides is directed against PDE7A.
10. The pharmaceutical composition of claim 9, wherein a second oligonucleotide of the at least two oligonucleotides is directed against PDE4B and PDE4D.
11. The pharmaceutical composition of claim 10, wherein the at least two oligonucleotides are selected from the group consisting of SEQ ID NOS. 41-88 and 115-126.

12. The pharmaceutical composition of claim 11 wherein the at least two oligonucleotides are SEQ ID NO. 2 and SEQ ID NO. 14.
13. The pharmaceutical composition of claim 11 wherein the at least two oligonucleotides are SEQ ID NO. 2 and SEQ ID NO. 24.
14. The pharmaceutical composition of claim 11 wherein the at least two oligonucleotides are SEQ ID NO. 2 and SEQ ID NO. 37.
15. The pharmaceutical composition of claim 11 wherein the at least two oligonucleotides are SEQ ID NO. 2 and SEQ ID NO. 40.
16. The pharmaceutical composition of claim 11 wherein the at least two oligonucleotides are SEQ ID NO. 2 and SEQ ID NO. 85.
17. The pharmaceutical composition of claim 11 wherein the at least two oligonucleotides are SEQ ID NO. 2 and SEQ ID NO. 122.
18. The pharmaceutical composition of claim 11 wherein the at least two oligonucleotides are SEQ ID NO. 53 and SEQ ID NO. 14.
19. The pharmaceutical composition of claim 11 wherein the at least two oligonucleotides are SEQ ID NO. 53 and SEQ ID NO. 24.
20. The pharmaceutical composition of claim 11 wherein the at least two oligonucleotides are SEQ ID NO. 53 and SEQ ID NO. 37.
21. The pharmaceutical composition of claim 11 wherein the at least two oligonucleotides are SEQ ID NO. 53 and SEQ ID NO. 40.
22. The pharmaceutical composition of claim 11 wherein the at least two oligonucleotides are SEQ ID NO. 53 and SEQ ID NO. 85.
23. The pharmaceutical composition of claim 11 wherein the at least two oligonucleotides are SEQ ID NO. 53 and SEQ ID NO. 122.
24. The pharmaceutical composition of claim 11 wherein the at least two oligonucleotides are SEQ ID NO. 54 and SEQ ID NO. 14.

25. The pharmaceutical composition of claim 11 wherein the at least two oligonucleotides are SEQ ID NO. 54 and SEQ ID NO. 24.
26. The pharmaceutical composition of claim 11 wherein the at least two oligonucleotides are SEQ ID NO. 54 and SEQ ID NO. 37.
27. The pharmaceutical composition of claim 11 wherein the at least two oligonucleotides are SEQ ID NO. 54 and SEQ ID NO. 40.
28. The pharmaceutical composition of claim 11 wherein the at least two oligonucleotides are SEQ ID NO. 54 and SEQ ID NO. 85.
29. The pharmaceutical composition of claim 11 wherein the at least two oligonucleotides are SEQ ID NO. 54 and SEQ ID NO. 122.
30. A method for treating a subject having a disease associated with reduced cAMP comprising administering a pharmaceutical composition of any one of claims 7 to 29.
31. A method for treating respiratory inflammation in a subject comprising administering a pharmaceutical composition of any one of claims 7 to 29.
32. The method of claim 31, wherein the respiratory inflammation is caused by any one of chronic obstructive pulmonary disease, asthma, eosinophilic cough, bronchitis, acute and chronic rejection of lung allograft, sarcoidosis, pulmonary fibrosis, rhinitis, sinusitis, viral infection or a neoplastic disease.
33. The method of claim 32 wherein the disease is chronic obstructive pulmonary disease.
34. Use of a pharmaceutical composition of any one of claims 7 to 29 for the preparation of a medicament for the treatment of a disease associated with reduced cAMP.
35. Use of a pharmaceutical composition of any one of claims 7 to 29 for the preparation of a medicament for the treatment of respiratory inflammation.

36. The use of claim 35, wherein the respiratory inflammation is caused by any one of chronic obstructive pulmonary disease, asthma, eosinophilic cough, bronchitis, acute and chronic rejection of lung allograft, sarcoidosis, pulmonary fibrosis, rhinitis, sinusitis, viral infection or a neoplastic disease.
37. The use of claim 36 wherein the disease is chronic obstructive pulmonary disease.
38. Use of a pharmaceutical composition of any one of claims 7 to 29 for the treatment of a disease associated with reduced cAMP.
39. Use of a pharmaceutical composition of any one of claims 7 to 29 for the treatment of respiratory inflammation.
40. The use of claim 39, wherein the respiratory inflammation is caused by any one of chronic obstructive pulmonary disease, asthma, eosinophilic cough, bronchitis, acute and chronic rejection of lung allograft, sarcoidosis, pulmonary fibrosis, rhinitis, sinusitis, viral infection or a neoplastic disease.
41. The use of claim 40 wherein the disease is chronic obstructive pulmonary disease.
42. A pharmaceutical composition comprising two oligonucleotides having SEQ ID NO. 54 and SEQ ID NO. 122 along with a pharmaceutically acceptable carrier.
43. An oligonucleotide that is at least 80% complementary to an mRNA of at least one of PDE3A, PDE3B, PDE4A, PDE4B, PDE4C, PDE4D, PDE7A1, PDE7A2, PDE7A3 and PDE7B, wherein at least one nucleotide of the oligonucleotide is a 2'-deoxy-2'-fluoroarabinonucleotide.
44. The oligonucleotide of claim 43 being at least 85% complementary to the mRNA.
45. The oligonucleotide of claim 43 being at least 90% complementary to the mRNA.

46. The oligonucleotide of claim 43 being at least 95% complementary to the mRNA.
47. The oligonucleotide of claim 43 being 100% complementary to the mRNA.
48. The oligonucleotide of claim 43 having a base sequence selected from the group consisting of (i) SEQ ID NOS. 1-92 and 113-126 and (ii) SEQ ID NOS. 1-92 and 113-126 containing a one base insertion, deletion or substitution.
49. A pharmaceutical composition comprising at least one oligonucleotide of any one of claims 43-48 in combination with a pharmaceutically acceptable carrier.
50. A pharmaceutical composition comprising at least two oligonucleotides of any one of claims 43-48 in combination with a pharmaceutically acceptable carrier.
51. A method for treating a subject having a disease associated with reduced cAMP comprising administering a pharmaceutical composition of any one of claims 49-50.
52. A method for treating a subject having respiratory inflammation comprising administering a pharmaceutical composition of any one of claims 49-50.
53. Use of a pharmaceutical composition of any one of claims 49-50 for the preparation of a medicament for the treatment of a disease associated with reduced cAMP.
54. Use of a pharmaceutical composition of any one of claims 49-50 for the preparation of a medicament for the treatment of respiratory inflammation.
55. Use of a pharmaceutical composition of any one of claims 49-50 for the treatment of a disease associated with reduced cAMP.
56. Use of a pharmaceutical composition of any one of claims 49-50 for the treatment of respiratory inflammation.

Figure 1A

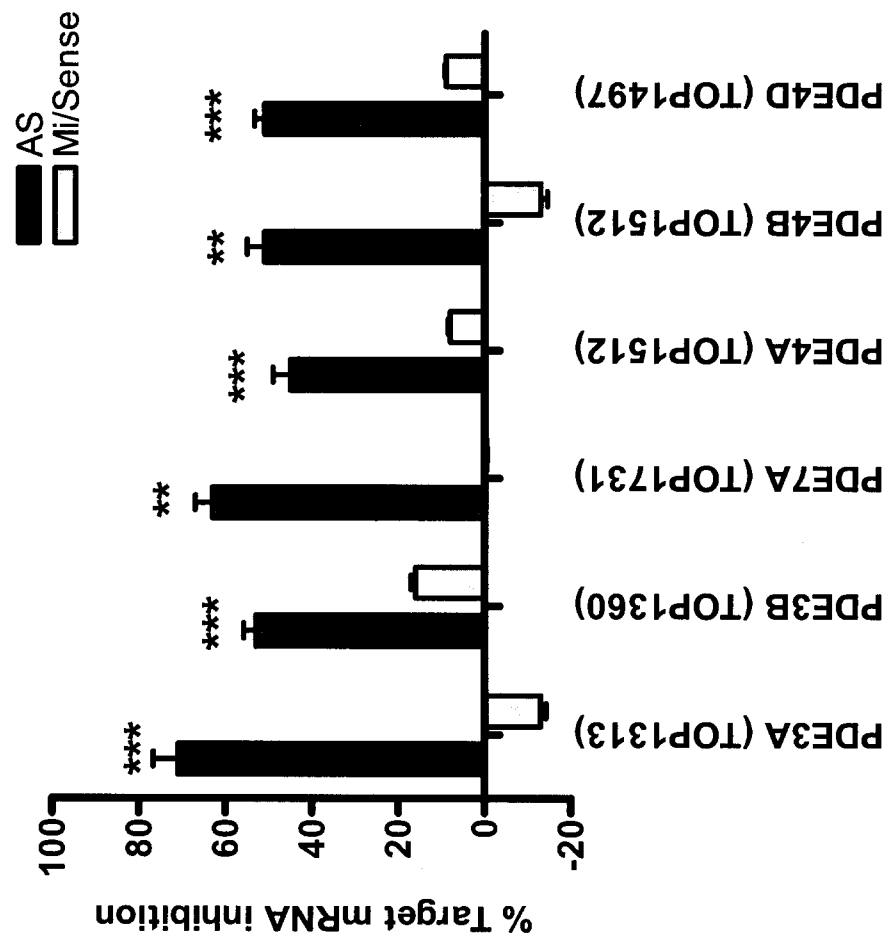


Figure 1b

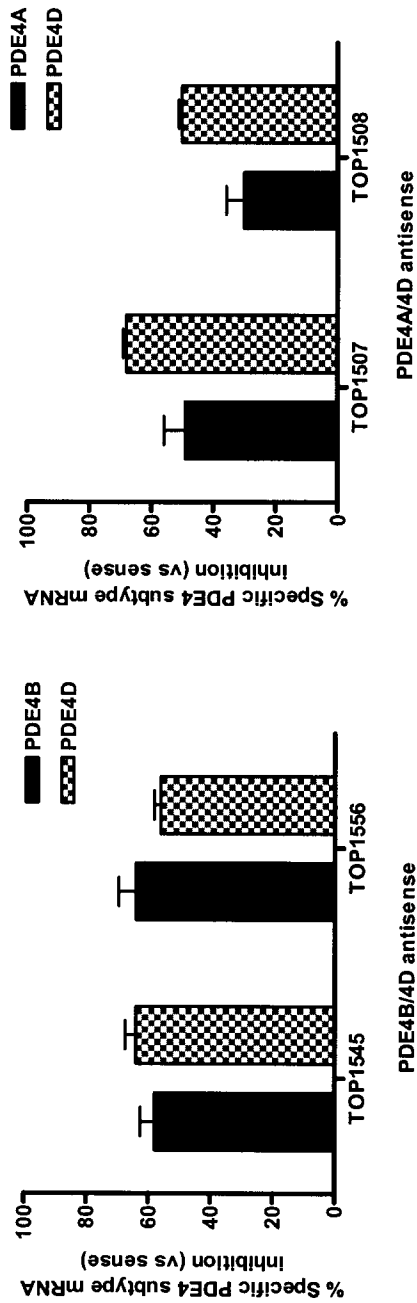
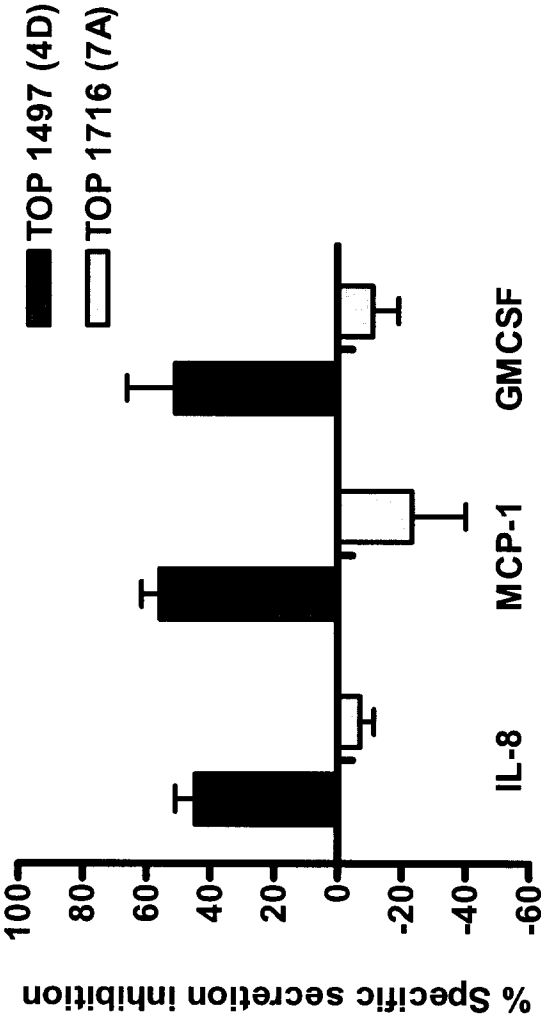


Figure 2
Lung epithelial cells (A549)



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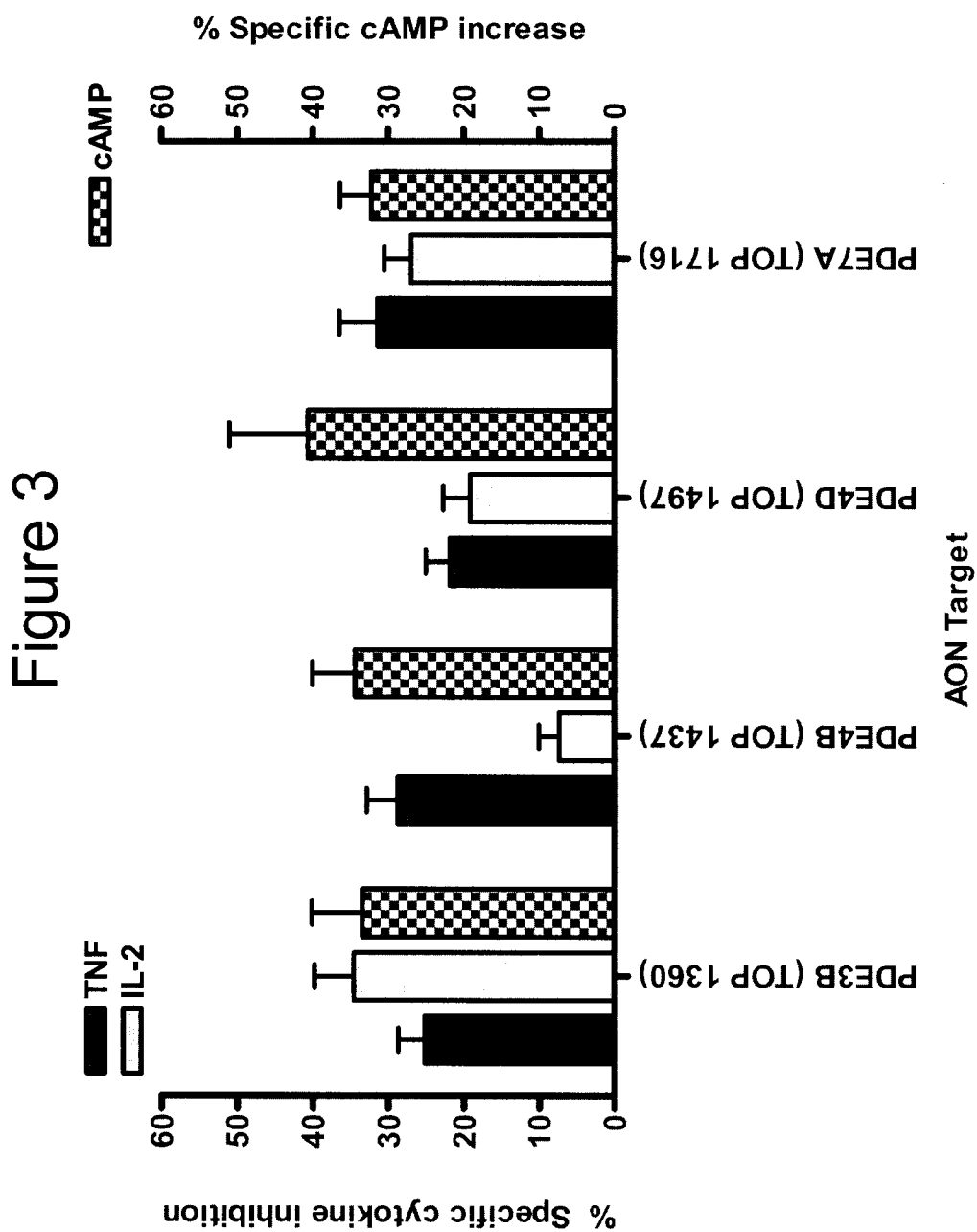
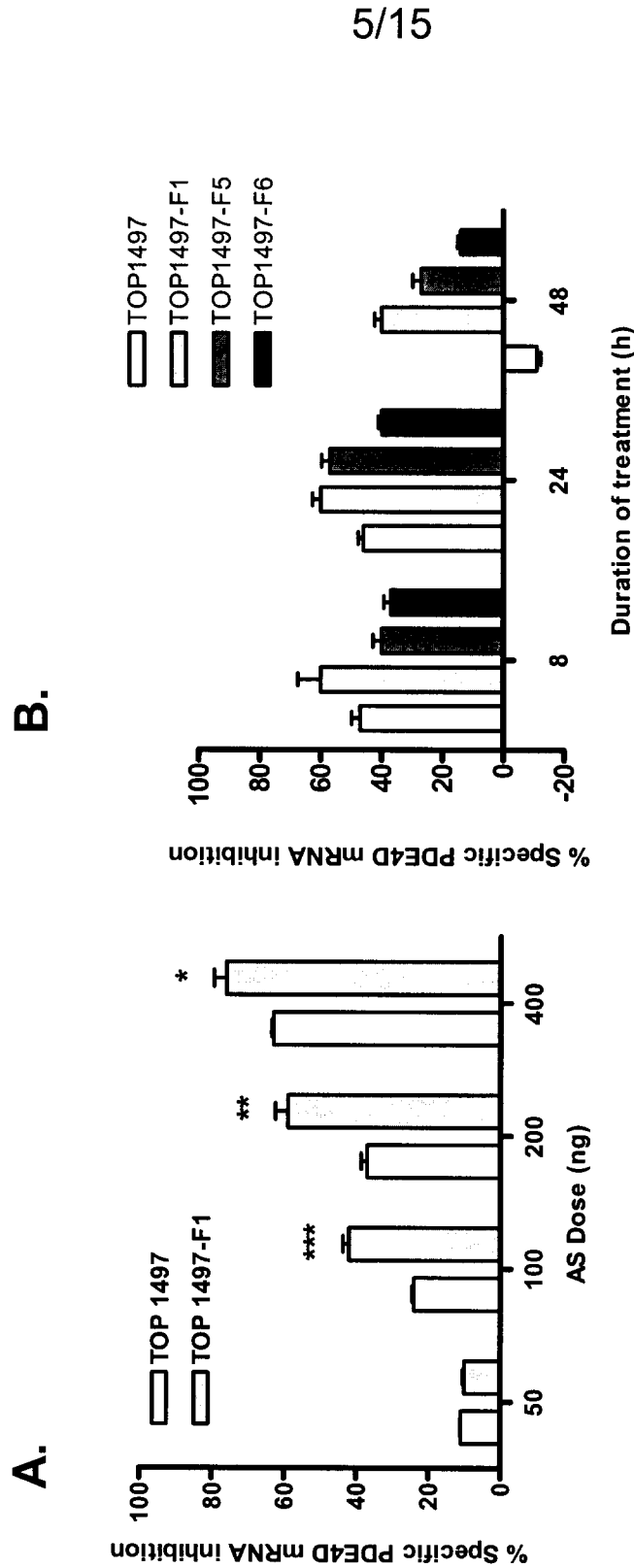


Figure 4



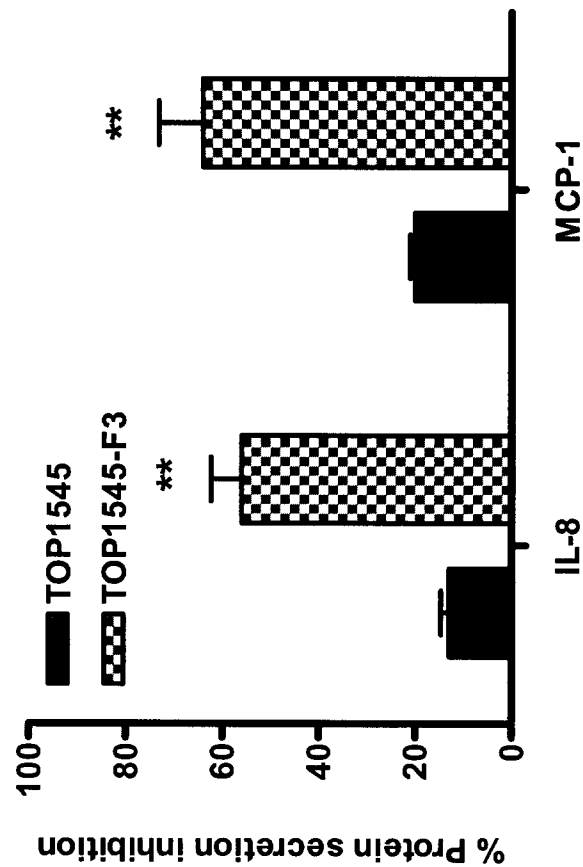
5/15

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TOP1497-F1	CTGCCCTcctctcttca AC CTG
TOP1497-F5	CTgcctcctctcttctca AC CTG
TOP1497-F6	ctgcctcctctcttctca AC CTG

Lower case=PS-DNA
UPPER CASE=PS-FANA

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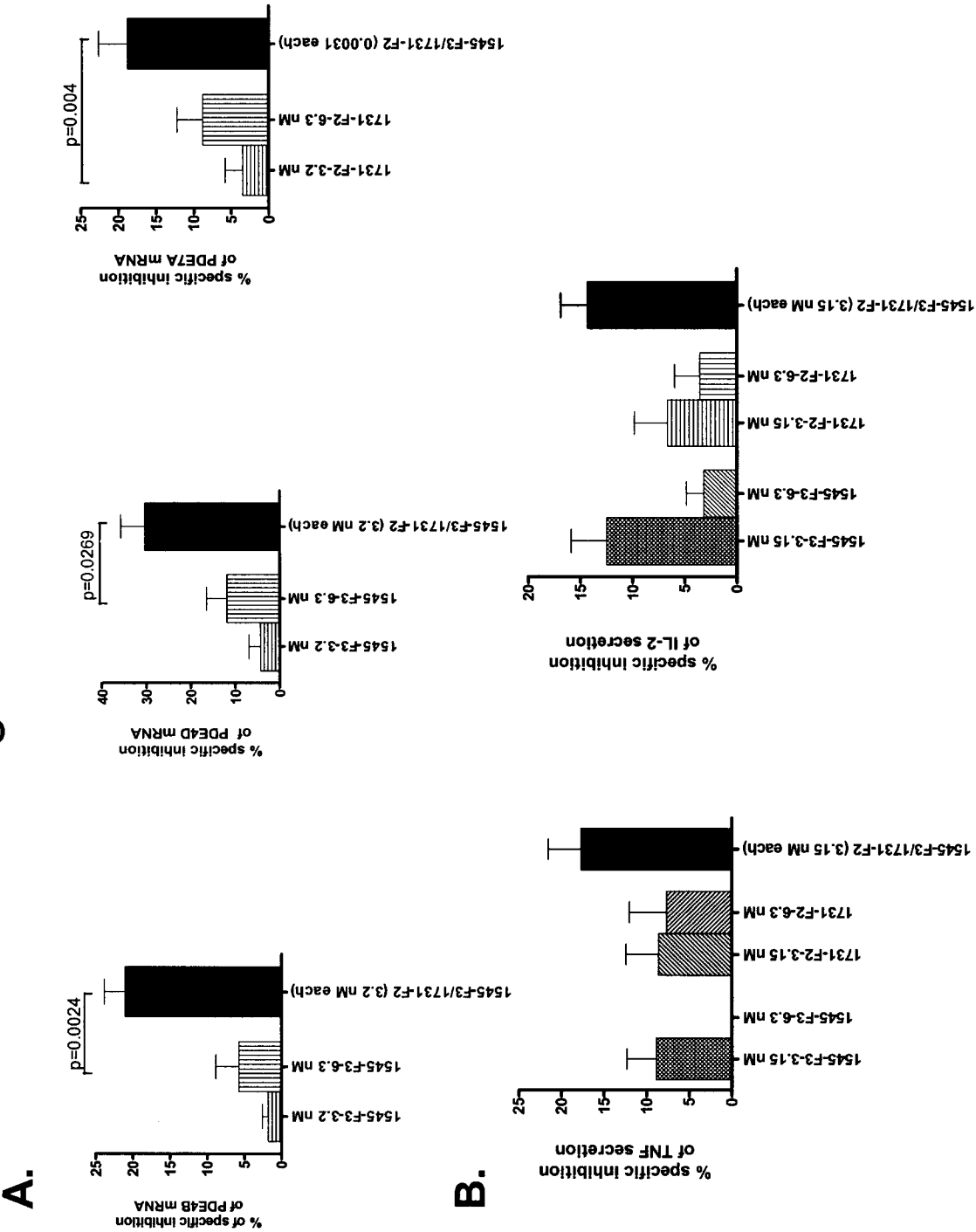
Figure 5



tctgcccatgtctccca	TOP 1545
Tctgcccatgtctccca	TOP 1545-F3

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Figure 6



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Figure 7



Figure 8

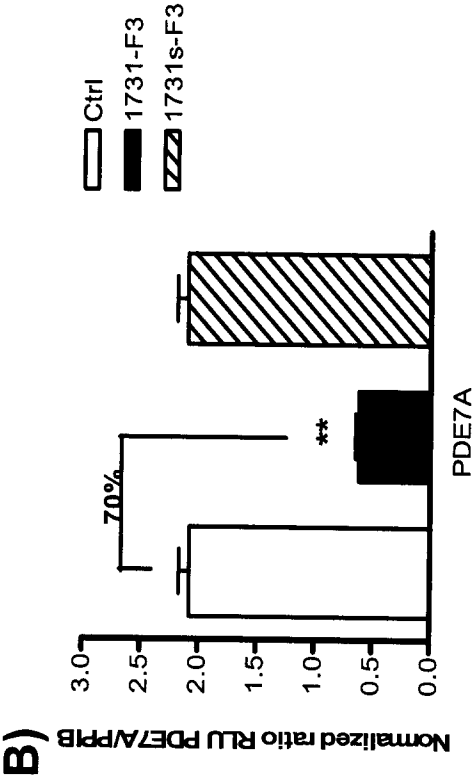
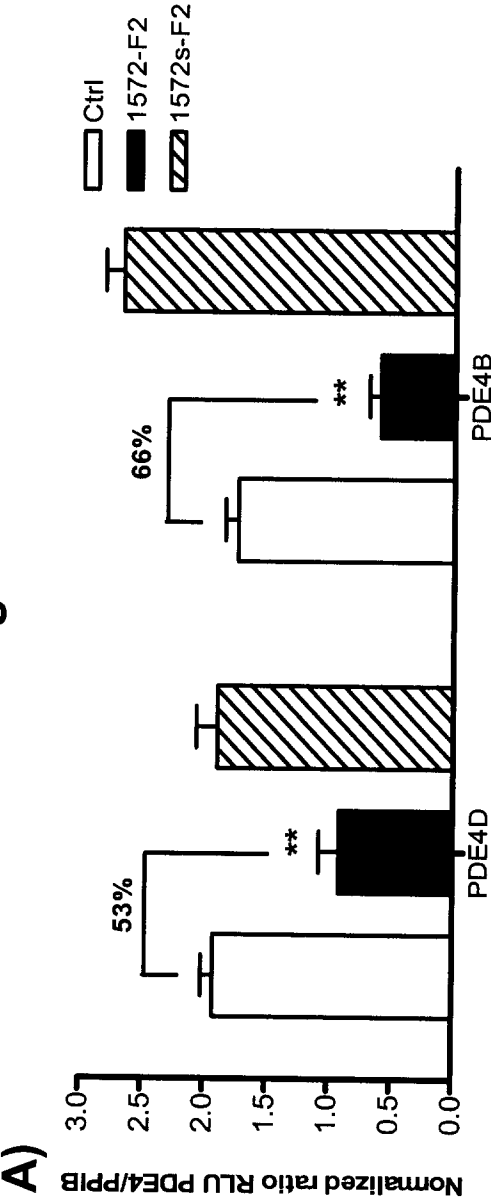
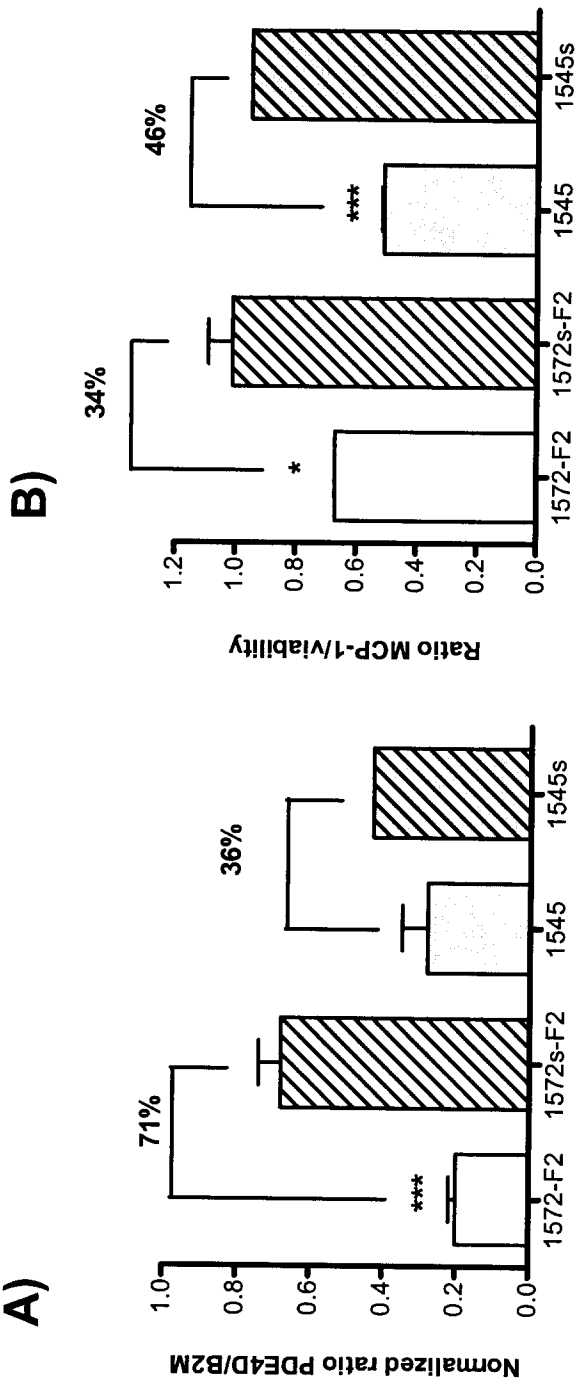


Figure 9



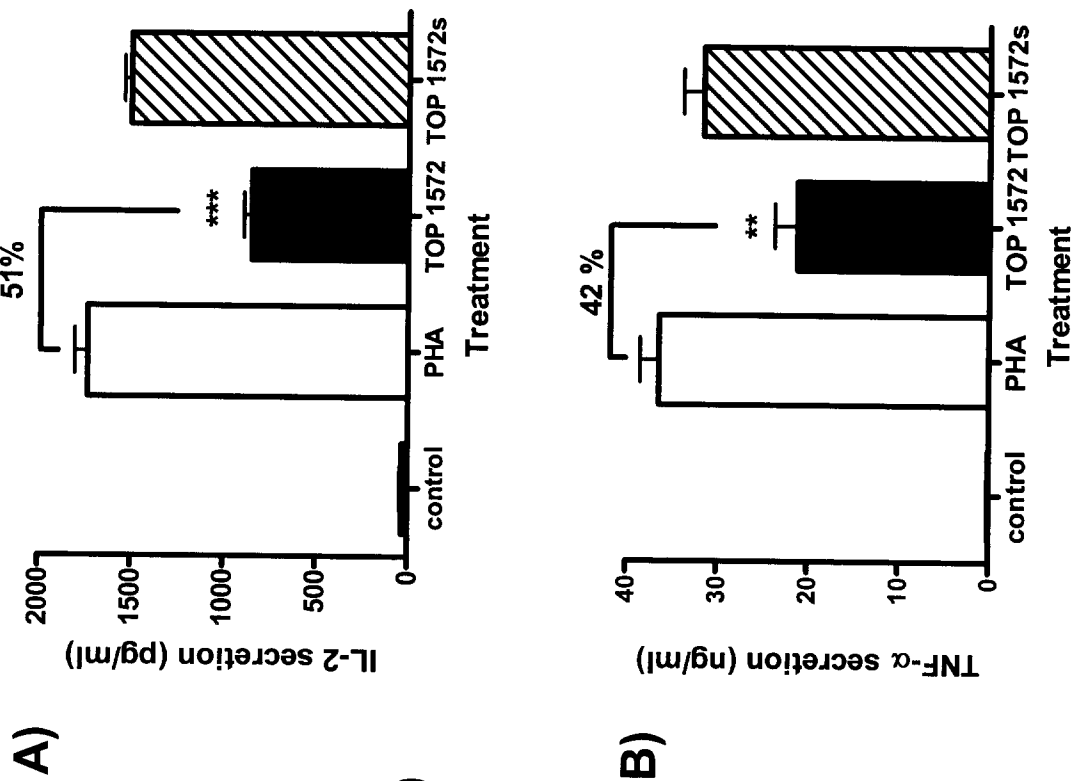


Figure 10

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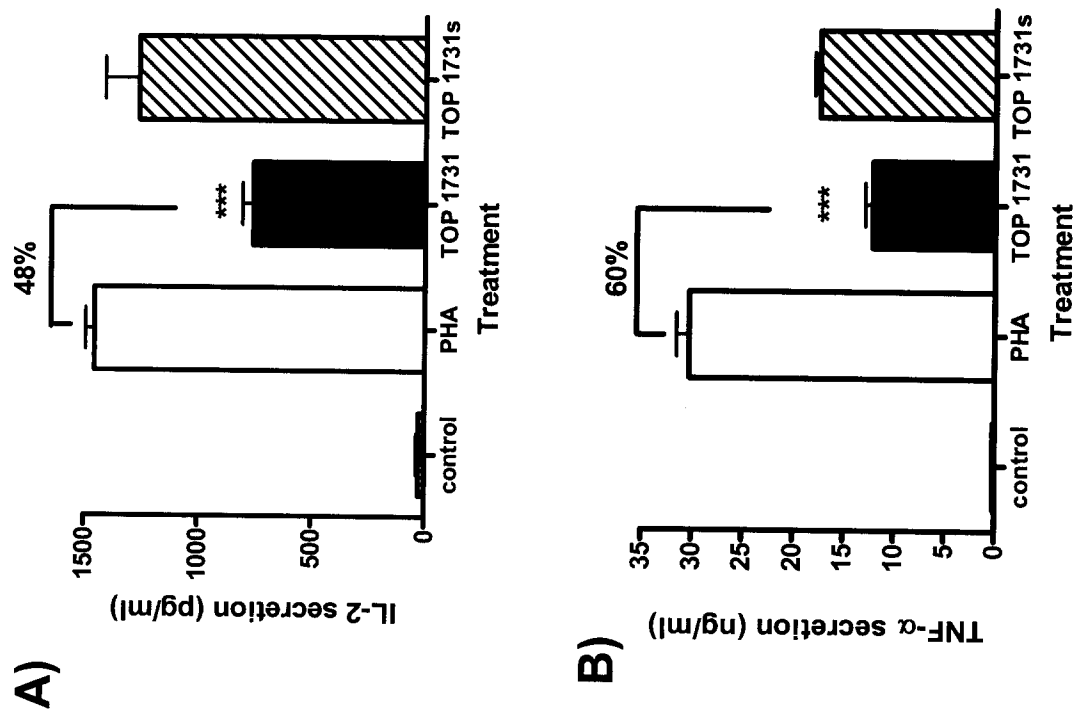


Figure 11

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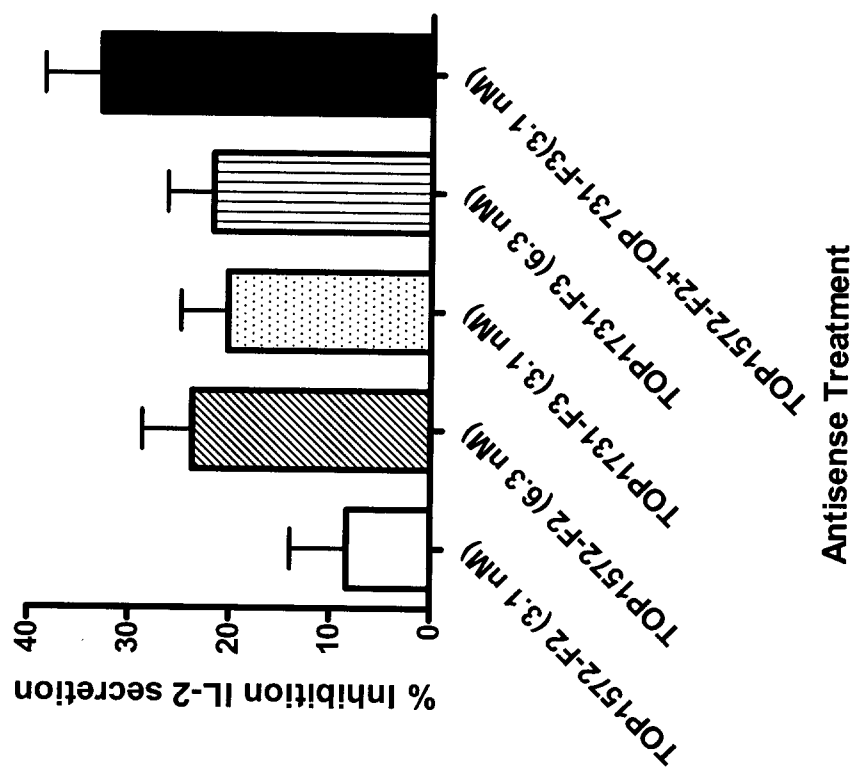
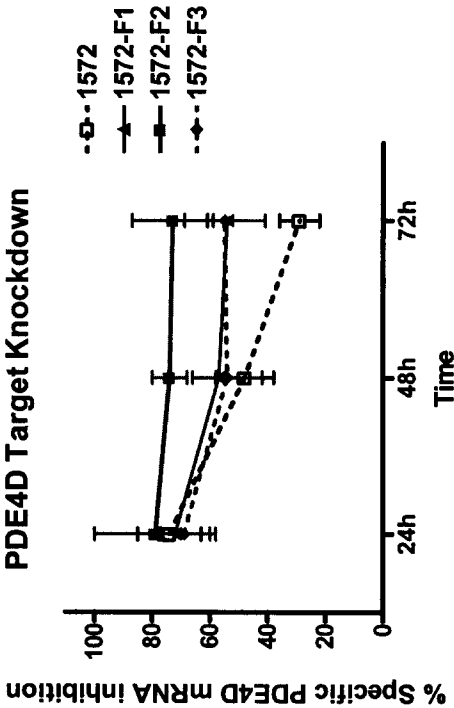


Figure 12

Figure 13

A)



B)

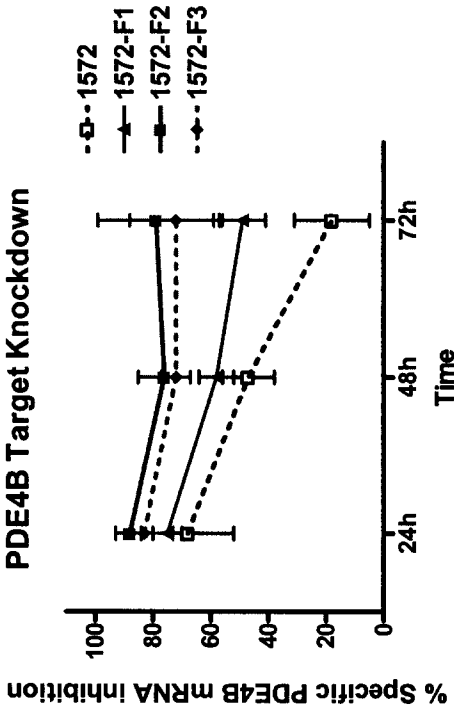
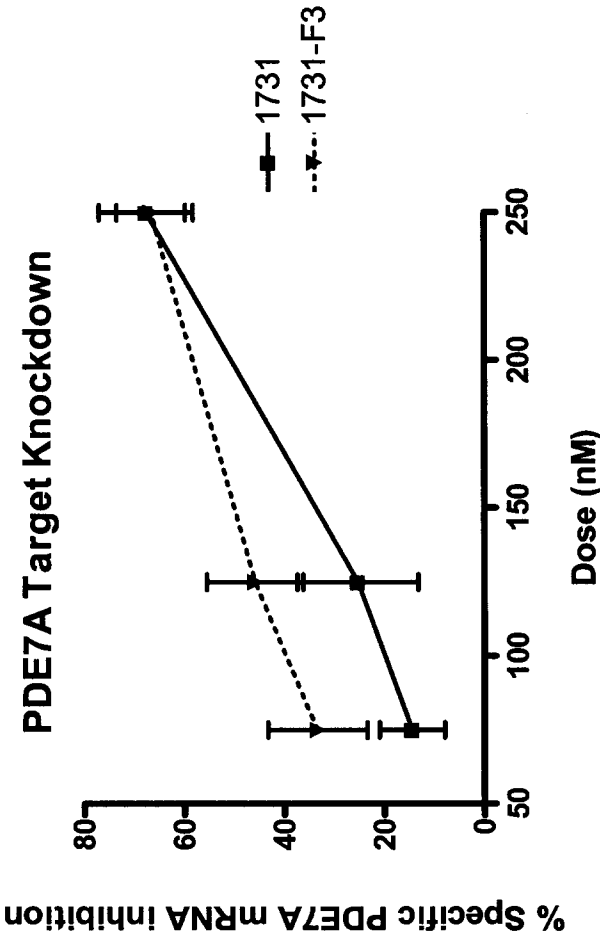


Figure 14



INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA2007/000902

A. CLASSIFICATION OF SUBJECT MATTER

IPC: **C07H 21/00** (2006.01) , **A61K 31/7088** (2006.01) , **A61P 11/00** (2006.01) , **A61P 29/00** (2006.01)
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07H (2006.01), **A61K** (2006.01), **C12N** (2006.01), **A61P** (2006.01)

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

Electronic database(s) consulted during the international search (name of database(s) and, where practicable, search terms used)
Delphion, PubMed, Scopus, GenomeQuest (GQPAT; DerwentGeneSeq; RefSeq mRNA; GB EST; GB HTG; GB GSS; GB STS; ENSEMBL mRNA; PDB Nucleotides; DrugBank Nucleotide)
Key words: phosphodiesterase, antisense, oligonucleotide, PDE4, PDE3, PDE7, expression, cAMP, fluoroarabinose, SEQ ID Nos: 1-92 and 113-126

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	WO 2005/030787 A2 (TOPIGEN PHARMACEUTIQUE INC.) 7 April 2005 Whole document	1-3, 7-10 and 30-41 4-6, 11 and 43-56
X Y	WO 2004/011613 A2 (EPIGENESIS PHARMACEUTICALS INC.) 5 February 2004 Abstract, claims 2-5 and SEQ ID NO: 1788	1-3, 7, 8, 31-33, 35-37 and 39-41 4, 5, 43-50, 52, 54 and 56
Y	KURRECK, J., "Antisense technologies: Improvement through novel chemical modifications", EUR. J. BIOCHEM., 2003, Vol. 270, pages 1628-1644, ISSN 0014-2956 Whole document	4-6, 11 and 43-56
Y	WILDS, C.J. AND DAMHA, M.J., "2'-Deoxy-2'-fluoro-b-D-arabinonucleosides and oligonucleotides (2'F-ANA): synthesis and physiochemical studies", NUCL. ACIDS RES., 2000, Vol. 28, pages 3625-3635, ISSN 0305-1048 Whole document	4-6, 11 and 43-56
X	US 2005/0244851 A1 (BLUME, J.E. and WILLIAMS, A.J.) 3 November 2005 SEQ ID NOS: 2782134 and 4996270	1-3, 7 and 8

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

☒ See patent family annex.

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

Date of the actual completion of the international search

16 July 2007 (16-07-2007)

Date of mailing of the international search report

10 August 2007 (10-08-2007)

Name and mailing address of the ISA/CA
Canadian Intellectual Property Office
Place du Portage I, C114 - 1st Floor, Box PCT
50 Victoria Street
Gatineau, Quebec K1A 0C9
Facsimile No.: 001-819-953-2476

Authorized officer

Christiane Hansen 819- 934-5144

INTERNATIONAL SEARCH REPORTInternational application No.
PCT/CA2007/000902**Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of the first sheet)**

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

1. ☒ Claim Nos. : 30-33, 51 and 52

because they relate to subject matter not required to be searched by this Authority, namely :

Claims 30-33, 51 and 52 are directed to a method for treatment of the human or animal body by surgery or therapy which this Authority is not required to search under Rule 39.1 (iv) of the PCT. Regardless, this Authority has carried out a search based on the alleged effect of the product defined in claims 7 to 29, 49 and 50

2. ☒ Claim Nos. : 1 and 43-48

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

Please see Supplemental Page

3. ☐ Claim Nos. :

because they are dependant claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

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

Please see Supplemental Page

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☒ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claim Nos. :
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim Nos. :

- Remark on Protest** ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

Continuation of Box II:

Claims 1 and 48, in so far as they relate to oligonucleotides having a base sequence defined by SEQ ID NOs. 1-92 and 113-126 and containing a one base insertion, deletion or substitution, and claims 43-47 relate to an extremely large number of possible compounds. Support within the meaning of Article 6 of the PCT and disclosure within the meaning of Article 5 of the PCT is found only for oligonucleotides consisting of SEQ ID NOs. 1-92 and 113-126. For all other oligonucleotides, the claims so lack support and the application so lacks disclosure that a meaningful search over the whole claimed scope is impossible. Consequently, the search in relation to claims 1 and 43-48 has been carried out only for the oligonucleotides consisting of SEQ ID NOs. 1-92 and 113-126.

Continuation of Box III:

Group A - Claims 1- 11, 30-41 and 43-56 (partially) and claims 12-29 and 42 (completely) featuring oligonucleotides directed against PDE7A, compositions containing oligonucleotides directed against PDE7A only, compositions containing an oligonucleotide directed against PDE7A in combination with an oligonucleotide directed against both PDE4B and PDE4D, methods employing said compositions and uses of said compositions.

Group B - Claims 1-8, 30-41 and 43-56 (partially) featuring oligonucleotides directed against PDE3A, compositions containing oligonucleotides directed against PDE3A only, methods employing said compositions and uses of said compositions.

Group C - Claims 1-8, 30-41 and 43-56 (partially) featuring oligonucleotides directed against PDE3B, compositions containing oligonucleotides directed against PDE3B only, methods employing said compositions and uses of said compositions.

Group D - Claims 1-8, 30-41 and 43-56 (partially) featuring oligonucleotides directed against PDE4A, compositions containing oligonucleotides directed against PDE4A only, methods employing said compositions and uses of said compositions.

Group E - Claims 1-8, 30-41 and 43-56 (partially) featuring oligonucleotides directed against PDE4B, compositions containing oligonucleotides directed against PDE4B only, methods employing said compositions and uses of said compositions.

Group F - Claims 1-8, 30-41 and 43-56 (partially) featuring oligonucleotides directed against PDE4D, compositions containing oligonucleotides directed against PDE4D only, methods employing said compositions and uses of said compositions.

Group G- Claims 1-5 featuring oligonucleotides directed against both PDE4A and PDE4B, compositions containing oligonucleotides directed against both PDE4A and PDE4B only, methods employing said compositions and uses of said compositions.

Group H- Claims 1-8, 30-41 and 48-56 (partially) featuring oligonucleotides directed against both PDE4A and PDE4D, compositions containing oligonucleotides directed against both PDE4A and PDE4D only, methods employing said compositions and uses of said compositions.

Group I- Claims 1-8, 30-41 and 48-56 (partially) featuring oligonucleotides directed against both PDE4B and PDE4D, compositions containing oligonucleotides directed against both PDE4B and PDE4D only, methods employing said compositions and uses of said compositions.

The claims must be limited to one inventive concept as set out in Rule 13 of the PCT.

Reference is made to the following documents:

D1: WO 2005/030787 A2 (TOPIGEN PHARMACEUTIQUE INC.) 7 April 2005

D3: KURRECK, J., EUR.J.BIOCHEM., 2003, Vol. 270, pages 1628-1644

D4: WILDS, C.J. and DAMHA, M.J., NUCL. ACIDS RES., 2000, Vol. 28, pages 3625-3635

Prior art document D1, for example, already discloses antisense oligonucleotides directed against various different phosphodiesterases, compositions containing said oligonucleotides, methods employing them and uses thereof. In addition, the presently disclosed oligonucleotides lack a common structural feature that could serve as a unifying element. As can be seen from documents D3 and D4, oligonucleotides with a phosphorothioate backbone and oligonucleotides containing a 2'-deoxy-2'-fluoroarabinonucleotide are known in the art. As a result, the oligonucleotides of the present application lack a unifying element and as such, each oligonucleotide may be considered to be a separate invention. To facilitate examination, the above groups of inventions are suggested. However, for the purposes of the present search report, all searchable claims, as indicated in Box II, have been searched.

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/CA2007/000902

Patent Document Cited in Search Report	Publication Date	Patent Family Member(s)	Publication Date
WO2005030787 A2	07-04-2005	AU2004275903 A1	07-04-2005
		BRPI0414886 A	12-12-2006
		CA2539752 A1	07-04-2005
		CN1918175 A	21-02-2007
		EP1668024 A2	14-06-2006
		JP2007506440T T	22-03-2007
		US2005153919 A1	14-07-2005
WO2004011613 A2	05-02-2004	AU2003268032 A1	16-02-2004
US2005244851 A1	03-11-2005	NONE	