Microdose DNA for the Treatment of Acute and Chronic Respiratory Diseases and Otitis Media

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Acute and chronic respiratory diseases such as common colds, allergic rhinitis, sinusitis, bronchitis, mucositis, asthma, emphysema, chronic obstructive pulmonary disease (COPD) and cystic fibrosis, as well as otitis media, have different etiologies. Causative agents include microorganisms such as viruses (colds) and bacteria (otitis), environmental insults such as cigarette smoke (COPD), radiation therapy (mucositis) and gene mutations (cystic fibrosis). However, these diseases share some common clinical features, including inflammation, bronchial and/or sinus congestion, obstructed airflow, and the production of large amounts of sputum and/or excessive nasal mucus. Many of these diseases may also have common physiological and immunological characteristics.[1-19]

Cystic fibrosis (CF) is an often-fatal genetic disorder of exocrine function characterized by abnormally viscous mucus secretions. Such secretions precede chronic pulmonary obstruction, pancreatic insufficiency and elevated sweat sodium and chloride levels. The viscosity of sputum produced by CF patients is believed to result from a high content (approximately 10% of the total sputum dry weight) of DNA released from necrotic neutrophils in the sputum.[2,12,14,16,20-22] This observation has led to the use of DNase (Dornase alfa; Pulmozyme) as a CF therapy in conjunction with the antibiotics, bronchodilators and corticosteroids regularly used in the treatment of CF.[23-25] The rationale for such therapy is that degrading DNA in sputum reduces the viscosity of the sputum and results in an increased ability of the patient to evacuate sputum from the lungs and nasal passages.[23-25]

As the presence of neutrophil DNA in the sputum of CF patients suggested an aberrant compensatory immune response, it was hypothesized that mammalian DNA itself could be employed as a neutralization therapy. This hypothesis became the basis for the development of a sublingually administered therapeutic composed of a proprietary formulation of DNA fragments derived, initially, from calf thymus DNA.[26] The hope was that, in the case of CF, sublingual dosing with DNA would ultimately lead to decreased neutrophil necrosis and DNA release into the sputum in the lungs, which would in turn result in decreased sputum viscosity.[26] In evidence-based clinical testing, the DNA therapeutic was successful at reducing mucus viscosity and/or decreasing mucus accumulation in the respiratory tracts of a number of CF patients. Subsequently, this sublingual therapeutic approach was extended to respiratory diseases other than CF. While the specific mechanism(s) of action are still being elucidated, it was found that the DNA therapeutic had broader application to a variety of acute and chronic respiratory diseases as well as to the treatment of otitis media.[27-31] Both calf thymus DNA and salmon sperm DNA have been used in clinical testing. The salmon sperm DNA was found to have therapeutic activity equivalent to that of the bovine derived DNA. In addition to eukaryotic vertebrate DNA, prokaryotic (bacterial) DNA and synthetic DNA have also been evaluated for activity in treating various respiratory ailments. It is speculated that the source of DNA may be less consequential than the method by which microdose DNA is formulated.

Milkhaus Laboratory, Inc., developed the original product, named HP-3 or ML-03, using eukaryotic vertebrate DNA. HP-3/ML-03 was active in three separate FDA-approved, placebo-controlled, double-blind Phase II clinical

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trials for the treatment of chronic bronchitis, COPD and CF. The current DNA-based therapeutic, derived from salmon testicle DNA, is being sold commercially as Mucolyxir.™ It has been referred to as microdose DNA because it is administered in microgram-range doses. The purpose of this article is to describe the product, discuss possible mechanisms of action and summarize clinical trial results. Evidence-based clinical experiences with microdose DNA as a treatment for a variety of respiratory conditions as well as otitis media have been described in detail in the patent literature and are summarized in a separate article.[26-32]

PRODUCT DESCRIPTION

DNA for use in Mucolyxir™ is eukaryotic DNA, specifically salmon testicular DNA obtained from a commercial source. The DNA is solubilized in sterile phenolated saline, and proprietary methods are used to generate a mixture of DNA fragments within a definable range of molecular weights. It is administered as sublingual drops. Although previous clinical investigations with microdose DNA employed solutions of 12 ug/ml (about 0.6 ug/dose based on a drop volume of about 50 ul), Mucolyxir™ is formulated in its liquid vehicle at a concentration of 6 ug/ml (approximately 0.3 ug/dose). A typical administration regimen is 1-2 sublingual drops one to four times daily.

PROPOSED MECHANISMS OF ACTION

Reduced congestion, decreased mucus viscosity and/or decreased inflammation in the upper and lower airways leading to improved respiratory functions are common observations in patients treated with microdose DNA. Based on dosage (low-level) and administration route (generally sublingual), microdose DNA may promote or restore homeostasis within the respiratory tract by an as yet uncharacterized signaling pathway(s) involving the body's regulatory systems, notably (though not limited to) the immune system. There have been several proposed hypotheses regarding specific mechanisms by which microdose DNA can alleviate various respiratory ailments. While each of these hypotheses is considered separately, one cannot exclude the possibility of multiple and/or interrelated and/or other mechanisms being responsible for the observed effects.

1. Immunotherapy via Desensitization, Hyposensitization or Neutralization

The original concept, proposed in the early 1990s, for utilizing a low dose of exogenous mammalian DNA as a treatment for CF was that it could generate an uncharacterized molecular signal that would trigger a localized immune response to alleviate the buildup of DNA in the sputum of afflicted individuals. The rationale for this approach was based on principles of allergy immunotherapy, in which repeated administration of a potential allergen would result in a decreased immune or inflammatory response.[33-38] The net effect would be reduced disease symptoms, i.e., reduced sputum/mucus production, inflammation and airway obstruction. It was hypothesized that the DNA released from necrotic neutrophils was itself acting as an allergen of sorts, promoting inflammation in CF patients. One problem with this hypothesis was that microdose DNA was found to be effective in treating respiratory diseases other than CF. Interestingly, however, the potential of microdose DNA as an immunotherapeutic substance may actually have been ahead of its time, as the concept of using specific immunostimulatory DNA sequences (CpG DNA) for the immunotherapy of acute and chronic respiratory diseases did not appear in the literature until the latter part of the decade and into the 21st century.[39-41]

2. Stimulation of Chloride Secretion and Mucociliary Clearance via Interaction with P2 Receptors

Although DNA in the sputum of CF patients increases its viscosity, exogenous purine and pyrimidine nucleosides such as ATP and UTP can, by interacting with P2 receptors and stimulating chloride secretion, improve mucociliary clearance in CF and other respiratory ailments characterized by excessive sputum production.[42-45] Subsequently, it was hypothesized that microdose DNA may alleviate respiratory conditions such as CF and bronchitis by interacting with P2Y receptors responsible for stimulating mucociliary clearance within the respiratory tract. P2Y receptors represent a family of 7-transmembrane G-protein–coupled receptors. Purine and pyrimidine nucleotides/nucleosides are known ligands for this family of receptors.[46-48] A specific P2Y receptor, P2Y(2), is present in a variety of airway epithelial cell types, such as the ciliated epithelial cells and goblet cells of the trachea and bronchi, as well as in middle ear ciliated epithelial cells.[49-51] Activation of P2Y(2) appears to result in increased mucociliary clearance in the lungs and other respiratory tract tissues and in the middle ear.[51-53] P2Y(2) receptor agonists, notably nucleoside triphosphates such as adenosine 5'-triphosphate (ATP) and uridine 5'-triphosphate (UTP) and related analogs, have been shown to promote a variety of activities related to improved mucociliary clearance. This includes stimulation of chloride secretion in human airway epithelial cell cultures, stimulation of mucin secretion in human nasal and tracheobronchial tissue explants, modulation of ion transport in an in vitro middle-ear epithelial cell line, increased cilia beat frequency in human airway epithelial cells in vitro, and increased tracheal mucus velocity, whole-lung mucociliary clearance and cough clearance in animals and in human respiratory disease patients.[42-43,45,51-60] However, when tested in vitro, it was determined that microdose DNA was not acting as a P2Y(2) receptor agonist (unpublished data). The possibility that microdose DNA could be affecting P2 receptors other than P2Y(2) requires further investigation.

3. Generation of Beneficial Immune Responses through Changes in Th1/Th2 Cytokine Ratios

Another viable hypothesis for the beneficial effects of microdose DNA in treating various respiratory diseases is that it is acting as an immunostimulant capable of altering certain cytokine imbalances. This hypothesis reflects on the original rationale for microdose DNA usage–the generation of a molecular signaling pathway that would result in a favorable immune response.

The immunostimulatory effects of bacterial extracts, specifically extracts of *Mycobacterium bovis* bacillus Calmette Guerin (BCG), and their application to host defense have been investigated since the late 19th century. Eventually (about a century later), it was determined that unmethylated nucleotides derived from the BCG DNA that contained a specific nucleotide sequence, CpG, were responsible for the observed beneficial immunostimulatory effects.[39-40, 61-63] Oligodeoxynucleotides (natural or synthetic) containing the CpG motif, referred to in the literature as immunostimulatory DNA sequences, can be readily detected by vertebrate innate immune receptors (toll-like receptors), including those on dendritic cells, macrophages, monocytes and neutrophils.[62,64-68] Toll-like receptor signaling by immunostimulatory DNA sequences leads to changes in cytokine production.[69-74] In respiratory ailments such as viral infections and allergen-induced respiratory inflammation, the ratio of type-1 and type-2 T-helper cells (Th1/Th2 ratio) is altered. Specifically, the Th2-type humoral immune response is enhanced, while the Th1-type cell-mediated immune response may be suppressed, leading to unfavorable alterations in cytokine balances that favor a given respiratory disease state.[75-80] Immunostimulatory DNA has been shown to balance and enhance the immune response via normalization of Th1/Th2 cytokine ratios. Consequently, the Th2 response is reduced and/or the Th1 response is enhanced to restore homeostasis in ailments such as respiratory syncytial virus infections, asthma and allergies that affect the respiratory tract.[61-62, 81-88] In vivo, immunostimulatory DNA administered mucosally or systemically has been shown to reduce inflammation and inhibit airway remodeling and airway hyper-responsiveness in a rodent allergic rhinitis model.[89] In a primate model of allergic asthma, airways from nucleotide-treated monkeys had thinner basement membranes, fewer mucus cells, fewer eosinophils and fewer mast cells than controls.[90]

A key issue with the hypothesis that microdose DNA has immunostimulatory effects analogous to that of CpG DNA is that the former is derived from eukaryotic DNA rather than from prokaryotic DNA. Not only does eukaryotic DNA contain significantly fewer CpG motifs than does prokaryotic DNA, there is more DNA methylation. The presence of relatively few unmethylated CpG motifs in eukaryotic DNA makes it less immunostimulatory than prokaryotic DNA.[63, 84, 91-95] However, it is speculated that the resulting combination of DNA sizes and sequences resulting from product preparation may be capable of generating a beneficial immune response to alleviate the above-named respiratory diseases in a manner analogous to that of Immunostimulatory DNA of prokaryotic origin. Thus, more in vitro and in vivo research with microdose DNA is required in order to validate the hypothesis that Mucolyxir™ can act as an immunostimulatory agent in the manner of CpG DNA.

4. Antiinflammatory Effects Such as Decreased Production of Proinflammatory Cytokines Increased Production of Antiinflammatory Cytokines

A fourth hypothesis is that microdose DNA can ameliorate various respiratory diseases by acting as an antiinflammatory agent. While immunostimulatory DNA sequences containing CpG motifs can have beneficial therapeutic effects, excessive stimulation of the innate immune system by bacterial and CpG DNA can cause detrimental effects, including inflammation, tissue damage and autoimmune diseases.[96-100] In fact, mammalian DNA, and specific sequences derived from mammalian DNA, have been shown to block activation of the immune system and decrease the production of proinflammatory cytokines caused by prokaryotic DNA or immunostimulatory DNA sequences with CpG motifs through an as yet uncharacterized mechanism(s).[96-99] Such antiinflammatory effects been demonstrated both in vitro and in an in vivo lung inflammation model.[99] As further evidence of the antiinflammatory effects of mammalian DNA, methylated calf thymus DNA was used to block immune activation by CpG DNA derived from the bacterium *Escherichia coli.*[101]

Antiinflammatory effects have also been demonstrated with exogenous nucleosides. Inosine, a purine nucleoside formed by the enzymatic deamination of adenosine, has been shown to have antiinflammatory activities in an in vivo murine model of acute lung inflammation induced by bacterial lipopolysaccharide.[102] Upon intratracheal instillation, inosine suppressed the production of proinflammatory cytokines such as IL-1-beta, IL-6 and TNF-alpha, while production of antiinflammatory cytokine IL-4 was enhanced.[102] Moreover, inosine had other antiinflammatory effects resulting in improved lung morphology, such as reduced polymorphonuclear neutrophil migration, edema, and nitric oxide production.[102] Inosine also had antiinflammatory effects on cultured human monocytes, neutrophils and epithelial cells, and it inhibited production of proinflammatory cytokines in vitro.[103-104] The mechanism of inosine action is unclear; one possibility suggested in the literature was signaling via A2 purinergic receptors.[102] Interestingly, it was noted that while relatively large doses of inosine were required in the in vivo experiments described, a local route of administration might achieve desired anti-inflammatory effects at markedly lower doses.[102]

5. Decreased Mucus Viscosity and Improved Mucociliary Clearance via Increased Production of Antiinflammatory Cytokine IL-4

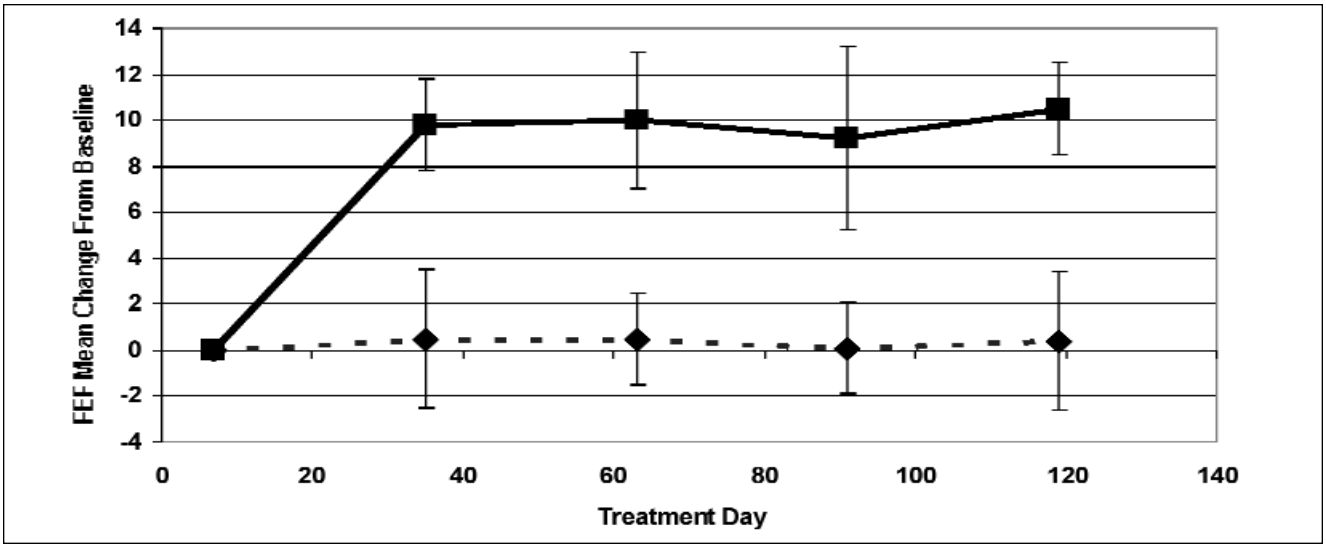
In addition to antiinflammatory effects, IL-4 has been shown to decrease sodium absorption while increasing chloride secretion in cultured lung cells.[105] Such changes in ion transport in vivo could facilitate improved airway surface liquid properties, notably decreased mucus viscosity and increased mucociliary clearance.[105-106] Accordingly, it is hypothesized that if microdose DNA can increase the production of IL-4, this would both decrease inflammation and beneficially increase hydration of airway surfaces. These effects could be important in respiratory diseases such as cystic fibrosis, in which there is a need to decrease the viscosity of the mucus and/or improve mucociliary clearance. However, IL-4 is not only an important antiinflammatory cytokine,[107-109] but also a key cytokine involved in Th2-type humoral immune response.[110-112] Cytokine imbalances favoring a Th2 immune response have been implicated in allergic respiratory diseases such as asthma.[77-80, 111-112] As microdose DNA has in fact been successfully used for treating asthma and other allergic respiratory diseases,[26-31] it is speculated that enhanced IL-4 production by microdose DNA could help restore a favorable balance between airway surface fluid absorption and secretion within the respiratory tract.

CLINICAL TRIALS OF MICRODOSE DNA (HP-3)

Microdose DNA has been tested in three FDA-authorized, double-blind, placebo-controlled Phase II clinical trials for efficacy in the treatment of a) chronic bronchitis, b) COPD and c) CF. The chronic bronchitis trial involved 49 eligible patients with the disease as defined by the American Thoracic Society. They were randomized to either microdose DNA (25 patients) or placebo (24 patients). After an initial 7-day placebo lead-in period, patients were treated with sublingually administered drops of either microdose DNA or placebo (vehicle control) daily for 90 days, with a subsequent evaluation one month after termination of treatment. Patients treated with microdose DNA showed a marked increase in sputum expectoration, clearing of the airways and significantly improved respiratory capacity. FEV_1 (forced expiratory volume in one second, a measure of airflow rate) increased over 5%, and $FEF_{25-75\%}$ (forced expiratory volume, a measure of small airway function) showed a statistically significant clinical improvement ($p = 0.007$; Figure 1). There were no serious adverse events related to treatment with microdose DNA, and the type and frequency of reported side effects were comparable in the treatment and placebo groups.

The COPD trial involved 48 eligible patients with the disease as defined by the American Thoracic Society. This study was conducted at a single site, with patients randomized to either microdose DNA (23 patients) or placebo (25 patients). Patients were treated with sublingually administered drops of either microdose DNA or placebo daily for 83 days subsequent to a 7-day placebo lead-in period. Initial and final evaluations involved a number of disease-related parameters. Significant improvement was seen in the blood oxygenation test (measured by pulse oximetry) and in the Distance Walked in 6 Minutes test ($p = 0.019$; Figure 2). Interestingly, many of the COPD patients had dry coughs with little or no sputum production; hence there was no increase in sputum output. As with the chronic bronchitis clinical trial, there were no serious adverse events related to treatment with microdose DNA. The type and frequency of side effects in the treatment and placebo groups were comparable.

Figure 1. Mean change in baseline values (mean +/- standard error of the mean) in the $FEF_{25-75\%}$ (forced expiratory volume in the middle half of the testing range) in chronic bronchitis patients treated with either microdose DNA (square) or placebo (diamond).



There were 37 patients (randomized to 17 treatment, 20 placebo) enrolled in the CF clinical trial. All patients in this trial had either mild or moderate disease. Patients were treated for 8 weeks with sublingual drops of microdose DNA or placebo. Evaluations were conducted at 1, 4 and 8 weeks after treatment was initialized. The main parameters evaluated were FEV_1, the FEV_1/FVC (forced expiration volume/forced vital capacity) ratio, a measure of airway obstruction, and the $FEF_{25-75\%}$. Patients treated with microdose DNA showed a trend toward clinical improvement in all 3 parameters (Figures 3A-3C). Statistical significance was not achieved owing to both the small number of subjects and the fact that this study was limited to patients with mild or moderate CF. However, these results, coupled with observed subjective increases in sputum clearance, underscored the potential of microdose DNA in improving pulmonary function in CF patients.

Figure 2. Mean change from baseline values (mean +/- standard error of the mean) in the Distance Walked in 6 Minutes test of COPD patients treated with either microdose DNA (square) or placebo (diamond).

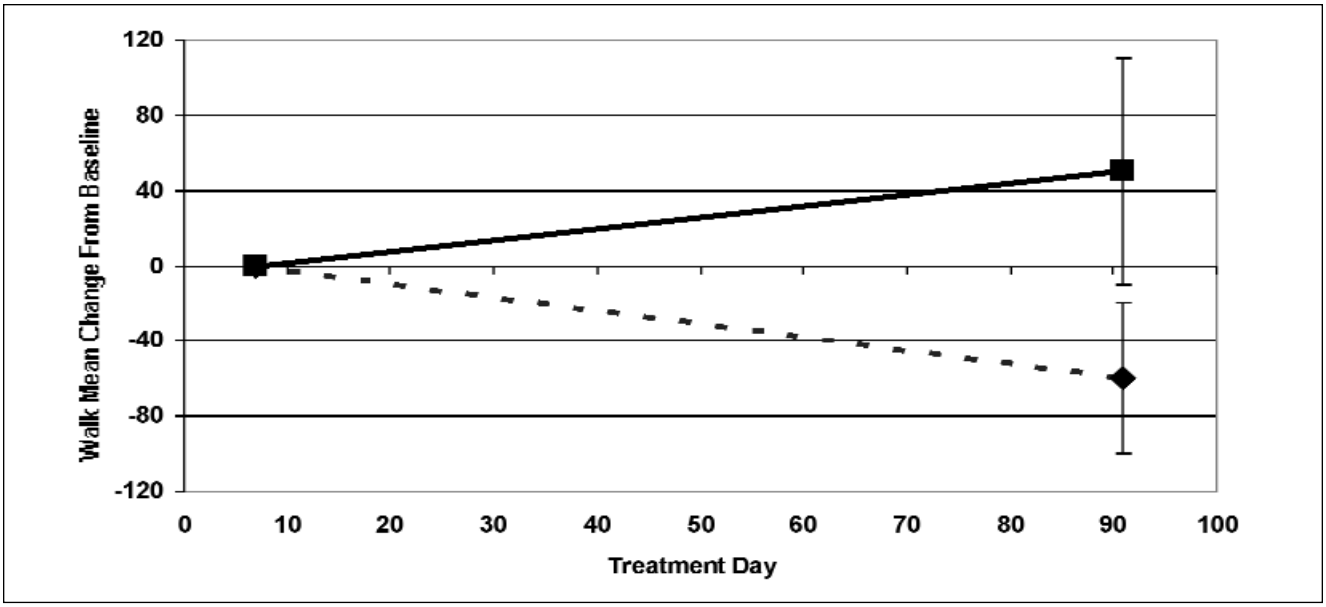


Figure 3A. Mean percent change from baseline values (mean +/- standard error of the mean) in pulmonary function tests of cystic fibrosis patients treated with either microdose DNA (square) or placebo (diamond). FEV_1= forced expiratory volume in one second.

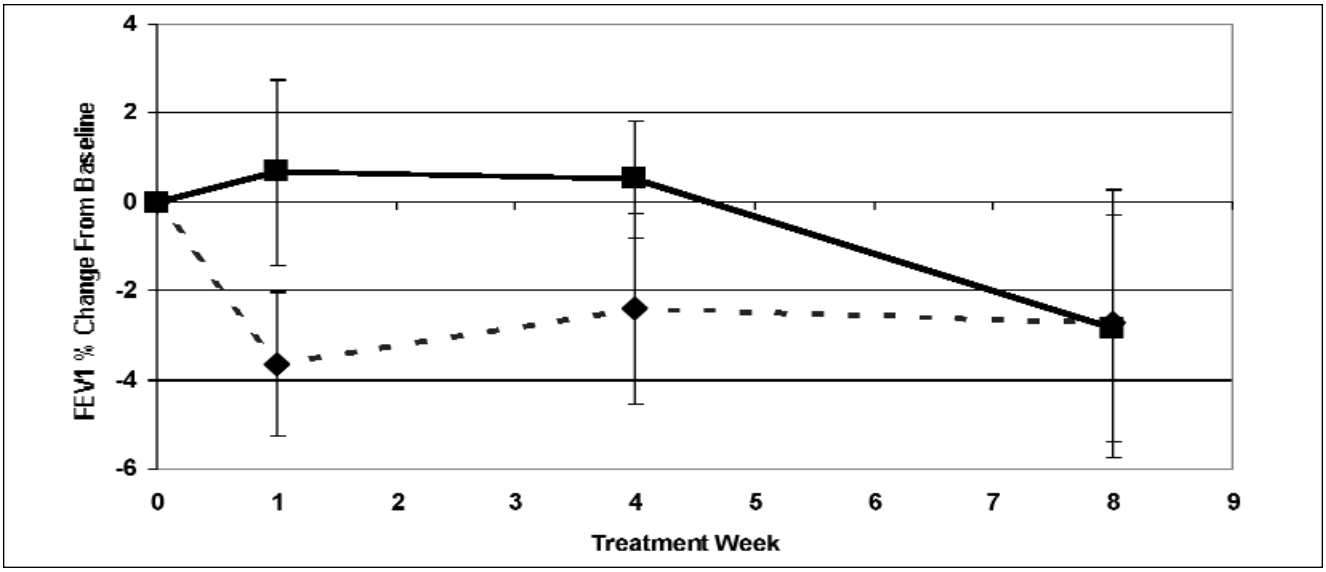


Figure 3B. Mean percent change from baseline values (mean +/- standard error of the mean) in pulmonary function tests of cystic fibrosis patients treated with either microdose DNA (square) or placebo (diamond). FEV_1/FVC = forced expiration volume/forced vital capacity ratio.

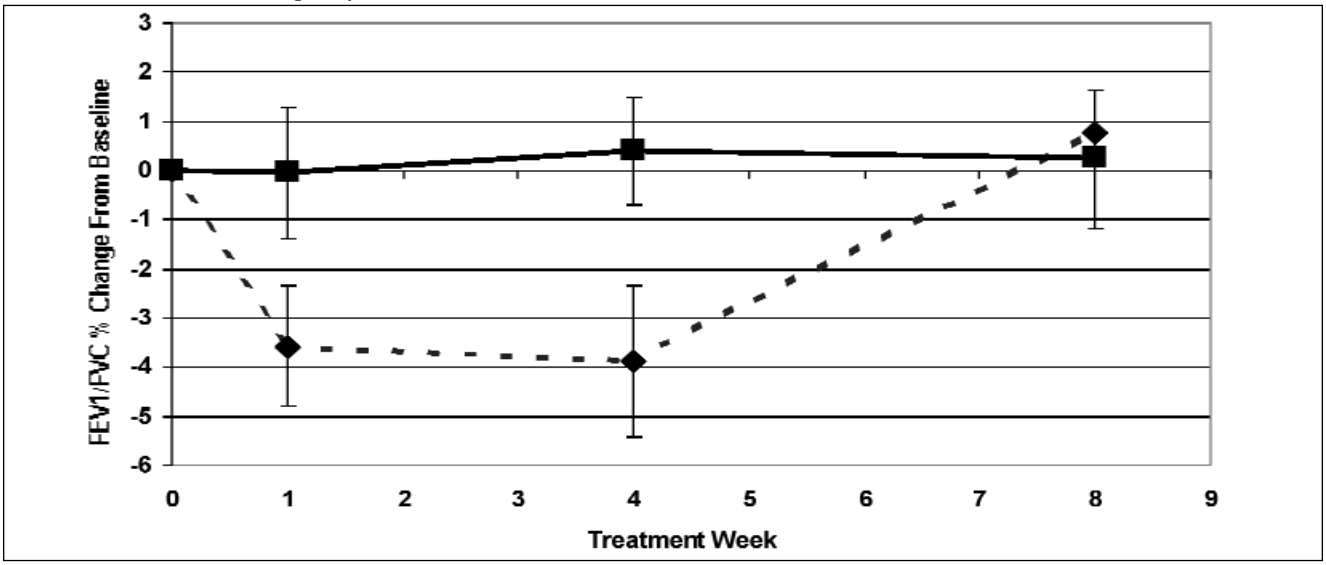
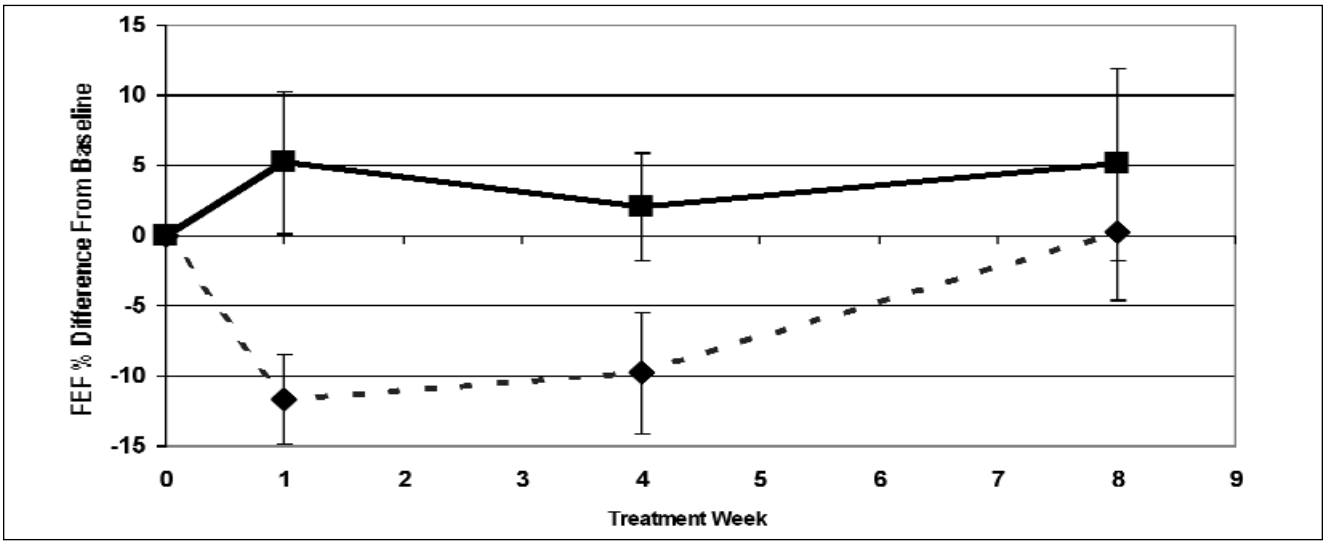


Figure 3C. Mean percent change from baseline values (mean +/- standard error of the mean) in pulmonary function tests of cystic fibrosis patients treated with either microdose DNA (square) or placebo (diamond). $FEF_{25-75\%}$ = forced expiratory volume.



EVIDENCE-BASED CLINICAL EXPERIENCES WITH MICRODOSE DNA

In addition to the clinical trials described above, the utility of microdose DNA in alleviating the symptoms of various respiratory diseases, as well as that of otitis media, has been demonstrated through evidence-based clinical testing. Examples of these clinical experiences, extracted from available patent literature, have been summarized in a separate publication.[32] The results of evidence-based testing suggest that microdose DNA has broad potential utility in relieving symptoms of a variety of respiratory ailments, including CF, COPD, chronic bronchitis, asthma, respiratory allergies, radiation-induced mucositis, respiratory disease resulting from occupational/environmental chemical exposure, chronic upper respiratory illness, respiratory congestion and otitis media.[32] Moreover, these results, along with the Phase II clinical trial data, indicate that microdose DNA has a good safety profile. It also appears that the product can be administered safely in conjunction with other medications.

Additionally, there have been numerous anecdotal reports that immediately upon the onset of cold symptoms, sublingual administration of one or two drops of microdose DNA 4-8 times daily for 5-10 days can alleviate the dura-

tion and/or severity of cold symptoms such as congestion, excessive nasal discharge and sore throat. (Some individuals administer as many as four sublingual drops per hour at cold onset and then reduce that number over the next several days). If meaningful reductions in the duration and severity of common cold symptoms can be demonstrated through formal clinical investigations, microdose DNA may have utility as a favored treatment for cold symptoms.

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