

Multitargeted Approach Using Antisense Oligonucleotides for the Treatment of Asthma

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ABSTRACT: Asthma is characterized by inflammation and hyperresponsiveness related to the accumulation of inflammatory cells, particularly eosinophils, within the airways. We tested the hypothesis that a multitargeted approach is better than a single-targeted approach in a rat model of asthma. We simultaneously delivered oligonucleotides (ODNs) targeting the chemokine receptor CCR3 and the common beta chain subunit of the receptors for IL-3, IL-5, and GM-CSF at the time of ovalbumin challenge in sensitized Brown Norway rats. Fewer eosinophils were detected in bronchoalveolar lavage (BAL) of rats treated with both ODNs as compared to each ODN alone. Moreover, airway responsiveness to LTD₄ was significantly decreased at lower doses in the 2 ODN-treated groups compared to a single ODN. As ODN therapy has raised concerns of toxicity we therefore examined ODNs prepared with modified DNA bases, specifically 2'-amino, 2'-deoxyadenosine (DAP) in place of adenosine. *In vivo*, administration of individual DAP-ODN was efficacious in inhibiting airway hyperresponsiveness, whereas delivery of 2 DAP-ODNs (targeting CCR3 and common beta chain) reduced the influx not only of eosinophils but also lymphocytes and macrophages in the lungs of rats as compared to the unmodified ODNs. Blocking multiple inflammatory pathways simultaneously is more effective in preventing eosinophilia and airway hyperresponsiveness than inhibiting either pathway alone. The challenges associated with the development of a product containing two oligonucleotides in humans are discussed.

KEYWORDS: asthma; common beta chain; CCR3; airway inflammation; airway hyperresponsiveness

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INTRODUCTION

While the potential of antisense oligonucleotides (AS-ODNs) to inhibit gene expression has been identified for over 25 years,¹ challenges have arisen in the practical application of antisense approaches as therapies to treat diseases. These challenges include issues of stability of ODNs *in vivo*, uptake of the ODN by the cellular targets, and toxicity of the ODN in systemic applications.² With these hurdles in mind, our approach was to develop a therapy for the respiratory disease, asthma, using AS-ODN that block the expression of specific genes present in overlapping pathways important in asthma. Characteristics of asthma include airway obstruction, airway hyperresponsiveness (AHR), and accumulation and persistence of inflammatory cells in the airways, specifically the accumulation and subsequent degranulation of eosinophils.^{3–5} Cytokines interleukin-3 (IL-3), IL-5, and granulocyte macrophage-colony stimulating factor (GM-CSF) are involved in this inflammatory process by mediating the survival and activation of eosinophils, as well as activating mast cells and macrophages to release proinflammatory cytokines, lipid mediators, and granules. These released products may then contribute to AHR, tissue damage, airway remodeling.^{6–8} Recruitment of eosinophils and other allergic inflammatory cells to the site of inflammation is mediated by chemokines, such as eotaxin, that bind to the chemokine receptor CCR3 expressed on the cell surface of eosinophils.^{9–12} CCR3 ligation has multiple effects including eosinophil recruitment, proliferation, and progenitor differentiation at the site of respiratory inflammation. Previously we demonstrated that delivery of AS-ODN targeting the common beta subunit of the IL-3/IL-5/GM-CSF receptors reduced eosinophilia and inhibited antigen-induced AHR in a rat model of asthma.¹³ Recently, using the same model we have demonstrated inhibition following the delivery of AS-ODN targeting CCR3 expression.¹⁴ With the results from these individual studies, we decided to determine whether the combined delivery of both AS-ODN together, one targeting the common beta chain of IL-3/IL-5/GM-CSF receptors and the second targeting the chemokine receptor CCR3, would function synergistically in reducing the asthma-like symptoms in the rat model. Such a combined antisense therapy required the determination of the optimal ratio of AS-ODNs, testing the possibility of duplex formation between the AS-ODN molecules in solution and assessing any increased risk of toxicity. We also pursued the advantages of chemically modifying the AS-ODN through the replacement of adenosine with 2' amino, 2' deoxyadenosine (DAP) to reduce potential toxic effects yet retain the efficacy of gene knockdown.

MATERIALS AND METHODS

Synthesis and Design of Rat AS-ODNs

AS-ODNs were designed in house following rules previously defined.¹⁵ AS-ODN 143 (5'- TGG CAC TTT AGG TGG CTG – 3') was found to be effective

at inhibiting mRNA expression of the rat common beta chain receptor for IL-3/IL-5/GM-CSF both *in vitro* and *in vivo*.¹³ For CCR3 antisense, AS-ODN ASA4 (5'- ACT CAT ATT CAT AGG GTG – 3') was found to be effective at inhibiting rat CCR3 mRNA expression *in vitro* and *in vivo* studies.¹⁴ Mismatch ODN for beta chain (MM143-ODN: 5'- GTG CCA TTT GAG TGG CTG – 3') and A4 (MM4-ODN: 5'- CAT CAT TAT CAT GAG GTG – 3') were also designed. All AS-ODNs were synthesized and cartridge (RP1) purified by SIGMA-Genosys (Oakville, ON, Canada). For some studies, in the synthesis of the sequences, adenosine bases were replaced by DAP, or tagged with FITC during synthesis.

Assessment of the Distribution of AS-ODNs Within the Lungs After Intratracheal (i.t.) Administration

Rats exposed i.t. with FITC-labeled ASA4 (200 µg) were sacrificed at different time points and immunohistochemistry was performed on cryosections from the left lungs as previously described.¹⁴ Sections were stained with polyclonal Ab to smooth muscle actin (1:50 dilution; Biomedica Corp., Foster City, CA), counterstained with Alexa Fluoro 633-labeled goat anti-rabbit IgG2a (Invitrogen, Burlington, ON, Canada) and the nuclear stain DAPI then mounted with Prolong Antifade (Invitrogen).

OVA Sensitization and Antisense Efficacy in Airways of Challenged Rats

The study was performed at the CHUM Research Center (Montréal QC, Canada) and was approved by the resident Animal Ethic Committee. Brown Norway rats 6 to 8 weeks old (Harlan Sprague-Dawley Inc., Walkerville, MD) were sensitized by subcutaneous injection of 1 mL of saline containing 1 mg of chicken egg OVA (Sigma-Aldrich, Oakville, ON, Canada) and 3.5 mg of aluminum hydroxide gel (Laboratoire MAT, Beauport QC, Canada). Fourteen days after OVA sensitization, rats were injected i.t. with either sterile saline (50 µL) or 200 µg of AS-ODN or their corresponding MM in 50 µL sterile saline. Rats were then challenged by exposure to OVA aerosols (5% in saline) for 15 min. Fifteen hours after OVA challenge, rats were anesthetized, intubated, and baseline R_L was measured to assess the effect of AS-ODNs on AHR to leukotriene D₄ (LTD₄) (Cayman Chemical, Ann Arbor MI). Rats were then exposed to incremental doses of LTD₄ in 50 µL of saline i.t. and measurements of R_L were obtained immediately after the administration of each dose.^{16,17} The concentration of LTD₄ required to double the baseline value was defined as EC₂₀₀LTD₄ and was calculated by linear interpolation between the two concentrations bounding the point at which R_L reached 200% of the baseline value.

Assessment of the effect of AS-ODNs on cellular infiltrate to the lungs was done in separate groups of rats treated as described above. Briefly, 15 h after OVA challenge, bronchoalveolar lavage (BAL) was performed with total and

differential cell counts on cytospin slides stained with Hema-3 stain kit (Fisher Scientific Canada, Ottawa, ON, Canada). At least 200 cells were counted under oil immersion microscopy.

T Melt Analysis

To ensure that the human equivalents of the two oligonucleotide strands did not interact in solution, AS-ODN selected against the human sequences for beta chain (19 mer TOP004: 5'-GGG TCT GCX GCG GGX TGG T – 3') and CCR3 (21 mer TOP005: 5'- GTT XCT XCT TCC XCC TGC CTG – 3') (X represents where DAP replaced A) were mixed at equimolar concentrations in 1X PBS and standard UV thermo-denaturation methods were conducted using a Beckman DU640 spectrophotometer (Mississauga, ON, Canada) with a T^m accessory. Change in absorbance was detected at 260 nm at each degree from 10°C to 90°C. Melting curves were fitted using MELTWIN 3.5 software (<http://www.meltwin.com/>) to determine thermodynamic parameters.

Efficacy of Human AS-ODN in Cynomolgus Monkey PBMCs

PBMCs were purified from fresh blood from cynomolgus monkeys (ITR Laboratories Canada Inc., Baie d'Urfé, Quebec, Canada) by Ficoll Paque density centrifugation and transfected with different concentrations of AS-ODNs. RNA was extracted 24 to 36 h later with the RNeasy Kit (Qiagen, Freemont, CA) and using equivalent amounts of RNA (0.2 g to 2 g) RT was performed followed by PCR with an equimolar amount of cDNA. The following gene-specific primers were used: for mkCCR3, 5' primer (5'- TGC TCT GTG AAA AAG CCG ATG -3') and 3' primer (5'- ACC AAA AGT GAC AGT CCT GGC -3'); for human beta chain, 5' primer (5'-AAG TCA GGG TTT GAG GGC TAT G-3') and 3' primer (5'- CAA GGG GGC AGA GAC AGG TAG-3'); and for the control GAPDH gene, 5' primer (5'- ACC ACA GTC CAT GCC ATC AC-3') and 3' primer (5'- TCC ACC ACC CCT GTT GCT GTA -3'). The amount of cDNA and the number of cycles were adjusted to be in the linear range of the assay. PCR products were analyzed by ethidium bromide-stained agarose gel and quantified by densitometry using a digital camera-equipped UltraLum gel documentation system (Claremont, CA) and TotalLab software (Nonlinear USA Inc., Durham, NC), with densitometric measurements corrected for background. Level of target gene expression was normalized to levels of the control gene GAPDH. Antisense efficacy was expressed as a percentage of the reduction of gene expression relative to control (untreated) cells.

Statistical Analysis

Differences between groups were determined by analysis of variance (ANOVA). Results are expressed as mean values for the group of animals analyzed $\pm$ standard deviation (SD). Statistical significance (unpaired *t*-test) was determined as $P < 0.05$.

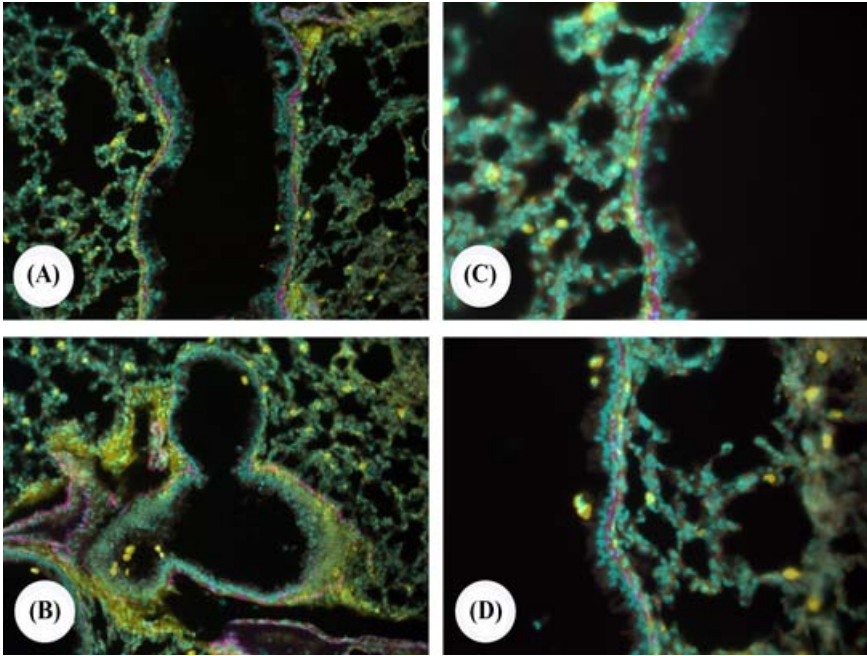


FIGURE 1. Topical delivery of tagged AS-ODN to the lungs of animals. Immunohistochemical sections of lungs of rats 24 h following i.t. exposure to FITC-labeled ASA4 (green). Smooth muscle actin (red) and nuclear label DAPI (blue) denote cells in the lung tissue. **A, B** at 200 $\times$ magnification; **C, D** at 400 $\times$ magnification.

RESULTS

Topical Delivery of AS-ODN to Target Tissue

As we are interested in asthma, we needed to assess that the AS-ODN can be successfully delivered to the target tissue, the lungs. Immunohistochemical sections of lungs from rats that had been exposed i.t. with fluorescently-tagged AS-ODN (200 μ g) clearly demonstrated AS-ODN present in the target tissue 24 h after exposure (FIG. 1). The pattern of fluorescence was different from that of FITC administered alone to the lungs and consisted of uptake in epithelial and endothelial cells, inflammatory cells, and the airway interstitium. There was also mild uptake in smooth muscle cells.

Airway Challenged Rat Model to Determine Combination Effect of ASA4 and AS143 on AHR to LTD₄

We have previously¹² shown effective blocking of the allergen-induced eosinophilia in the lungs and reducing the AHR to LTD₄ following topical

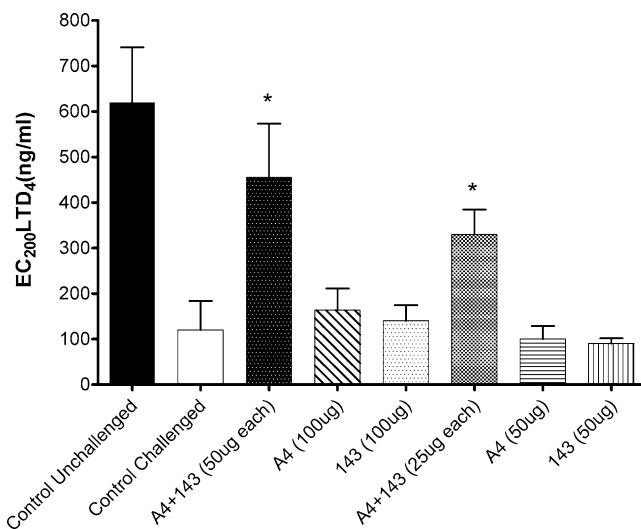


FIGURE 2. Synergistic effect of AS-ODNs targeting the common beta chain for IL-3/IL-5/GM-CSF receptor and the chemokine receptor CCR3 on antigen-induced AHR. OVA-sensitized BN rats ($n = 8$ per group) were OVA challenged and 15 h later baseline R_L was measured. Rats were exposed (i.t.) to incremental doses of LTD₄ to determine EC₂₀₀LTD₄ values. Before OVA challenge, animals received saline, AS-ODN against beta chain (AS143), AS-ODN against CCR3 (ASA4), or both AS-ODNs at indicated doses (i.t.). * $p < 0.05$.

delivery of AS-ODN (200 μ g i.t.) targeting either the common beta chain of the IL-3/IL-5/GM-CSF receptor (AS143)¹³ or the chemokine receptor CCR3 (ASA4).¹⁴ In order to evaluate whether combined delivery of both AS-ODN could act synergistically and affect AHR we determined the effect of pretreatment with both AS-ODN at doses that were shown previously to have no effect (50–100 μ g).¹³ As shown in FIGURE 2, OVA challenge resulted in a significant decrease in the effective dose of LTD₄ required to double the resistance baseline value (EC₂₀₀LTD₄) when compared to unchallenged rats. Single i.t. pretreatments with ASA4 or AS143 at a dose of 100 μ g had no effect on the EC₂₀₀LTD₄, yielding values similar to the challenged group, however a combined pretreatment with both AS143 and ASA4 of 50 μ g each together for a total dose of 100 μ g significantly increased the LTD₄ effective dose required to double the resistance baseline value. This synergistic effect was observed at an even lower total combined dose of only 50 μ g (25 μ g each of ASA4 and AS143) although animals treated with 50 μ g of either antisense alone showed no change in their EC₂₀₀LTD₄. A ratio of 1:1 of AS-ODN appeared to be effective.

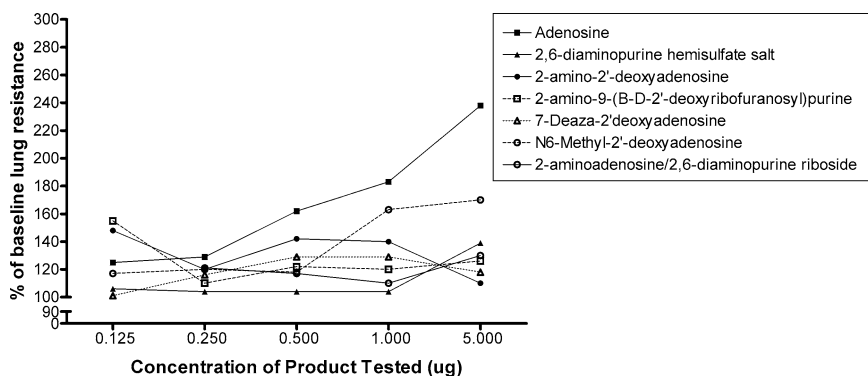


FIGURE 3. Airway responsiveness to adenosine, DAP, and DAP analogs. At 5-min intervals sensitized but unchallenged rats (≥ 6 per group) were administered incremental doses of adenosine (*closed squares*), DAP, or DAP analogs. The average percentage baseline lung resistance is presented.

Modification of AS-ODN with DAP

A number of chemical modifications of the nucleotides have been used in antisense development to reduce degradation by nucleases and limit toxicity. To this purpose, we compared the effects of i.t. administration of adenosine with DAP or analogs thereof on the change in baseline lung resistance in unchallenged BN rats. As shown in FIGURE 3, as the dose of adenosine increased, the average percentage baseline lung resistance in the animals also increased, whereas very little change in the average percentage baseline lung resistance was observed for increasing doses of DAP or any of its analogs. These results indicate that contrary to adenosine, DAP does not significantly affect lung resistance.

Airway Challenged Rat Model to Determine the Effects of DAP-Modified AS-ODN Combination Treatment on AHR to LTD₄ and Pulmonary Inflammation

Replacing adenosine with DAP in ASA4 and AS143 showed similar effects on AHR in the airway challenged rat model as reported in FIGURE 2. When delivered in combination, the DAP-modified ASA4 and AS143 were as effective at inhibiting AHR after allergen challenge. The increase in AHR was inhibited by 50% with the combined delivery of unmodified ASA4 and AS143 (50 μ g total) and by 48% with the combination of DAP-modified ASA4 and AS143 (50 μ g total). As reported with the unmodified oligonucleotides, DAP-modified AS-ODN delivered alone at a 50 μ g dose had no effect on AHR in the rats (data not shown).

The effect of DAP-modified AS-ODN upon cellular influx was also studied. OVA challenge leads to an increase in the number of eosinophils in the BAL (FIG. 4) and this OVA-induced eosinophilia was inhibited in rats treated with the combination of ASA4 and AS143 when compared to rats that received no AS-ODN. Treatment with the combination of ASA4-DAP and AS143-DAP also reduced the OVA-induced pulmonary eosinophilia similar to its unmodified counterparts, these chemically modified AS-ODN also decreased the recruitment of lymphocytes and macrophages when compared to rats that did not receive any AS-ODN indicating a broader anti-inflammatory effect of the DAP-modified AS-ODN.

Testing for Duplex Formation when Combining the Selected Human AS-ODN

To ensure that the human equivalents of the two AS-ODNs did not interact in solution leading to the formation of duplexes, thermodynamic evaluations were conducted on the AS-ODN that had been selected to target the human sequences for the common beta chain and CCR3 (TOP004 and TOP005, respectively). The AS-ODNs were mixed at equimolar concentrations and standard UV thermodenaturation methods were performed to measure the absorbance at each degree change. As shown in TABLE 1, no sudden shift in the absorbance

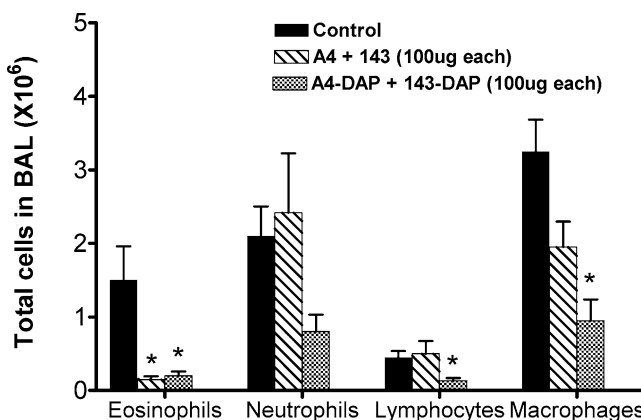


FIGURE 4. AS-ODNs containing DAP are more effective at inhibiting lung inflammation *in vivo* after challenge. Sensitized BN rats ($n = 8$ per group) were OVA challenged and 15 h later BAL was harvested and differential cell counts were assessed. Before OVA challenge, animals received (i.t.) saline, AS-ODN against beta chain composed of either only phosphorothioate (AS143) or containing DAP modifications (AS143-DAP), AS-ODN against CCR3 composed of either only phosphorothioate (ASA4), or containing DAP modifications (ASA4-DAP) or both sets of AS-ODNs at indicated doses. Data expressed as mean $\pm$ SD of specific cell types. * $p < 0.05$ versus control.

TABLE 1. T melt analysis of human AS-ODN when combined in solution

Concentration (M)	dH (kcal/mol)	dS (cal/k-mol)	dG (kcal/mol, 37°C)	T _m (°C)
8.61e-6	-46.14	-135.02	-4.27	13.64
6.77e-6	-46.01	-134.32	-4.35	13.12
5.82e-6	-54.5	-159.85	-4.92	18.99
4.34e-6	-45.44	-130.83	-4.86	14.23
3.1e-6	-53.22	-157.27	-4.45	14.19
1.41e-6	-50.76	-145.57	-5.61	15.13

TOP004 and TOP005 mixed at equimolar concentrations in 1X PBS at different concentrations of total AS-ODN were subjected to increasing temperature and change in absorbance was detected at 260 nm at each degree from 10°C to 90°C. Melting curves were fitted using MELTWIN 3.5 software to determine thermodynamic parameters. Stability of the melting temperatures suggests that these two AS-ODNs do not form duplexes when in solution.

was observed for any of the concentrations tested, indicating that for these AS-ODNs there was no formation of duplexes at any of the temperatures or concentrations tested.

Efficacy of Combination AS-ODN in Monkey PBMCs

To verify that DAP-modified AS-ODN of the human sequence retain efficacy, TOP004 and TOP005 were tested alone and combined in a 1:1 ratio (ASM8) in cynomolgus monkey PBMCs for their ability in knocking down target gene expression. Monkey PBMCs exposed to various concentrations of AS exhibited significant inhibition in gene expression of the beta chain and CCR3 (FIG. 5). A lower dose of combined ASM8 (2.5–5 μ M) was needed for maximal inhibition of gene expression (*hatched and shaded bars*, FIG. 5) when compared to cells transfected with either TOP004 (beta chain, *solid bars*) or TOP005 (CCR3, *open bars*) alone. This increased potency (synergy) of ASM8 at blocking the expression of the targets, was also observed in human cell lines (data not shown).

DISCUSSION

The two current approaches in the treatment of respiratory disease involve either the inhibition of a specific target in a single pathway or the broad and nonspecific inhibition of multiple pathways (i.e., corticosteroids). In humans, asthma is associated with the accumulation of eosinophils and other inflammatory cells.^{18,19} The results presented here show effective inhibition of cellular influx and AHR following delivery of a combination of our two selected AS-ODNs: one that targets the common beta chain receptor for IL-3/IL-5/GM-CSF, and the other, the chemokine receptor CCR3, both of which

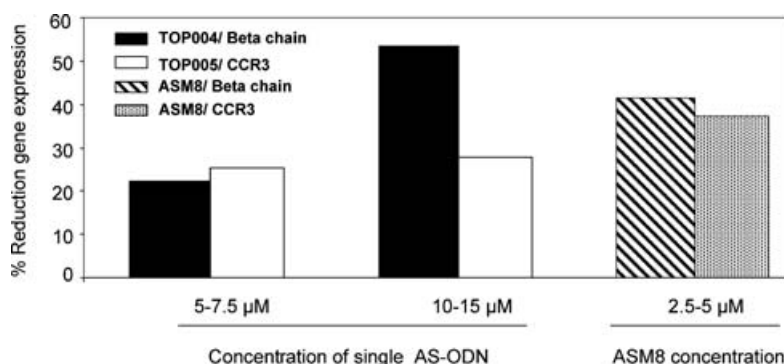


FIGURE 5. Reduced beta chain and CCR3 mRNA expression by ASM8 in cynomolgus monkey PBMCs. Purified monkey PBMCs were incubated with different concentrations of TOP004, TOP005, or ASM8 (TOP004/TOP005 in 1:1 ratio) then gene expression of either beta chain or CCR3 was assessed by RT-PCR. Results are the mean changes in gene expression obtained from experiments performed on at least five bloods of monkeys.

are mediators of allergic inflammation in asthma. The efficacy of the combined AS-ODN was observed at doses lower than either AS-ODN when delivered alone, demonstrating a synergistic effect when sets of pathways are specifically targeted.

Administration of a monoclonal antibody (mAb) targeting CCR3 was reported to partially decrease eosinophilia, but multiple doses of mAb were required for this effect and no effects on airway responsiveness were reported.²⁰ In another study several concurrent administrations (systemic/local) of a rat anti-mouse CCR3 mAb were required to deplete eosinophils from the lungs of OVA-sensitized/challenged mice.²¹ In a clinical trial, administration of an IL-5 blocking mAb prevented blood and lung eosinophilia but had little effect on the AHR in humans with asthma.²² As IL-3 and GM-CSF are also increased in the airways of patients with asthma, targeting IL-5 alone may be insufficient to treat asthma. We have previously reported that i.t. administration of IL-5 is only partially responsible for the physiologic changes encountered in asthma.²³ Thus limitations exist when blocking a single ligand as a therapeutic approach for asthma. In contrast, results from the present study demonstrate that a single topical administration of a low dose of combined AS-ODNs (ASA4 and AS143) was sufficient to significantly reduce both pulmonary inflammation and AHR following antigen challenge in the animal model, demonstrating the effectiveness of our approach in targeting the expression of multiple pathways rather than directly blocking a single ligand.

AS-ODNs are being developed as therapeutic agents against various diseases.²⁴ However, the therapeutic application of these agents may be limited as some class-related toxicity (phosphorothioate) has been described. In particular, AS-ODNs of the phosphorothioate class can bind with low affinity to a large number of proteins, which may contribute to organ distribution, accumulation,

and toxicity associated with systemic delivery of AS-ODNs.² Thus topical delivery of AS-ODNs directly to the lungs, as performed and demonstrated in the present study, is one approach to limiting the systemic availability of AS-ODNs. Furthermore, the efficacy of these AS-ODNs delivered to the lungs may be enhanced as they are now in a position to act locally on the infiltrating immune cells and those cells margined in lung vessels.

Many developing strategies for AS-ODN therapy focus on chemically modifying the nucleotides, sugars, or phosphate backbone to enhance stability of the AS-ODN *in vivo*, limit nonspecific binding and reduce toxicity.² Replacing adenosine with DAP we demonstrated similar levels of efficacy as the phosphorothiate AS-ODN on AHR in the animals as well as a broader anti-inflammatory effect suggesting that DAP modifications would enhance the overall efficacy of this therapy.

When developing a combined AS-ODN therapy using two AS-ODNs, there are additional concerns to address. The optimal ratio of each AS-ODN needs to be determined and it is important to ensure that the selected AS-ODNs are available and do not form duplexes in solution, as we demonstrated in our T^omelt studies. Furthermore, in developing a human therapy it is important to be able to measure the pharmacokinetics of the AS-ODNs following delivery *in vivo*. By selecting AS-ODN of different sizes (19 mer for TOP004 and 21 mer for TOP005), we were successful in determining the presence of each AS-ODN and their n-1 metabolites in the plasma of rats and monkeys even after delivery of the combined product (data not shown).

In summary, our results clearly demonstrate the possibility of combining at least two oligonucleotides into a single product and maintaining efficacy in target knockdown. Furthermore, we demonstrate that topical delivery of two AS-ODNs synergizes for potent efficacy at low doses. The modifications using DAP in this combined product not only retain the efficacy but also appear to provide a broader coverage against inflammation. We are optimistic that our AS-ODNs could be developed as a novel therapeutic for asthma.

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