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Pharmacological Characterization of a Novel ENaCα siRNA (GSK2225745) With Potential for the Treatment of Cystic Fibrosis

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Lung pathology in cystic fibrosis is linked to dehydration of the airways epithelial surface which in part results from inappropriately raised sodium reabsorption through the epithelial sodium channel (ENaC). To identify a small-interfering RNA (siRNA) which selectively inhibits ENaC expression, chemically modified 21-mer siRNAs targeting human ENaCα were designed and screened. GSK2225745, was identified as a potent inhibitor of ENaCα mRNA (EC_{50} (half maximal effective concentration) = 0.4 nmol/l, maximum knockdown = 85%) and protein levels in A549 cells. Engagement of the RNA interference (RNAi) pathway was confirmed using 5′ RACE. Further profiling was carried out in therapeutically relevant human primary cells. In bronchial epithelial cells, GSK2225745 elicited potent suppression of ENaCα mRNA (EC_{50} = 1.6 nmol/l, maximum knockdown = 82%). In human nasal epithelial cells, GSK2225745 also produced potent and long-lasting (≥72 hours) suppression of ENaCα mRNA levels which was associated with significant inhibition of ENaC function (69% inhibition of amiloride-sensitive current in cells treated with GSK2225745 at 10 nmol/l). GSK2225745 showed no evidence for potential to stimulate toll-like receptor (TLR)3, 7 or 8. *In vivo*, topical delivery of GSK2225745 in a lipid nanoparticle formulation to the airways of mice resulted in significant inhibition of the expression of ENaCα in the lungs. In conclusion, GSK2225745 is a potent inhibitor of ENaCα expression and warrants further evaluation as a potential novel inhaled therapeutic for cystic fibrosis.

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Introduction

More than 20 years have passed since the landmark discovery that the faulty gene in cystic fibrosis is the cystic fibrosis transmembrane regulator (CFTR). This discovery catalyzed major research efforts into how mutations in CFTR lead to cystic fibrosis disease and how to use this emerging knowledge to advance novel therapeutics. Significant progress has been made on all fronts and it is heartening that Kalydeco (VX-770, Ivacaftor), a small molecule potentiator of CFTR, has now been approved in both Europe and the United States for the treatment of patients who carry the CFTR G551D mutation.[1] However, there remains a significant unmet need for novel therapies which can impact on patient morbidity, mortality, and reduce treatment burden.

One important function of CFTR is to transport chloride and water across epithelia in a basolateral to apical direction. In the airways, this is critical for hydration of the epithelial surface, normal ciliary function, and mucociliary clearance. In healthy airway epithelium, CFTR activity is counterbalanced by the epithelial sodium channel (ENaC) which stimulates sodium and water absorption. Working in concert, CFTR and ENaC regulate airways epithelial hydration in an appropriate manner to support effective mucociliary clearance. However, in cystic fibrosis, the defective CFTR not only leads to inappropriately low chloride transport and impaired hydration but also to enhanced activity of ENaC driving sodium absorption and further dehydration of the airways epithelial surface. This results in chronically impaired mucociliary clearance, predisposes the patient to opportunistic bacterial pathogens and contributes to a vicious, destructive cycle of infection and chronic inflammation.[2]

Therapeutically, replacement or correction of the faulty CFTR gene is a compelling approach. In addition, however, inhibition of sodium hyperabsorption may also be therapeutically beneficial and small molecule ENaC inhibitors such as amiloride and benzamil have undergone clinical evaluation *via* inhalation (reviewed by Hirsh, 2002).[3] These molecules showed some short-term beneficial effects on mucociliary clearance but their short duration of action and narrow therapeutic index means that they could not be used to fully test the concept that sustained ENaC inhibition would lead to clinical benefit. We propose that inhaled therapeutic oligonucleotides represent an attractive approach to overcome the challenges which small molecule inhibitors of ENaC have faced. Previous

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workers have already demonstrated that ENaC function in airway epithelial cells can be inhibited with both small-interfering RNAs (siRNAs) and antisense oligonucleotides (ASOs) with a long duration of action.[4,5] This long duration of target suppression bodes well for the development of medicines which can be dosed infrequently and thereby reduce patient treatment burden. Moreover, clinical data with inhaled oligonucleotides suggests that systemic exposure is minimal after inhalation.[6,7] This contrasts with small molecules which are likely to readily access the systemic circulation following inhalation and potentially evoke undesirable effects through inhibition of ENaC in the kidneys. These combined attributes indicate that inhaled oligonucleotides targeting ENaC have the potential to show a better therapeutic index and significantly improved duration of action than small molecule inhibitors.

This paper details our efforts to identify and pharmacologically characterize, GSK2225745 (**Figure 1**), a potent siRNA-targeting ENaCα. Our findings indicate that GSK2225745 warrants further evaluation as a novel potential therapeutic for the treatment of cystic fibrosis.

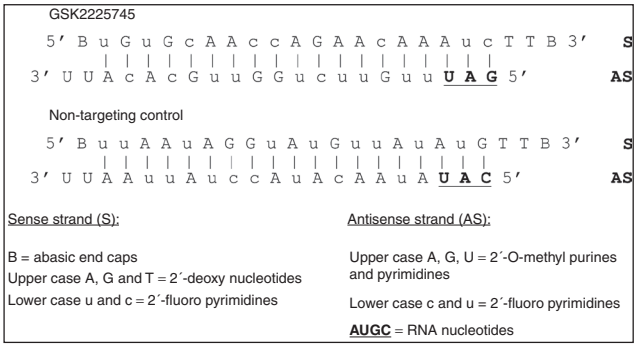

Figure 1 Sequence and chemical modifications of the siRNA, GSK2225745 (designed to target human ENaCα), and the nontargeting control (NTC) siRNA used in these studies. ENaC, epithelial sodium channel; siRNA, small-interfering RNA.

Results

Identification of GSK2225745A and evaluation in A549 cells

Using the human ENaCα nucleotide sequence (accession no. NM_001038) and a proprietary design algorithm, 32 siRNAs were designed, synthesized, and screened in triplicate in A549 cells. All siRNAs were 21-mers and displayed the same chemical modification pattern of the sequences described in **Figure 1**. The design and chemical modification pattern of the siRNAs was based on previous literature,[8–10] the objective being to identify molecules suitable for potential development with a combination of stability, potency, and minimized potential for immunostimulation.

BLAST analysis of the AS strand of the nontargeting control (NTC) sequence against all human sequences in

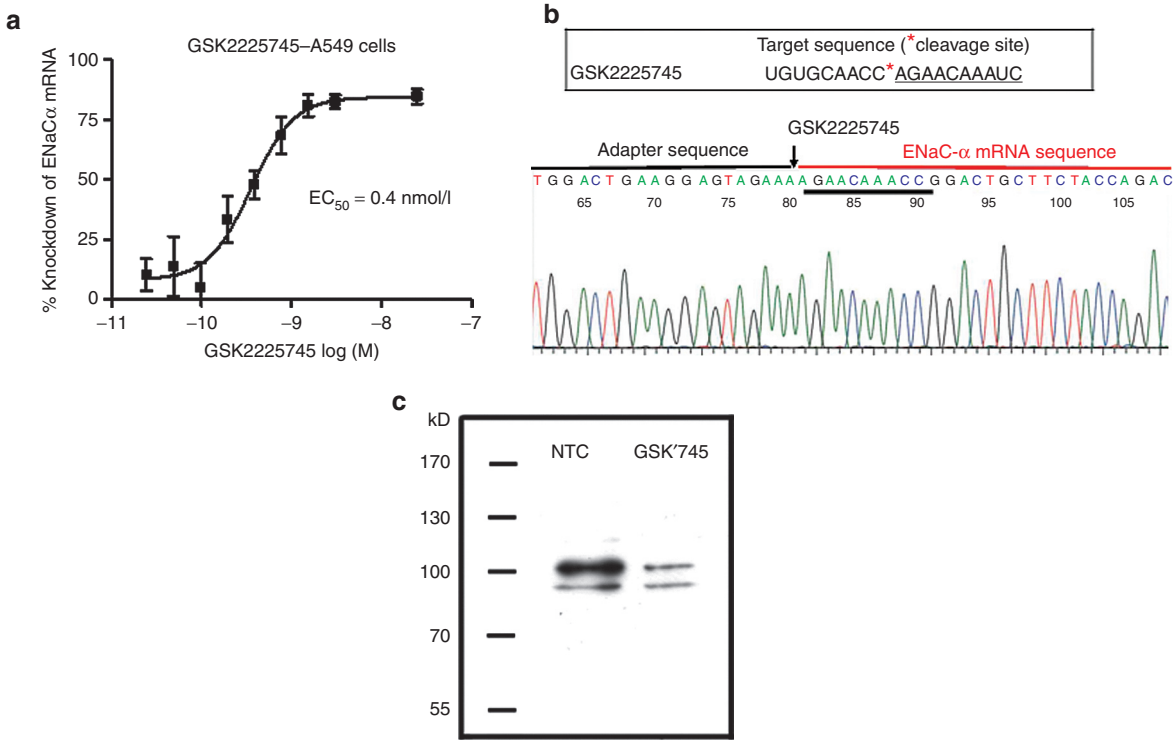

Figure 2 Profile of GSK2225745 in A549 cells. (**a**) Effects of GSK2225745 on ENaCα mRNA levels. Values are mean ± SD ($n = 3$) and are expressed as percentage inhibition relative to the nontargeting control (NTC) siRNA. (**b**) 5′ RACE-PCR confirms the predicted cleavage product arising from engagement of the RNAi pathway. The small box shows the target mRNA sequence of GSK2225745A with the predicted cleavage point indicated by the red asterisk. Immediately below is a sequencing analysis from the 5′ RACE-PCR demonstrating that the predicted cleavage product is present in RNA derived from cells treated with 25 nmol/l GSK2225745. (**c**) Western blot analysis from cells treated with either 100 nmol/l GSK2225745 or 100 nmol/l NTC siRNA. The 100 kD band corresponding to ENaCα is inhibited in cells treated with GSK2225745. A representative blot is shown from three independent experiments. EC_{50}, half maximal effective concentration; ENaC, epithelial sodium channel; RNAi, RNA interference; siRNA, small-interfering RNA.

the National Center for Biotechnology Information RefSeq database revealed no significant homology to any mRNA sequence. That is, there were no identical hits over the entire sequence and no hits showing an identical match over 15 or more contiguous nucleotides in a "plus/minus" orientation.

Initial screening of the ENaC-targeting siRNAs in A549 cells was carried out at 25 nmol/l followed by dose–response assessment of the more effective sequences to confirm their ability to suppress ENaCα mRNA levels and to facilitate selection of the most potent siRNA.

In these cells, the NTC siRNA (25 nmol/l) evoked no significant suppression of ENaCα (data not shown). In contrast, in this initial screen GSK2225745 (25 nmol/l) suppressed ENaCα mRNA expression by >50% and was therefore selected for detailed evaluation. In subsequent dose–response evaluation, GSK2225745 caused dose-dependent suppression of ENaCα mRNA (**Figure 2a**) with an estimated EC_{50} (half maximal effective concentration) of 0.4 nmol/l. Further experiments applied 5′ RACE-PCR in A549 cells 24 hours post-treatment with GSK2225745 (25 nmol/l). A PCR product of the predicted size (211 bp) was observed and sequencing confirmed that the predicted cleavage product of the ENaCα transcript was present (**Figure 2b**), indicating that GS2225745 was acting specifically, *via* engagement of the RNA interference (RNAi) machinery. Finally, a custom antibody was generated and used to demonstrate that treatment of A549 cells with GSK2225745 (100 nmol/l) also resulted in significant reduction in ENaCα protein levels (**Figure 2c**). Densitometric quantification of blots from three independent experiments indicated that ENaCα protein levels were reduced by 63% relative to levels observed in cells treated with the NTC siRNA.

Specificity of GSK2225745

BLAST analysis of the AS strand of GSK2225745 against the National Center for Biotechnology Information human RefSeq database confirmed 100% identity to the three transcript variants for ENaCα (accession nos. NM_001038, NM_001159576, and NM_001159575). No additional hits with a contiguous match over 15 or more nucleotides were reported. Transfection studies with GSK2225745 (25 nmol/l) showed no reduction in levels of ENaCβ mRNA in A549 cells (data not shown). Combined, these observations confirm GSK2225745A as a sequence-specific inhibitor of the α-isoform of ENaC having low homology with the other ENaC isoforms (β, γ, and δ).

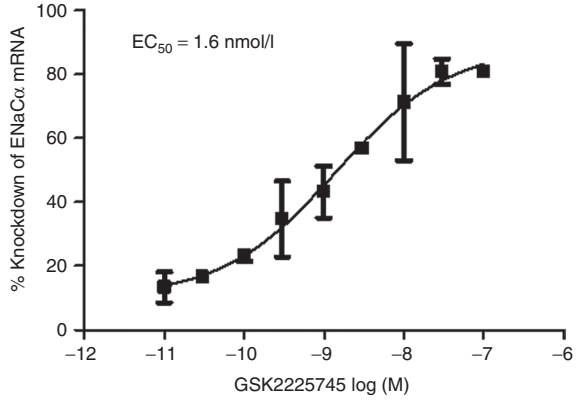

Figure 3 Effects of GSK2225745 on ENaCα mRNA levels in human primary bronchial epithelial cells. Values are mean ± SD ($n = 3$) and are expressed as percentage inhibition relative to the non-targeting control siRNA. EC_{50}, half maximal effective concentration; ENaC, epithelial sodium channel; siRNA, small-interfering RNA.

Effects of GSK2225745 in human primary bronchial epithelial cells

In human bronchial epithelial cells, the effects of GSK2225745 on ENaCα mRNA levels were assessed 48 hours after exposure to the siRNA. The NTC siRNA (100 nmol/l) evoked no significant suppression of ENaCα (data not shown). In contrast, GSK2225745 potently inhibited ENaCα expression in a dose-dependent fashion (**Figure 3**) with an EC_{50} of 1.6 nmol/l.

Effects of GSK2225745 in human primary nasal epithelial cells

The primary goal of these experiments was to measure the effects of GSK2225745 on the function of the ENaC channel in human primary epithelial cells. Amiloride-sensitive sodium current was used as a direct marker of ENaC function. Cells were grown on specialized filters such that they could be

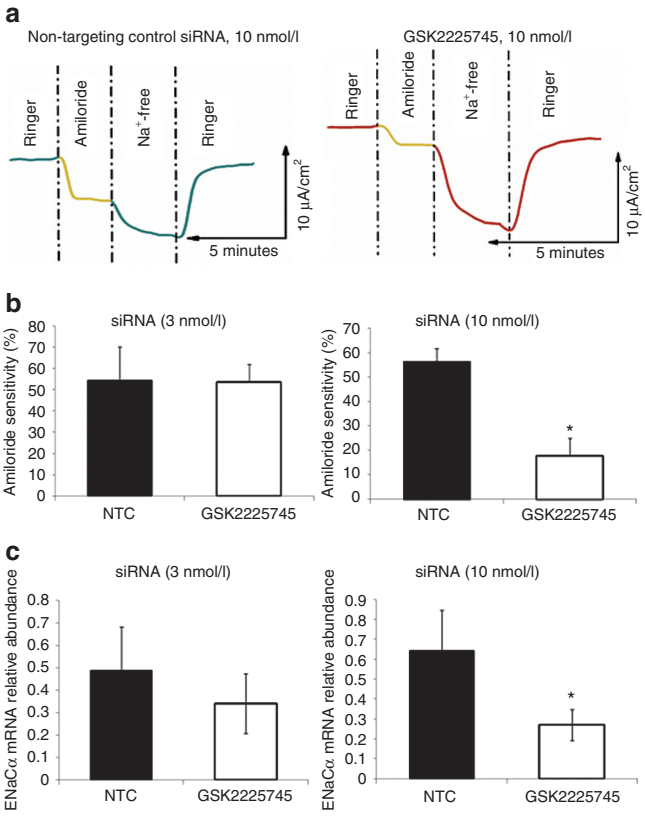

Figure 4 Profile of GSK2225745 in human primary nasal epithelial cells. (**a**) Example of short-circuit current (I_{sc}) recordings from representative experiments in monolayers 24 hours post-treatment with either nontargeting control (NTC) siRNA or GSK2225745. (**b**) Measurements of amiloride sensitivity in cells 24 hours post-treatment with either GSK2225745 or NTC siRNA. Values are mean ± SD (n = 6–8 donors). (**c**) Measurements of ENaCα mRNA levels relative to GAPDH in cells 24 hours post-treatment with either GSK2225745 or NTC siRNA. Values are mean ± SD (n = 4–7 donors). *$P < 0.05$ versus NTC siRNA. ENaC, epithelial sodium channel; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; siRNA, small-interfering RNA.

placed in Ussing chambers for electrophysiological measurements. However, following these electrical measurements, RNA was extracted from the cells for quantification of ENaCα mRNA in order that an attempt could be made to correlate functional change with mRNA suppression.

Figure 4 indicates that GSK2225745 elicited a dose-dependent inhibition of both the expression of ENaCα mRNA and the function of the ENaC channel in human nasal epithelial cells at 24 hours. Therefore, at a concentration of 3 nmol/l, GSK2225745 produced a trend towards suppression of ENaCα mRNA (**Figure 4c**), although this was not statistically significant. Consistent with this observation, no inhibition of amiloride-sensitive current was observed (**Figure 4b**). However, when the concentration of GSK2225745 was increased to 10 nmol/l, significant inhibition (69%) of amiloride-sensitive current was observed (**Figure 4b**) and this was associated with a 60% reduction in ENaCα mRNA expression relative to cells treated with NTC siRNA (**Figure 4c**).

In further experiments, the duration of effect of GSK2225745 (10 nmol/l) was evaluated. Significant inhibition of amiloride-sensitive current was observed 48 and 72 hours post-exposure to GSK2225745 with little apparent reduction of effect compared with the 24-hour measurements (**Figure 5**). Similarly, significant suppression of ENaCα mRNA levels was also observed at the 48- and 72-hour timepoints (data not shown). It was difficult to maintain the cells for longer periods than 72 hours to allow for the determination of the timepoint at which recovery from the effects of GSK2225745 occurred.

Screening for evaluation of potential immunostimulatory effects

Nucleic acids have the potential to stimulate the innate immune system *via* activation of discrete classes of intracellular and cell surface receptors.[11] Recognition of double-stranded 21-mer siRNAs is most likely to occur *via* activation of toll-like receptors (TLRs) and in particular, TLR3, TLR7, and TLR8. While TLR3, 7, and 8 have an intracellular endosomal localization, TLR3 has also been reported to occur on the cell membrane of airway epithelial cells.[12] This is of significance to GSK2225745 as the ultimate goal is to deliver the molecule *via* inhalation. Therefore, it could rapidly reach the epithelial membrane at relatively high concentrations.

A simple pattern-matching computational analysis was performed whereby GSK2225745A was screened *in silico* to ensure that no known or putative TLR7 or TLR8 immunostimulatory sequence motifs[13,14] were present in the siRNA

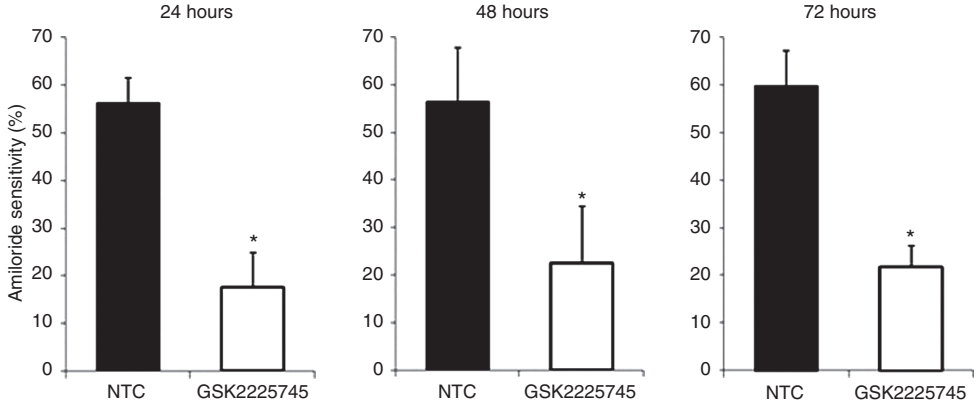

Figure 5 Duration of effect of GSK2225745 in human primary nasal epithelial cells. Measurements of amiloride sensitivity in cells 24, 48, and 72 hours following treatment with either GSK2225745 or nontargeting control (NTC) siRNA. Values are mean ± SD (n = 4–8 donors). *P < 0.05 versus NTC siRNA. siRNA, small-interfering RNA.

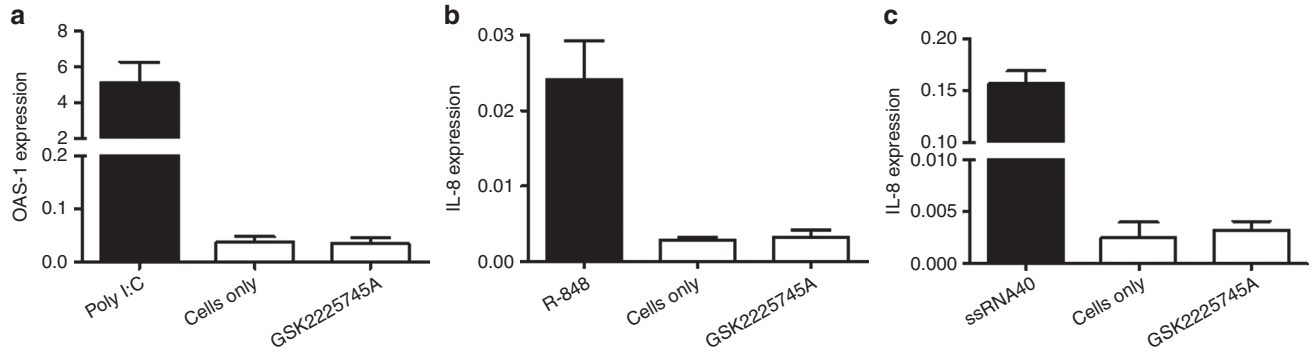

Figure 6 Screening of GSK2225745 for evidence of TLR stimulation *in vitro*. Values are mean ± SD (n = 3) and show mRNA expression relative to GAPDH. (**a**) Effects of the TLR3 agonist, poly I:C (2.5 µg/ml) on the interferon responsive gene, 2′,5′-oligoadenylate synthetase 1 (OAS1), in human bronchial epithelial cells. No induction was observed in response to GSK2225745 (100 nmol/l). (**b**) Effects of the TLR7 agonist, R-848 (30 µmol/l) on IL-8 in U2OS cells stably expressing TLR7. No induction was observed in response to GSK2225745 (100 nmol/l). (**c**) Effects of the TLR7/8 agonist, ssRNA40 (0.6 µmol/l) on IL-8 in U2OS cells stably expressing TLR8. No induction was observed in response to GSK2225745 (100 nmol/l). GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IL, interleukin; TLR, toll-like receptor.

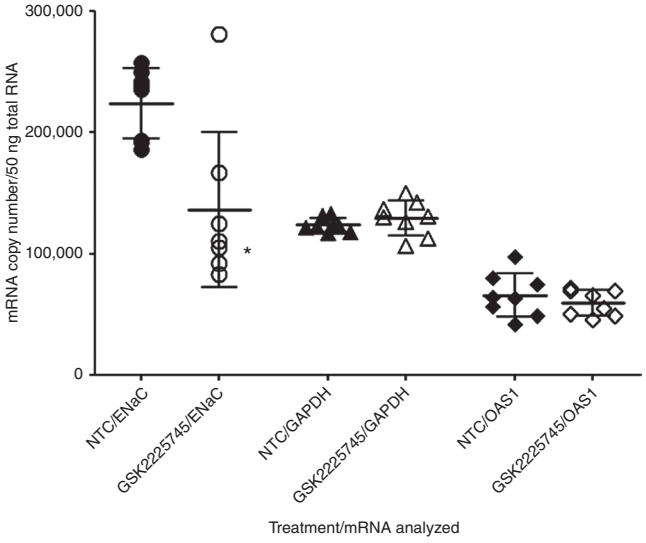

Figure 7 Effect of topical pulmonary delivery of GSK2225745 or nontargeting control (NTC) siRNA on the expression of ENaCα in the lungs of mice. GSK2225745 or NTC siRNA were prepared in a lipid (C12-200) nanoparticle formulation and administered intranasally (1.6 mg/kg) in a dose volume sufficient to deliver the drug substance to the lungs; 72 hours after dose, whole lungs were taken for mRNA extraction and qRT-PCR. The lung mRNA expression levels of GAPDH and the interferon response gene, OAS1 (2′,5′-oligoadenylate synthetase 1), are also shown. Target mRNA expression is quantified as copy number per 50 ng of lung total RNA. Horizontal bars define the mean, vertical bars define the SD of the mean (n = 8/treatment group). *P < 0.05 GSK2225745 versus NTC siRNA. ENaC, epithelial sodium channel; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; OAS1, 2′,5′-oligoadenylate synthetase 1; qRT-PCR, quantitative reverse transcription-PCR; siRNA, small-interfering RNA.

sequence. In addition, cell culture studies were performed in human cells in order to confirm that GSK2225745 does not cause stimulation of TLRs.

To evaluate the potential of GSK2225745 to stimulate TLR3, human primary bronchial epithelial cells were used. Exposure of the cells to the TLR3 agonist poly I:C (2.5 µg/ml) produced a marked induction of the interferon response gene OAS1 (2′,5′-oligoadenylate synthetase 1) (**Figure 6a**). In contrast, exposure of the cells to GSK2225745 (100 nmol/l) induced no induction of OAS1. To screen for potential immunostimulatory effects mediated by either TLR7 or TLR8, U2OS cells were engineered to stably express either TLR7 or TLR8 linked to a nuclear factor-κB reporter construct such that TLR stimulation resulted in induction of the nuclear factor-κB responsive gene, interleukin (IL)-8. In U2OS cells expressing TLR7, the selective agonist R-848/Resiquimod induced a significant induction of IL-8. In contrast, GSK2225745 (100 nmol/l) induced no induction of IL-8 (**Figure 6b**). Similarly, in cells overexpressing TLR8, the positive control agonist, ssRNA40 induced a significant induction of IL-8 while no such effect was observed in cells treated with GSK2225745 (100 nmol/l) (**Figure 6c**).

Evaluation of GSK2225745 *in vivo*

Previous experience in our laboratory indicates that topical delivery of GSK2225745 to the lungs in a simple vehicle (phosphate-buffered saline (PBS) or water) is ineffective at achieving knockdown of ENaCα (data not shown). Therefore, GSK2225745 was formulated into lipid nanoparticles containing the lipid C12-200, which has previously been reported[15] to facilitate siRNA delivery to mouse liver *in vivo*. When GSK2225745 was formulated with C12-200, the resultant particles had a diameter of ~120 nm with a polydispersity <0.12 and a siRNA entrapment of ~97% when prepared in 0.1X PBS.

Lipid nanoparticle-formulated GSK2225745 was administered to mice *via* the intranasal route using a dose volume (50 µl) known from previous experience and previous reports[16] to result in effective delivery of drug substance to the lungs; 72 hours after a single administration of GSK2225745 (1.6 mg/kg), significant suppression (P = 0.003) of ENaCα mRNA levels was observed in the lung (**Figure 7**). The mRNA levels of the interferon response gene, OAS1, were also measured as a marker of any possible immunostimulatory response. No evidence of OAS1 induction was observed (**Figure 7**).

Discussion

Increased understanding of the molecular pathophysiology of cystic fibrosis is progressively leading to the introduction of novel pharmacological therapies. Nevertheless, the pulmonary manifestations of the disease still lead to a heavy burden in terms of patient morbidity and mortality, and there remains a significant need for the introduction of novel therapeutics. In that regard, inhaled oligonucleotides represent a new and promising class of potential therapeutics. Such molecules have already shown promise in clinical trials in asthma[6] and respiratory syncytial virus infection[7] and offer the prospect of engaging targets known to be important in disease pathology but which are not readily addressed with small molecule or antibody therapeutics. In addition, after inhalation in humans, oligonucleotides do not appear to readily access the systemic circulation,[6,7] a property which may help confer a greater therapeutic index relative to small molecule therapeutics. Finally, the ability of oligonucleotides to suppress target proteins for long periods of time after a single dose[4,17] offers the prospect of infrequent dosing. This would be a significant advantage in diseases like cystic fibrosis which carry a heavy treatment burden for the patient. Although overactivity of the ENaC channel is implicated in the pulmonary pathophysiology of cystic fibrosis, it has proven difficult to identify small molecule therapeutics with the desirable potency, duration of action, and lung selectivity to determine whether real clinical benefit can be derived from its inhibition. Therefore, this paper describes our efforts to identify a potent siRNA-targeting ENaCα and characterize its pharmacology *in vitro*.

siRNAs were designed to target the α-subunit of the ENaC channel since it is known that this subunit is critical for channel function.[18] The siRNAs were chemically modified to improve stability and reduce propensity for stimulation of the innate immune system. Screening in human A549 cells led to the identification of GSK2225745 as a very potent inhibitor of ENaCα mRNA levels. Engagement of the RNAi pathway results in RNA-induced silencing complex-mediated cleavage of the target transcript between the nucleotides 10

and 11 complementary to the AS strand of the siRNA being evaluated. Therefore, 5′ RACE-PCR was used to confirm that in A549 cells treated with GSK2225745, ENaCα mRNA cleavage product could be identified, consistent with the molecule acting *via* activation of the RNAi pathway. Evidence was also obtained suggesting that a reduction in ENaCα protein expression occurs in GSK2225745-treated A549 cells.

Having gathered data from studies in a cell line that GSK2225745 is a promising lead, we progressed to evaluate the molecule in greater detail using more physiologically relevant primary cell assays. In addition, to progress GSK2225745 as a potential therapy for cystic fibrosis we felt it is essential to demonstrate that it can achieve potent and long-lasting suppression of ENaCα mRNA levels and that this is associated with inhibition of channel function. Experiments in human bronchial epithelial cells confirmed that GSK2225745 potently suppresses ENaCα mRNA levels in physiologically relevant primary cells. To evaluate effects on the function of the ENaC channel, we turned our attention to electrophysiological measurements in human nasal epithelial cells, a system which has previously been used successfully to study the effects of ENaC-targeting ASOs on the function of the channel.[5] Therefore, when human nasal epithelial cells are grown as monolayers and placed in modified Ussing chambers, it is possible to measure changes in transepithelial current through the ENaC channel. Using this system, we were able to demonstrate that GSK2225745-induced suppression of ENaCα mRNA was also associated with marked inhibition of ENaC channel function. Close evaluation of the ENaC mRNA suppression data would suggest that GSK2225745 was less potent in human nasal epithelial cells than in human bronchial epithelial cells. This likely reflects the different transfection conditions which were used, with those in the human nasal epithelial cells having been optimized for the evaluation of channel function. Importantly, the functional inhibition of ENaC by GSK2225745 had a long duration, with significant suppression of both ENaCα mRNA and function observed for at least 72 hours following a single treatment. Technical limitations made it difficult to study the cells significantly beyond 72 hours to fully define the duration of action of GSK2225745 and further studies will be required in the future for that purpose.

For most prospective therapeutic oligonucleotides, it is important to determine whether there is a potential to stimulate the innate immune system *via* engagement of TLRs. Screening of GSK2225745 in primary cells and engineered cell lines suggests no propensity at the concentrations tested for stimulation of either TLR3, TLR7 or TLR8, the three receptors of the innate immune system which are most likely to be activated by a short double- or single-stranded RNA. Therefore, as described by Judge and co-workers,[10] it seems likely that the 2′O-methyl modifications which have been made to the AS strand of GSK2225745 have successfully abrogated the potential for immune stimulation.

Having demonstrated that GSK2225745 is a potent suppressor of ENaC expression and function in human airway epithelial cells in culture, the next key challenge is to try and translate these findings to the clinic. The first step in that process is to question whether the molecule can work *in vivo*. Initial attempts to demonstrate *in vivo* activity of GSK2225745 evaluated "naked" delivery, *i.e.,* siRNA preparation in either saline or distilled water before local delivery to the nasal passages or lungs of rodents. Unfortunately, following naked delivery of GSK2225745 by either the inhaled, intranasal or intratracheal routes, we were unable to see evidence for reduction in ENaCα in rodent lungs (data not shown). This suggests that there are significant barriers to the effective delivery of siRNAs to the airway epithelium and is consistent with studies in airway epithelial cells in culture where transfection reagents are required to reveal the effects of GSK2225745. Barriers to inhaled delivery of naked siRNA are likely to reflect a formidable combination of mucociliary clearance, inability to penetrate the layer of mucus and surfactant lining the airway epithelium, binding to mucins tethered to the epithelial apical surface, degradation of the oligonucleotides by pulmonary enzymes, and inability to cross the epithelial apical membrane. Given the lack of efficacy observed with naked delivery, we reasoned that for effective delivery of GSK2225745 to airway epithelium *in vivo*, a specialized delivery formulation would be required. For the current studies, we chose a lipid nanoparticle formulation utilizing the cationic lipid, C12-200. This formulation has previously been reported to successfully deliver siRNA to the liver.[15] Utilizing a C12-200 nanoparticle formulation of GSK2225745, we were able to demonstrate significant suppression of ENaCα mRNA in the lungs of mice 72 hours after a single dose was administered. The formulation was well tolerated with no evidence for induction of the immunostimulation marker gene, OAS1. Overall this data is encouraging as it suggests GSK2225745 is effective *in vivo* and with an appropriate optimized formulation could be further developed. Future studies will need to fully evaluate the time course of ENaCα suppression by GSK2225745 although significant suppression of ENaCα 72 hours post-dose bodes well for a long duration of action. It will also be key to demonstrate efficacy following nebulized delivery since the current studies used the intranasal route.

The studies reported herein are in good agreement with recently published work on oligonucleotides targeting ENaC. For example, Caci and co-workers[4] evaluated the effects of ENaC siRNAs independently targeting the α, β, and γ subunits of the channel in human primary bronchial epithelial cells in culture. They demonstrated significant suppression of each of the subunits at the mRNA level and importantly that this led to significant suppression of ENaC channel function out to at least 8 days post-treatment. Similar data have previously been published from studies in human-cultured nasal epithelial cells. Therefore, Sobczak and colleagues[5,19] evaluated the effects of an ASO targeting the α-subunit of ENaC. They reported that this ASO was able to significantly inhibit the function of the ENaC channel with duration of action of at least 3 days. Importantly, significant inhibition of ENaC function in response to the ASO was demonstrated in cells from both healthy and cystic fibrosis donors. Finally, encouraging *in vivo* data has also recently been reported from a study in mice using a siRNA-targeting ENaCγ.[20] In this study, evidence was found for suppression of ENaCγ mRNA levels in lung and nasal epithelium following repeated intranasal doses of siRNA. Therefore, taken together with the work described in this paper, this collective body of evidence confirms that ENaC is an attractive and viable target for oligonucleotides

and that such an approach could yield potential therapeutics with a long duration of action. Efforts now must focus on optimization of safe and efficient delivery to the upper airways *in vivo* such that an ENaC-targeting oligonucleotide can be advanced to clinical evaluation in cystic fibrosis.

In summary, through a sequential process of screening, *in vitro* characterization in human primary cells, and *in vivo* evaluation in mice, we have identified and characterized GSK2225745, as a potent siRNA which induces long-lasting inhibition of ENaCα expression and function. The pharmacological profile of GSK2225745 supports its further evaluation as a potential novel inhaled therapeutic for the treatment of cystic fibrosis.

Materials and methods

Target selection. ENaC is a multimeric ion channel commonly composed of α, β, and γ subunits.[21] In addition, in respiratory epithelia, a δ subunit can also play a role in the formation of active channels.[22] Previous workers have shown that the α-subunit is critical for the function of the channel in respiratory epithelia.[4,5,18,23] Therefore, this work focused on the identification of a siRNA-targeting ENaCα.

Oligonucleotides. All oligonucleotides were 21-mers and were synthesized at either Sirna-Merck Therapeutics (West Point, PA) or ST Pharm (Seoul, Korea) by standard procedures. Complementary strands were annealed in PBS, desalted and lyophilized. Modifications were introduced into both the sense and AS strands as previously described to improve stability, enhance potency, and avoid immunostimulation.[8–10] The sequence and chemical modifications of GSK2225745 and the NTC sequence used in the studies described herein are shown in **Figure 1**. Neither GSK2225745 nor the NTC contained any phosphorothioate linkages.

Purity of all oligonucleotides used was confirmed using chromatographic techniques and mass spectrometry.

Evaluation in A549 cells

Screening of siRNAs. A549 were obtained from ATCC (Manassas, VA) (human, ATCC cat. no. CCL-185). Cells were cultured at 37 °C in the presence of 5% CO_2 and grown in Ham's F12K medium with 2 mmol/l L-glutamine adjusted to contain 1.5 g/l sodium bicarbonate and supplemented with fetal bovine serum at a final concentration of 10% and 100 U/ml penicillin. All transfections were performed using Lipofectamine 2000 (LF2K), Invitrogen (Carlsbad, CA) cat. no. 11668-027 as per the manufacturer's instructions.

Cells were plated in the center 60 wells of a tissue culture treated, 96-well plate at a final count of 7,500 cells/well in 100 µl of appropriate culture media. The outer wells of the plate were filled with 100 µl of Dulbecco's PBS to prevent evaporation. Cells were cultured for 24 hours after plating at 37 °C in the presence of 5% CO_2. After 24 hours, complexes containing siRNA and LF2K were created as follows, a solution of LF2K in Opti-MEM was prepared containing LF2K at a final concentration of 14 µg/ml. In parallel, solutions of the siRNAs for testing were prepared to a final concentration of 150 nmol/l in Opti-MEM. After incubation of both solutions at 20 °C for 20 minutes, an equal volume of the siRNA solution and the LF2K solution were added together for each of the siRNAs. Mixing resulted in a solution of siRNA/LF2K where the final concentration of siRNA was 75 nmol/l and the final concentration of LF2K was 7 µg/ml. This solution was incubated at 20 °C for a further 20 minutes. After incubation, 50 µl of the solution was added to each of the relevant wells (each siRNA was screened in triplicate in each experiment). The final concentration of siRNA in each well was 25 nmol/l and the final concentration of LF2K in each well was 2.33 µg/ml. The time of incubation with the LF2K–siRNA complexes was 48 hours, unless otherwise indicated. There was no change in media between the transfection and harvesting of the cells unless otherwise indicated. Dose–response curves were carried out in a similar fashion in 96-well plates, with each concentration of siRNA being performed in triplicate and maintaining a constant amount of LF2K in each transfection. RNA was extracted using standard methodology. Quantitative PCR (TaqMan) was used to measure the effects of the siRNAs on levels of ENaCα mRNA. The sequences of the primer-probes sets used are described below. The TaqMan data was analyzed by standard methods on an ABI 7500 instrument (Applied Biosystems, Carlsbad, CA). For detection of the predicted RNAi cleavage product by 5′ RACE, the GeneRacer kit was obtained from Invitrogen and the manufacturer's protocol was followed. The primers used were as follows: GeneRacer 5′ primer (5′-CGACTGGAGCACGAGGACACTGA), GeneRacer 5′ nested primer (5′-GGACACTGACATGGACTGAAGGAG TA), ENaCα primer 1 (5′-GGAAGACATCCAGAGGTTGG), ENaCα primer 2 (5′-GGTTGCAGGAGACCTGGTT).

Protein biochemistry. For western blot analysis, human A549 cells were seeded on T-75 cell culture flasks (3×10^6 cells per flask) and transfected the next day. Therefore, one flask was transfected with 100 nmol/l GSK2225745 and a second flask with 100 nmol/l NTC according to the Effectene transfection manual (Qiagen, Valencia, CA). After 24 hours, proteins were isolated using the ProteoExtract Transmembrane Protein Extraction Kit (EMD Millipore, Darmstadt, Germany) and separated into a cytosolic and a membrane fraction. The protein concentration was measured using the bicinchoninic acid test. For the detection of ENaC, 30 µg of membrane protein was separated *via* sodium dodecyl sulfate-polyacrylamide gel electrophoresis (7.5% acrylamide) and transferred to a polyvinylidine fluoride membrane. Nonspecific binding sites were blocked by 2 hours incubation in 5% non-fat dry milk in Tris-buffered saline/Tween (TBST) (10 mmol/l Tris HCl, pH 7.4; 140 mmol/l NaCl; 0.1% Tween 20). ENaC was detected with a custom anti-ENaCα antibody with a concentration of 1:500 diluted in 5% non-fat dry milk/TBST at 4 °C overnight. After washing in TBST, the membrane was incubated for 1 hour at room temperature with the goat anti-rabbit IgG conjugated with horseradish peroxidase (Dianova, Hamburg, Germany) diluted 1:10,000 in 5% non-fat dry milk/TBST. The membrane was washed again in TBST and the chemiluminescent detection was detected on a radiographic film. To determine the protein amounts of the bands detected by the specific ENaC antibody, the film membranes were digitized and protein bands were analyzed semi-quantitatively by densitometry using the ImageJ analysis software 1.36 (NIH, Bethesda, MD).

The custom ENaCα antibody used in these studies was generated by Cambridge Research Biochemicals using a peptide (Ac-LIEFHRSYRELFEFFC-amide) designed by GlaxoSmithKline (Stevenage, UK). This peptide corresponds to amino acids 49-64 of human ENaCα and was chosen based on sequence alignments which highlighted significant variation in this region between ENaCα and ENaCβ, γ, and δ. Antibody generation was achieved using a 12-week, two rabbit rapid 77-day immunization procedure, incorporating six separate immunizations, four bleeds/test ELISAs with affinity purification of the final samples.

In silico assessment of GSK2225745 target specificity. The GSK2225745 AS strand sequence was used to computationally determine putative "siRNA-like" and "miRNA-like" interactions by searching for sequence complementarity matches to human mRNAs. Given the chemical modifications made to the sense strand that effectively inhibit its incorporation into RNA-induced silencing complex,[24] only those hits identified with the AS strand in the "plus/minus" orientation were considered biologically relevant. BLAST analysis of the AS sequence was conducted against the National Center for Biotechnology Information human RefSeq database (http://www.ncbi.nlm.nih.gov/Refseq/), with a hit defined as a high-scoring segment pair of ≥15 contiguous nucleotides between the siRNA sequence and nontarget sequence.

Evaluation in human primary bronchial epithelial cells. Normal human bronchial epithelial cells (nonsmokers; Lonza, Slough, UK) were cultured in basal medium plus supplements (Lonza). Cells (12000/well) were transfected for a total of 48 hours using Gemini lipid surfactant (Example 37, Camilleri *et al.*)[25] (5 µg/ml) with varying concentrations of both the nontargeting siRNA and GSK2225745. Target gene expression was analyzed using TaqMan quantitative reverse transcription-PCR, using GAPDH (glyceraldehyde 3-phosphate dehydrogenase) as the housekeeping gene.

Evaluation in human primary nasal epithelial cells
Patients and ethical consent. We obtained nasal specimens from patients undergoing nasal surgery. The specimens were typically nasal polyps or nasal turbinates of patients suffering from chronic sinusitis. The study was approved by the committees for human studies of the University of Muenster (*Ethik Kommission Muenster*). Declaration of Helsinki protocols were followed and the patients provided their written, informed consent.

Cell culture. Primary cell culture of human nasal epithelia cells was performed as described previously.[26] Briefly, nasal epithelial cells were isolated by enzymatic digestion for 24–48 hours and afterwards seeded on permeable collagen filters with a diameter of 14 mm (Cellagen TM dics CD 24; MP Biomedicals, Eschwege, Germany) for Ussing chamber experiments. The cells were cultured with serum-free F-12 Nutrient Mixture (Ham) (PAA Laboratories, Coelbe, Germany) supplemented with the following agents: insulin (2 µg/ml) (Life Technologies, Karlsruhe, Germany), epidermal growth factor (13 ng/ml) (Sigma-Aldrich, Schnelldorf, Germany), endothelial cell growth supplement (7.5 µg/ml) (Becton Dickinson, Heidelberg, Germany), triiodo-thyronine (3 nmol/l) (Sigma-Aldrich), hydrocortisone (100 nmol/l) (Sigma-Aldrich), gentamycin (10 µg/ml) (Biochrom AG, Berlin, Germany), penicillin/streptomycin (100 U/ml) (Biochrom AG), L-Glutamine (2 mmol/l) (Biochrom AG), and transferrin (4 µg/ml) (Life Technologies). Previously, it was shown that these supplements had no effect on the electrical parameters of the human nasal epithelium.[27] Cells were incubated in 95% air and 5% CO_2 at 37 °C.

Transfection procedures. Transfection was carried out following the manufacturer's standard protocol (Effectene transfection reagent (Qiagen, Hilden, Germany)) for transfection in a 48-well format using the collagen filter or for RNA isolation in a 6-well format using culture dishes. For the electrophysiological measurements, cell monolayers were transfected either with GSK2225745 or with the NTC. For dose–response analyses, final siRNA concentrations of 3 and 10 nmol/l were used and measurements performed after 24 hours. For timepoint measurements, the effective concentration of 10 nmol/l was used for the transfection and monolayers were measured after 24, 48, and 72 hours of incubation with the respective siRNA. For RNA isolation, cells were transfected on cell culture dishes in the same way as described before and also isolated after the same incubation conditions. Therefore, cells were scraped off with 350 µl RLT buffer (according to the protocol of the RNeasy Plus Mini Kit; Invitrogen, Hilden, Germany). Cells for these analyses were only isolated if the respective filter (with cells of the same tissue origin) showed positive results in the electrophysiological Ussing chamber measurements.

Transepithelial measurements. After reaching confluence (7–9 days after seeding), the nasal epithelial cells were mounted in modified Ussing chambers. The two compartments of the chamber were continuously perfused with cell culture Ringer at 37 °C. Voltage and current electrodes (AgCl wires) were used and were electrically connected to the chamber by KCl-agar bridges. The current electrodes held the transepithelial potential (V_t), which is established by an active transport of ions to zero. The required current of compensation, called short-circuit current (I_{sc}), depends on changes in conductance of the epithelium, which is generated by perfusion of different solutions. Na^+ absorption through ENaC was assessed as I_{sc} and conductance (G_t) in the presence and absence of amiloride (100 µmol/l). To determine the overall Na^+ absorption of the cells, we removed Na^+ from the apical Ringer in a second step. I_{SC} was continuously monitored using the computer program ImpDsp1.4 (Prof Willy Van Driessche, KU Leuven, Leuven, Belgium). The measured area had a size of 0.5 cm^2, while the electrical parameters were normalized to an area of 1 cm^2.

Solutions. Cell culture Ringer was composed as follows: 130 mmol/l NaCl, 5 mmol/l KCl, 1 mmol/l $CaCl_2$, 2 mmol/l $MgCl_2$, 5 mmol/l glucose, 10 mmol/l HEPES. In the sodium-free solution, NaCl was replaced by an equivalent amount of tetramethylammonium chloride.

Evaluation of immunostimulatory potential. Potential TLR3-mediated stimulatory effects of GSK2225745 were monitored by incubating normal human bronchial epithelial cells

for 48 hours with varying concentrations of the siRNA, both in the presence and absence of Gemini surfactant. PolyI:C (2.5 µg/ml; InvivoGen, San Diego, CA) was included as a positive control. Expression of the immunostimulatory marker OAS1 was measured by TaqMan assay. Recombinant U2OS cells stably expressing either TLR7 or TLR8 receptors were utilized to further monitor potential immunostimulatory effects of GSK2225745. The cells were transfected with varying concentrations of GSK2225745 and a nontargeting siRNA using Dharmafect 1 (Dharmacon, Lafayette, CO), for 24 hours. Resiquimod (R848)(30 µmol/l) and ssRNA40 (0.6 µmol/l) were included as positive controls for TLR7 and TLR8 stimulation, respectively. Levels of expression of IL-8 mRNA were measured by TaqMan assay and expressed relative to the housekeeping gene, GAPDH.

Evaluation of GSK2225745 in vivo. GSK2225745 was formulated using a cationic lipid, C12-200, previously reported[15] to be able to effectively facilitate siRNA delivery to mouse liver *in vivo*. Briefly, C12-200, distearoylphosphatidylcholine (part number 850365P; Avanti-Lipids, Alabaster, AL), Cholesterol (part number C86675G; Sigma-Aldrich, St Louis, MO), and mPEG-2000-DMG were prepared as a solution in a ethanol-water mixture (90/10 vol/vol), in a 50:10:38.5:1.5 molar ratio respectively; 5.05×10^{-8} mol of the C12-200 lipid were used in 1 ml of the ethanol-water mixture. The GSK2225745 siRNA was dissolved in 10 mmol/l pH 3 citrate buffer to give a concentration of ~29.4 µmol/l and an initial measurement of the ultraviolet absorbance of this solution at 260 nm was made. Equal volumes of the lipid and siRNA solutions were transferred into vials and pumped in opposition through a small plastic T-piece at a rate of 130 ml/minute to spontaneously form lipid nanoparticles. The output from the T-piece immediately underwent further mixing using an in-line 24-element static-mixer before secondary dilution and stirring in an incubation vessel containing an equivalent total volume of the citrate buffer. The suspension of lipid nanoparticles was allowed to incubate for a period of 1 hour with gentle stirring. Tangential flow filtration was performed with a Vivaflow 50 cartridge (50 kDa, part number VF05P3; Sartorius Stedim, Bohemia, NY) to exchange the aqueous citrate buffer-ethanol mixture for a 0.1X PBS. Six volumes of permeate were collected and the absorbance measured to provide a preliminary estimate of the amount of siRNA that had not been incorporated in the lipid nanoparticles. The lipid nanoparticles were then concentrated by a factor of four by detaching the buffer inlet. Control of the concentration of the final lipid nanoparticle suspension was achieved by measuring the permeate volume during the concentration step. The suspension of lipid nanoparticles was then sterile filtered through 0.22 µm polyethersulfone membranes into glass vials with silanized lids. The lipid nanoparticles were stored under a nitrogen headspace at refrigerated conditions until further use.

Prior to use of the lipid nanaoparticles, the size and polydispersity of the systems were measured using dynamic light scattering. The percentage siRNA entrapped in the lipid nanoparticles was determined by using a centrifugal filter methodology (Centrisart filters 100 kDa MWCO, part number 13269E; Sartorius Stedim).

All *in vivo* studies were carried out using female BALB/c mice supplied by Charles River Laboratories, Margate, UK. Test materials were administered under a brief period of isoflurane (2% in oxygen) anesthesia. The anesthetized animals were dosed intranasally using a Gilson pipette (total volume administered was 50 µl/mouse) while being held vertically, in order to assist distribution of dosing solution to the lower airway.

Mice were euthanized by intraperitoneal injection of pentobarbitone 72 hours after administration of siRNA. The lung lobes were removed, quickly sliced using fine dissecting scissors and divided to provide two samples per animal which were placed in Qiagen RNAlater (each sample in 1.5 ml) Tissue Protect Tubes at room temperature. The Qiagen RNAlater solution was aspirated from the samples on the following day (at least 22 hours after collection) and the samples were then stored frozen (–80 °C) before RNA extraction and TaqMan analysis.

All animal studies were ethically reviewed and carried out in accordance with Animals (Scientific Procedures) Act 1986 and the GSK Policy on the Care, Welfare, and Treatment of Laboratory Animals.

Sequences of primers and probes used for quantitative reverse transcription-PCR. TaqMan primers and probe sets were designed using the Applied Biosystems primer express 3.0. TaqMan primers were supplied by Sigma-Aldrich and probes by Biosearch Technologies (Novato, CA).

GAPDH
Forward: 5′-CAAGGTCATCCATGACAACTTTG-3′
Reverse: 5′-GGGCCATCCACAGTCTTCT-3′
Probe: 5′d FAM-ACCACAGTCCATGCCATCACTGCCA-TAMRA 3′

ENaCα
Forward: 5′-ACATCCCAGGAATGGGTCTTC-3′
Reverse: 5′-ACTTTGGCCACTCCATTTCTCT-3′
Probe: 5′dFAM-TGCTATCGCGACAGAACAATTACACCGTC-TAMRA 3′

OAS1
Forward: 5′-ACCTAACCCCCAAATCTATGTCAA-3′
Reverse: 5′-TGGAGAACTCGCCCTCTTTC-3′
Probe: 5′d FAM-CTCATCGAGGAGTGCACCGACCTG-TAMRA 3′

IL-8
Forward: 5′-CTGGCCGTGGCTCTCTTG-3′
Reverse: 5′-CCTTGGCAAAACTGCACCTT-3′
Probe: 5′d FAM-CAGCCTTCCTGATTTCTGCAGTCTGTG-TAMRA 3′

Data analysis. Where mean data are shown, they are presented as mean ± SD. When appropriate, statistical analysis between treatment groups was carried out using Student's *t*-test for unpaired data. $P < 0.05$ was taken as evidence of a statistically significant difference.

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