Development of Pre-Clinical Models for Evaluating the Therapeutic Potential of Candidate siRNA Targeting STAT6

Gareth D. Healey¹,², Jennifer A. Lockridge³, Shawn Zinnen⁴, Julian M. Hopkin¹, Ivan Richards⁵, William Walker¹

¹College of Medicine, Swansea University, Swansea, United Kingdom, ²Allema Therapeutics Ltd, Swansea University, Swansea, United Kingdom, ³Lockridge Pharmaceutical Consulting LLC, Westminster, Colorado, United States of America, ⁴Zincyte Consulting, Denver, Colorado, United States of America, ⁵Sunapten Therapeutics, Kalamazoo, Michigan, United States of America

Abstract

Developing siRNA therapeutics poses technical challenges including appropriate molecular design and testing in suitable pre-clinical models. We previously detailed sequence-selection and modification strategies for siRNA candidates targeting STAT6. Here, we describe methodology that evaluates the suitability of candidate siRNA for respiratory administration. Chemically-modified siRNA exhibited similar inhibitory activity (IC₅₀) against STAT6 in vitro compared to unmodified siRNA and apical exposure testing with Caco-2 cell monolayers showed modification was not associated with cellular toxicity. Use of a modified RNA extraction protocol improved the sensitivity of a PCR-based bio-analytical assay (lower limit of siRNA strand quantification = 0.01 pg/µl) which was used to demonstrate that lung distribution profiles for both siRNAs were similar following intra-tracheal administration. However, after 6 hours, modified siRNA was detected in lung tissue at concentrations >1000-fold higher than unmodified siRNA. Evaluation in a rat model of allergic inflammation confirmed the persistence of modified siRNA in vivo, which was detectable in broncho-alveolar lavage (BAL) fluid, BAL cells and lung tissue samples, 72 hours after dosing. Based upon the concept of respiratory allergy as a single airway disease, we considered nasal delivery as a route for respiratory targeting, evaluating an intra-nasal exposure model that involved simple dosing followed by fine dissection of the nasal cavity. Notably, endogenous STAT6 expression was invariant throughout the nasal cavities and modified siRNA persisted for at least 3 days after administration. Coupled with our previous findings showing upregulated expression of inflammatory markers in nasal samples from asthmatics, these findings support the potential of intranasal siRNA delivery. In summary, we demonstrate the successful chemical modification of STAT6 targeting siRNA, which enhanced bio-availability without cellular toxicity or reduced efficacy. We have established a robust, sensitive method for determining siRNA bio-distribution in vivo, and developed a nasal model to aid evaluation. Further work is warranted.

Introduction

The mainstay of treatment for allergic asthma continues to rely on the use of anti-inflammatory corticosteroid drugs [1] that can have undesired side-effects [2], indicating the need for more selective therapeutics. In this regard, interleukin (IL)-13, acting through the signal transduction molecule - STAT6, has been implicated as a major driver of bronchial inflammation [3–5], prompting efforts to develop therapeutics that specifically inhibit this pathway [6–8]. Furthermore, consistent with the increased recognition that epithelial cells play a fundamental role in asthma pathogenesis [9,10], it is notable that STAT6 expression in lung epithelial cells was shown to be exclusively required for IL-13-mediated pathology [11]. We therefore developed a refined therapeutic approach utilising small interfering RNA (siRNA) to specifically suppress STAT6 expression in lung epithelial cells and demonstrated that siRNA treatment can ablate the ongoing inflammatory response initiated in epithelial cells after in vivo exposure to IL-13 [12]. We further expanded this work by developing methodology for screening the STAT6 gene, permitting the identification of candidate therapeutic siRNA. In particular, we were able to identify cross-species active siRNA molecules that did not produce interferon responses and showed that stabilising chemical modifications could be applied to certain candidates without loss of targeting efficacy [13].

The development of new therapeutics involves significant hurdles, which with regard to asthma are particularly challenging...
given the limitations of current animal models [14–16] and the paucity of relevant biomarkers [17,18]. Variables including pharmacokinetics and the status of target gene expression within respiratory tissue pre- and post-exposure are particularly pertinent when considering the preclinical evaluation of STAT6 siRNA. To address the latter, we recently evaluated primary human nasal epithelial cells, based on the premise that respiratory allergy is an integrated disorder of the respiratory tract [17,19,20], and found that such cells from asthmatic donors indeed possessed inflammatory changes consistent with disease phenotype [21]. Furthermore, biopsied nasal epithelium exhibited invariant STAT6 expression that was readily targetable with siRNA in vivo, pointing to the potential utility of nasal epithelium in the pre-clinical testing of STAT6 siRNA. Challenges particularly relevant to the siRNA molecules themselves as drug candidates include accurate analysis of bio-distribution within target tissues in relation to the persistence of molecules engineered to act in vivo with minimal side effects [22]. Therefore, to address these issues, we describe the pre-clinical characterisation of STAT6 targeting respiratory siRNA with potential as novel therapeutics, placing particular focus on the comparative in vivo analysis of unmodified versus chemically-stabilised siRNA. Utilising an optimised candidate siRNA with complete homology to various mammalian species (see Methods) and a sensitive PCR-based bio-analytical assay, we compare siRNA bio-distribution within the lung of normal and allergen sensitised animals following intra-tracheal delivery and a model involving intra-nasal administration of siRNA. This approach of comparing traditional in vivo methodologies alongside the development of novel drug-testing platforms provides valuable, predictive pre-clinical data that should assist the optimal development of siRNA-based therapeutics intended for respiratory administration.

### Materials and Methods

#### Small interfering RNA

STAT6-specific siRNA (372u) and mismatch (scrambled 372 sequence) control siRNA were synthesised using standard chemistry and annealed by Agilent Technologies, Inc. (Delaware, USA). Chemically-modified siRNA (372 m) and mismatch control (MMC) siRNA were similarly synthesised with specific modifications consisting of: deoxy-ribonucleotides substitutions, 2’O-methyl-modified adenosine/ guanine, internal 2’ fluoro-modified cytosine/ uracil and phosphorothioate linkage as detailed in Table 1. Scrambled (SC) siRNA used within the A549 lung epithelial cell experiments was Silencer Select Negative Control No. 1 siRNA (Life Technologies, Paisley, UK). Cross-species activity of 372 (u, m) siRNA is defined as complete sequence homology with human, mouse, rat, rhesus macaque and bovine STAT6 [13].

#### Gene expression analysis

Real-time RT-PCR was performed using TaqMan gene expression assays: STAT6 Hs00598625_m1; GAPDH Hs99999905_m1; β-actin Hs99999903_m1 (Life Technologies). The expression of each test gene was normalised against expression of housekeeping genes, GAPDH & β-actin. Quantification was performed using a standard curve of recombinant human STAT6 and results expressed as absolute values or percentage STAT6 mRNA remaining. Percent mRNA remaining
was calculated by multiplying the fold change value, derived using the method described by Pfaffl et al [23], by 100.

**Western blotting**

Western Blotting for STAT6 and GAPDH was carried out as previously described [12]. Protein densitometry analysis was carried out using the Quantity One 1-D analysis software (Bio-Rad Laboratories Ltd, Hemel Hempstead, UK).

**Evaluation of cellular toxicity using Caco-2 cell monolayers**

Cellular toxicity/permeability screening was performed as previously described [24,25] with minor modifications. Briefly, Caco-2 cells were grown to confluence for 16 days on 1 μm filters in 24-well plates. Confluent monolayers were then exposed on their apical surface to siRNA diluted in HBSS (Sigma-Aldrich Ltd, Gillingham, UK) at the indicated concentrations (400 μl/well, in triplicate) for 6 hours at 37°C. Control monolayers consisted of cells exposed to 1% Triton x-100 (Sigma-Aldrich) in HBSS, or calcium-free phosphate buffered saline (PBS; Life Technologies) in both compartments. Following incubation, apical and basolateral chambers were sampled for siRNA quantification. To assess monolayer integrity, Lucifer Yellow was added to basolateral chambers (HBSS in apical chambers) and incubated for 30 minutes at 4°C with results reported as percent Lucifer Yellow transfer. To permit RNA analysis, cells were washed, and harvested from the filters using TRI-Reagent (Sigma-Aldrich).

**Bio-analytical assay**

The siRNA-specific assay was adapted and modified from published methodology [26] to enable quantification of individual siRNA strands in biological samples using separate (sense and anti-sense) stem-loop reverse transcription followed by TaqMan PCR. Plasma or lavage fluid samples were diluted (1:50) in 1× Tris-EDTA (TE) buffer (Life Technologies) prior to stem-loop primer annealing and subsequent cDNA synthesis. To isolate siRNA from tissues, cells were lysed with TRI-Reagent (Sigma-Aldrich), and the first step (chloroform) phase separation then carried out. The aqueous phase from this separation was then diluted (1:50) in 1× TE buffer and samples treated the same as the plasma and liquid samples. Standard curves were constructed using sense and anti-sense strands of known concentration diluted in 1× TE buffer. Stem-loop primer annealing was performed by heating the samples to 95°C for 5 min followed by stepped temperature ramping to 42°C at a rate of 10°C per 2 minutes. Stem-loop primers used for the detection of 372 (u, m) are shown in Table 1. Synthesis of cDNA was performed using the TaqMan microRNA RT synthesis kit (Life Technologies). Real-time PCR was then performed on cDNA samples in triplicate (20 μl reactions) using specific forward primers to the sense and anti-sense strands and a common reverse primer (Table 2). Detection of the PCR product was achieved using Universal library probe #77 (Roche Diagnostics, West Sussex, UK). Percent PCR efficiency was calculated using the formula: (10^(1/slope)-1)×100%.

**Animal models**

All procedures were approved by the South west Wales local research ethics committee and conducted in accordance with the Animal (Scientific Procedures) Act of 1986.

**Lung bio-distribution**

Adult (10 weeks old) Brown Norway male rats (BN/RijHsd; Harlan Laboratories Inc.) were anaesthetised with Isoflurane vapors utilising a precision vapouriser (3–5%, for approximately 5–10 min). Each rat received a single dose of siRNA in sterile saline via the intra-tracheal route (1 mg/kg). At the indicated time points blood was collected by closed cardiac puncture into a 5 ml syringe containing 100 μl of 1000 U/ml heparin (APP Pharmaceuticals, Illinois, USA). The needle was removed from the syringe and the blood added to a sterile, RNase-free polypropylene tube, gently mixed, and kept on ice until plasma was separated by centrifugation (within 30 min of blood collection). Animals were then euthanised with CO₂ followed by decapitation before dissection to remove the lungs for further processing. Plasma was stored at –80°C and lung tissue was insuflated with, and then stored in RNAlater (Life Technologies).

**Model of allergic inflammation**

Brown Norway male rats were pre-sensitised to allergen by intraperitoneal injections of ovalbumin (10 μg) in aluminium hydroxide (10 mg) at days 0, 7 and 14. Animals, anaesthetised as described above, were treated with siRNA (2 mg/kg) in sterile saline via the intra-tracheal route on 3 consecutive days (10 μl volume, days: 18, 19 and 20 of the study). Dexamethasone (0.3 mg/kg) was used as the control reference drug. Sensitised/drug-treated animals were then exposed to aerosolised ovalbumin (10 mg/ml) using a nose-only inhalation system, for 1 hour on day 21 of the study. Plasma and broncho-alveolar lavage (BAL) were harvested 72 hours (day 24) after exposure to aerosolised ovalbumin from anaesthetised animals. Cytospin (Fisher Scientific, Loughborough, UK) preparations of cells recovered from lavage fluid were adjusted to 1×10⁶ cells/ml and stained with Speedy-Diff (Clin-Tech Ltd, Guilford, UK) for differential enumeration of eosinophils, neutrophils, monocytes and lymphocytes. Following lavage, animals were euthanised with CO₂ followed by decapitation; lungs were removed, insuflated and fixed with 10% formalin prior to standard processing for paraffin-wax histopathology. Inflammation scoring of haematoxylin- & eosin-stained tissue sections was conducted in a randomised/ blinded fashion by assessing the presence of: goblet cell hyperplasia, peribronchial inflammation and eosinophil infiltration. Each characteristic was scored as either: 0 (not present), 1 (mild), 2 (moderate) or 3 (severe) and the mean scores for each lobe then added to give an overall characteristic score for the whole lung of each animal. The total lung inflammation score was determined by summation of the individual characteristic scores for each animal.

**Intra-nasal exposure model**

Anaesthetised, as described above, adult male Brown Norway rats (BN/RijHsd) were administered 0.2, 1.0 or 2.0 mg/kg of siRNA in sterile saline using an Eppendorf pipette: total dose volume/rat/day = 60 μl (two 15 μl doses within 1 minute to each nare). Tissue samples were collected from euthanised animals, as described above, 72 hours after the final dose by removing the skin and lower jaw from the skull and splitting at the medial suture. Separate structures collected from the exposed nasal cavity included right and left: nasal septum lining, maxillo-turbinate, naso-turbinate, ethmoid tumour and lateral lining. For subsequent gene expression/siRNA quantification, excised nasal tissue samples were immediately placed into 1 ml of TRI-Reagent and processed accordingly.

**Statistical and data analysis**

Statistical analyses were performed using IBM SPSS 19.0 [SPSS Inc, Chicago, IL, USA]. Data were analysed using analysis of variance (ANOVA) with post hoc analysis performed using...
Bonferroni correction. Data are expressed as mean ± S.E.M and in all cases, P values <0.05 were considered significant.

**Results**

Effect of chemical modification on the in vitro efficacy of STAT6-targeting siRNA

Prior to in vivo testing, the specific inhibitory activity of unmodified (372u) and chemically-stabilised (372 m), cross-species (see Materials and Methods), STAT6 siRNA were verified through in vitro testing in human A549 lung epithelial cells as previously described [12]. Total RNA was harvested from cells 72 hours after transfection with a range of siRNA concentrations (0.1 – 100 nM) and quantitative RT-PCR used to determine the expression of STAT6 mRNA after each treatment. Data analysis showed that 372u and 372 m siRNA were comparable in their ability to suppress human STAT6 mRNA expression, exhibiting 50% inhibitory concentration (IC\textsubscript{50}) values of 0.27 nM and 0.35 nM respectively (Figs. 1a, b). Western blot analysis of protein extracts from siRNA-treated cells confirmed that inhibition of STAT6 protein expression was observed at all concentrations of 372u tested (c) and at concentrations ≥ 5 nM for 372 m (d). SC = Silencer Select Negative Control No. 1 siRNA, vehicle = transfection reagent only. Values presented are mean ± S.E.M (n = 3).

doi:10.1371/journal.pone.0090338.g001

Figure 1. Small interfering RNA suppression of STAT6 expression in vitro. A549 lung epithelial cells were transfected with a range of siRNA concentrations (0.1 – 100 nM) and total RNA and protein extracted 72 hours later. The percent STAT6 mRNA remaining at each of the concentrations tested was used to calculate the 50% inhibitory concentration of 372u (a) and 372 m (b); 0.27nM and 0.35nM, respectively. Western blot analysis was then used to confirm STAT6 protein ablation, which was observed at all concentrations of 372u tested (c) and at concentrations ≥ 5 nM for 372 m (d). SC = Silencer Select Negative Control No. 1 siRNA, vehicle = transfection reagent only. Values presented are mean ± S.E.M (n = 3).
Figure 2. Optimisation of the bio-analytical assay. (a-d): Maintenance of bio-analytical assay sensitivity within different biological matrices. 372u or 372 m siRNA were spiked into 1 x TE buffer (●), plasma (□) or rat lung tissue extract (△) at a range of concentrations (0.032 – 100 pg/μl) and then analysed using an RT-PCR-based bio-analytical assay. No significant difference in the threshold cycle of detection (Ct) was noted between the three matrices at any of the siRNA concentrations of tested. Shown are representative standard curves obtained for sense (a, b) and anti-sense (c, d) strand measurement. Values presented are mean (n = 3) with errors bars omitted for clarity. Percent PCR efficiency for 372u (sense) in TE buffer, plasma, or lung extract = 94.2%, 86.3% and 102.8% respectively. Percent PCR efficiency for 372 m (sense) = 124.2%, 101.5%, 114.8% respectively. (e, f): To compare the effect of RNA extraction methods on bio-analytical assay sensitivity, rat lung tissue was spiked with 372u (e) or 372m (f) (125 ng) and homogenised in 4M guanidine isothiocyanate (GITC) or Tri-reagent. Tri-reagent homogenised tissue was processed through the manufacturer’s recommended procedure for the extraction of RNA, either up to the chloroform phase separation step (TR-AQ) or through the entire process (TR-F). Recovery of siRNA from TR-AQ processed tissue was comparable to GITC, recovery from TR-F processed tissue was significantly lower. Values presented are mean ± S.E.M (n = 3), and represent % siRNA recovered in comparison to GITC.

doi:10.1371/journal.pone.0090338.g002
Figure 3. In vitro Caco-2 cell monolayer toxicity testing of STAT6 siRNA. Caco-2 cell monolayers were exposed to a range of siRNA concentrations (50–5000pM) for 6 hours and membrane integrity monitored by the transfer of Lucifer Yellow dye between the basolateral and apical compartments. No significant transfer of Lucifer Yellow dye was noted following treatment with either 372u (a) or 372m (b) compared to the vehicle control (saline). Analysis of siRNA recovered from the apical (c, d) and basolateral (e, f) compartments following treatment, showed that both 372u (c, e) and 372m (d, f) were present at ≤4pg/μl, LLOQ = 0.01 pg/μl. Values presented are mean ± S.E.M (n = 3), and P-values represent comparison to the vehicle (saline) control.

doi:10.1371/journal.pone.0090338.g003
Development and validation of a STAT6 siRNA-specific bio-analytical assay

In order to accurately quantify bio-distribution of siRNA we required a method that was conducive to high-throughput sample screening, capable of detecting individual siRNA strands and, given the general efficiency of siRNA-mediated gene silencing, robust enough to detect small (pico-gram) quantities in harvested tissue samples. The method used was adapted from Chen et al 2005 [26] and utilises separate (sense and anti-sense) stem-loop reverse transcription to detect specific siRNA strands followed by TaqMan PCR. Prior to experimental use, we performed validation testing of the assay to confirm its specificity for STAT6 siRNA and its capability to perform robustly without interference in the presence of plasma or material extracted from animal tissue. Analysis of spiked samples showed that the wide dynamic range and sensitivity ($LLOQ = 0.01 \text{ pg/µl}$) of the assay was not significantly affected by biological matrices such as plasma or lung tissue extract (Fig. 2a-d). Furthermore, in order to develop a simpler protocol that was compatible with simultaneous measurement of gene expression (protein and mRNA) in tissue homogenates we compared guanidine isothiocyanate (GITC) extraction with a modified TRI-Reagent extraction protocol. In this novel, truncated TRI-Reagent method, avoidance of isopropanol precipitation steps were shown to produce RNA yields equivalent to that obtained with GITC (Fig. 2e, f), we therefore employed this modified protocol in all subsequent experiments requiring siRNA and simultaneous gene expression quantification.

Cellular toxicity analysis of 372 and 372m siRNA

Having confirmed that chemical modification did not significantly affect the suppressive activity of 372u siRNA, we wished to extend our in vitro analyses to include evaluation of cellular penetration and toxicity. To measure this we utilised the Caco-2 cell monolayer model which has become well established in drug permeability studies [27]. Apical exposure of cells to either unformulated siRNA (delivered in saline solution) did not disrupt the cell monolayer sufficiently to permit dye transfer between the basolateral and apical compartments, indicating that tight-junction integrity of the epithelial monolayer was maintained (Fig. 3a, b). In addition to Lucifer Yellow transfer, we utilised our PCR-based bio-analytical assay to determine the concentration of siRNA present in the apical, basolateral and cellular fractions of the Caco-2 cell assay. Consistent with the Lucifer Yellow results, bio-analytical analysis revealed that the majority of detectable siRNA was present in the apical chamber at 6 hours post-treatment (Fig. 3c, d), with very small amounts ($\leq 4 \text{ pg/µl}$) detectable within the basolateral compartment (Fig. 3e, f) and neither siRNA being detected within the cellular compartment (data not shown). Therefore, unformulated 372u or its chemically-modified counterpart 372m, did not penetrate or have any significant detrimental effect on epithelial membrane integrity.

Bio-distribution of STAT6 siRNA following lung exposure in normal and allergen sensitised rats

In initial bio-distribution experiments, siRNA quantification in plasma and lung tissue was performed after normal animals had received a single intra-tracheal dose of siRNA (372u or 372m, 1 mg/kg). In the absence of a suitable delivery vehicle and to avoid potential confounding factors, these experiments were performed with unformulated siRNA. Lungs were dissected into nine separate sections representing the right accessory lobe (1), right cranial lobe (2), right middle lobe (3), upper left lobe (4), mid left lobe (5), lower left lobe (6), upper right caudal lobe (7), mid right caudal lobe (8) and lower right caudal lobe (9) (Fig. 4a). Five minutes after administration both siRNA’s were distributed throughout the rat lung with notable accumulation within the right cranial lobe (2). Both sense and anti-sense strands from 372u and 372m were detectable at similar levels by bio-analytical assay (Fig. 4c, d). After 6 hours, whole lung analysis showed that 372m sense and antisense strands were detectable at a concentration >1000-fold higher than 372u (Fig. 4b; $372m = 6.5 \times 10^6 \text{ pg/mg} \pm 3.3 \times 10^6$, $372u = 90.1 \text{ pg/mg} \pm 37.5$). Consistent with this observation, only 372m was detectable in plasma 6 hours post-administration (Fig. 4f).

To evaluate bio-distribution in the presence of allergic lung inflammation, siRNA were administered to ovalbumin-sensitised rats on 3 consecutive days with subsequent aerosol airway challenge with ovalbumin 24 hours after the last siRNA dose. Bio-analytical analysis of broncho-alveolar lavage (BAL) obtained from rats 72 hours after aerosolised ovalbumin challenge (6 days after first siRNA dose, 2mg/kg) showed that 372m was detectable at significantly higher levels in both BAL (Fig. 5a) and harvested cells (Fig. 5b). Histological analysis of lung sections showed that only dexamethasone treatment significantly reduced lung inflammation (goblet cell hyperplasia, peribronchial inflammation, eosinophil infiltration) following allergen challenge (Fig. 5c). Analysis of BAL cellularity corroborated this finding in that monocyte (Fig. 5d), neutrophil (Fig. 5e) and eosinophil (Fig. 5f) numbers were only significantly reduced following treatment with dexamethasone.

Intra-nasal exposure as a pre-clinical model for assessing STAT6 siRNA

Given our recent findings correlating asthma with biomarker gene expression in primary human nasal epithelial cells [21], we hypothesised that intra-nasal exposure would provide a simpler, relevant approach to the analysis of STAT6 siRNA activity in vivo. As rat nasal cavities are predominantly lined with respiratory as well as olfactory epithelium [28], we first carried out fine dissection to determine STAT6 expression in the various nasal sections (Lateral lining, LL; inferior ethmoid turbinate, IE; median ethmoid turbinate, ME; superior ethmoid turbinate, SE; maxillary turbinate, M; naso-turbinate, N & septum lining, SL: Fig. 6f). RT-PCR and Western blotting analysis revealed that endogenous STAT6 mRNA and STAT6 protein expression was invariant throughout the nasal cavities of untreated rats (Fig. 6a, b), consistent with our findings in both human epithelial cell lines [12] and nasal cells from human donors [21]. To allow accumulation and retention of siRNA solution within the nasal cavities, anaesthetised rats were administered small volumes of unformulated siRNA in saline using a positive displacement pipette (15 µl doses, 60 µl maximum/ animal, 2 mg/kg). Bioanalytical analysis...
Figure 4. Bio-distribution and persistence of STAT6 targeting siRNA in rat lungs following intra-tracheal delivery. Normal rats were administered 372u or 372m (1 mg/kg, intra-tracheal) and siRNA quantification performed in lung tissue and plasma. Whole lung analysis showed the presence of significantly more 372m after 6 hours compared to 372u (b). 372u (c) and 372m (d) were both distributed throughout the lung 5 minutes after intra-tracheal administration. In addition, plasma concentrations of 372m (f) were significantly higher and persisted for longer than 372u (e). 1 = right
of the nasal cavities 72 hours post-administration revealed the presence of 372u and 372m at <0.1% of the original dose (372u = 4.3 ±2.7 ng, 372m = 14.2 ±8.2 ng, initial dose = 500 µg) throughout the nasal cavities (Fig. 6c, d). Of note, 372m was present at significantly (P<0.001) higher levels in the nasal septum lining (SL) compared to 372u. Consistent with this, 372m was also detectable at significantly (P<0.001) higher levels in plasma at this time point (Fig. 6e). A comparative analysis of STAT6 expression in the various nasal cavities 72 hours after administration of either 372u or 372m siRNA was also performed. When compared with saline treatment, there was a trend toward lower detectable levels of STAT6 mRNA within the inferior ethmoid turbinate of 372m or 372u. Statistical analysis revealed the lack of significant (P = 4.3

2.7 ng, 372m = 14.2

m

372m and 372u.

Accurate measurement of siRNA levels during pre-clinical testing is essential to their successful development as suitable drugs and we therefore modified the methodology of Chen et al 2005 [26] to develop a PCR-based bio-analytical assay with specificity to our candidate siRNA (372u, 372m). This highly sensitive assay was robust in its ability to detect either form of STAT6 siRNA to our candidate siRNA (372u, 372m). This highly sensitive assay [26] to develop a PCR-based bio-analytical assay with specificity [33,34]. However, we previously demonstrated that application of a chemical modification strategy favouring sense strand degradation produces siRNA which retain silencing ability without immune stimulation [13]. Here, we extend this work by showing that modified STAT6 siRNA (372m) exhibited comparable suppressive activity (IC50) to its unmodified counterpart (372u) and that modification was not detrimental to cells, in that neither siRNA disrupted epithelial monolayer tight-junction integrity. Furthermore, bio-distribution analysis demonstrated that both siRNAs were equally distributed throughout the lungs and that chemical modification significantly enhanced bioavailability of 372m in both lung tissue and plasma, a critical barrier to overcome in the development of siRNA therapeutics [35–37]. This improved bio-availability was also notable in rats with inflamed airways in that higher concentrations of 372m were recoverable from BAL. Notably, the BAL cellular fraction which consisted predominantly of inflammatory cells also contained significant amounts of 372m siRNA. Whether the detected siRNA is of actual intracellular origin or represents contaminating siRNA adhered to BAL cell surfaces, is debatable, given that unformulated siRNA exhibit poor cellular penetration characteristics [38,39], as demonstrated by our findings showing a lack of significant siRNA transfer across Caco-2 cell monolayers. However, it is notable that macrophages, which are more receptive to siRNA uptake [40,41], predominated in the BAL from asthmatic rats and these cells may therefore represent the source of 372m. Although unformulated 372m siRNA treatment did not attenuate allergen-induced lung inflammation in our rat model, a previous study utilising a mouse model of allergic inflammation showed that intranasal administration of unformulated STAT6 siRNA could attenuate allergic airway inflammation [42]. The reasons for this discrepancy are unclear but may reflect differences including, distinct animal models, siRNA sequences, dose and routes of administration. Furthermore, previous studies using formulated siRNA to target IL-13 in a mouse model of allergic inflammation, did demonstrate efficacy (and hence cellular penetration) in that airway hyper-reactivity was ameliorated without concomitant attenuation of lung inflammation [43]. This suggests that at least in the animal models used, lung inflammation per se may not be a reliable indicator of targeted suppression within the IL-13 signalling pathway.

Given our recent findings of candidate siRNA activity in primary human nasal epithelium, which illustrated the potential utility of human nasal epithelium sampling in respiratory siRNA development [21]. We developed an animal model for the pre-clinical testing of SiRNA targeting STAT6
Figure 5. Effectiveness of STAT6 targeting siRNA within a rat model of allergic inflammation. Ovalbumin-sensitised animals were administered 372u, 372m, MMC siRNA (2 mg/kg, intra-tracheal) or dexamethasone (0.3 mg/kg) on 3 consecutive days and then aerosol challenged with ovalbumin 24 hours after the final dose. Seventy-two hours after challenge, 372m was present at significantly higher concentrations in BAL than 372u (a, b). Treatment with 372u or 372m did not reduce lung inflammation compared to the saline treated control, as evidenced by histological scoring (c) and inflammatory cell enumeration (d-f). Treatment with dexamethasone however, did significantly reduce lung inflammation following allergen challenge. Values presented are mean ± S.E.M (n = 10), and P-value represents comparison to the saline treated control.

doi:10.1371/journal.pone.0090338.g005
clinical evaluation of STAT6 siRNA, that incorporated fine dissection of the nasal cavity with bio-analytical analysis to obtain a high-resolution picture of siRNA distribution. As allergic, IL-13 driven, asthma is the primary indication for therapeutic STAT6 siRNA, our rationale for this approach was based on findings that respiratory allergy is reflective of an integrated, systemic inflammatory disorder [17,19,20]. Furthermore, in contrast to lung delivery in which inhaled aerosol is the preferred methodology [44], intra-nasal administration provided a simpler pipette method of delivery that produced uniform siRNA distribution throughout the nasal cavities (<0.1% original dose) with 372m present at significantly higher (P<0.001) levels in the SL compared to 372u (c, d). 372m persisted longer and was detected at significantly higher levels in rat plasma compared to 372u (e). Values presented are mean ± S.E.M (n = 6), and P-value represents comparison to 372u. Nasal sections = Lateral lining, LL; inferior ethmoid turbinate, IE; median ethmoid turbinate, ME; superior ethmoid turbinate, SE; maxilla-turbinate, M; naso-turbinate, N & septum lining, SL.

doi:10.1371/journal.pone.0090338.g006
70% of the dose accumulated in the liver or spleen and the administration. However, also notable was that approximately knockdown within the lungs of mice following intravenous et al. airway disease [19,46]. Given the accessibility of the nasal passages [45], consistent with the concept of respiratory allergy as a single compared with low doses of bronchial steroids for asthma therapy it is notable that nasal steroids are equally efficacious when measurement of siRNA distribution and target gene status, should be a useful pre-cursor to lung delivery in evaluating new delivery technologies for respiratory administration. Regarding the general relevance of intra-nasal exposure as a surrogate pre-clinical model, it is notable that nasal steroids are equally efficacious when compared with low doses of bronchial steroids for asthma therapy [45], consistent with the concept of respiratory allergy as a single airway disease [19,46]. Given the accessibility of the nasal passages compared to the lower airways, delivering siRNA-based drugs via the nasal route may be a productive treatment strategy. Recent work by McCaskill et al [47] demonstrated successful target knockdown within the lungs of mice following intravenous administration. However, also notable was that approximately 70% of the dose accumulated in the liver or spleen and the majority of cells targeted within the pulmonary tissues, were endothelial cells. We would therefore argue that intravenous delivery, although attractive as a means to avoid problems associated with delivery to diseased or inflamed airways, is an inefficient method for targeting cells of the respiratory tract and does not aid patient compliance, an important factor in the treatment of chronic respiratory disease.

In summary, we have demonstrated the successful chemical modification of STAT6-targeting siRNA, which led to significantly enhanced bio-availability without loss of efficacy or cellular toxicity. In addition, we have established a robust and highly sensitive method for determining the bio-distribution of our siRNA in vivo, and developed a nasal model to further aid evaluation. Further work is warranted, encompassing the optimisation of a suitable delivery modality to improve cellular uptake in vivo and further characterisation of cellular as well as tissue distribution.

Acknowledgments
During the execution of this study, Jennifer Lockridge, Shawn Zinnen, Ivan Richards, Julian Hopkin and William Walker acted as consultants to Allerna Therapeutics Ltd. The proprietary siRNA used in this study (372) is protected by U.S. patent: U.S.7,566,700 (Allerna Therapeutics Ltd.).

Author Contributions
Conceived and designed the experiments: GDH JAL SZ JMH IR WW. Performed the experiments: GH WW. Analyzed the data: GDH JAL SZ JMH IR WW. Wrote the paper: GDH JAL SZ JMH IR WW.

References
1. Adams NP and Jones PV (2006) The dose-response characteristics of inhaled corticosteroids when used to treat asthma: an overview of Cochrane systematic reviews. 100: 1297–1306.
2. Gros C, Wu EQ, Ray S and Colice GL (2009) Inhaled corticosteroids or long-acting beta-agonists alone or in fixed-dose combinations in asthma treatment: a systematic review of fluticasone/budesonide and formoterol/salmeterol. Clin Ther 31: 2779–2003.
3. Akimoto T, Numata F, Tamura M, Takata Y, Higashila N, et al. (1998) Abrogation of bronchial eosinophilic inflammation and airway hyperreactivity in signal transducer and activators of transcription (STAT)-deficient mice. J Exp Med 187: 1537–1542.
4. Kuperman DA and Schleimer RP (2008) Interleukin-4, interleukin-13, signal transducer and activator of transcription factor 6, and allergic asthma. Curr Mol Med 187: 1537–1542.
5. Wills-Karp M (2004) Interleukin-13 in asthma pathogenesis. Immunol Rev 202: 175–190.
6. Inghara K, Arima K, Kanaji S, Ohta S and Kanaji T (2006) IL-13: a promising therapeutic target for bronchial asthma. Curr Med Chem 13: 2291–2298.
7. Inghara K, Shirakawa T, Astra CN, Hamasaki N and Hopkin JM (1999) Emerging therapeutic targets in allergy: IL-4Ralpha and Stat6. Emerging Therapeutic Targets 3: 381–389.
8. Oh CK, Geba GP and Mollino N (2010) Investigational therapeutics targeting the IL-4/IL-13/STAT6 pathway for the treatment of asthma. Eur Rev Med 115.
9. Holgate ST (2007) The epithelium takes centre stage in asthma and atopic dermatitis. Trends Immunol 28: 248–251.
10. Kato A and Schleimer RP (2007) Beyond inflammation: epithelial cells are at the interface of innate and adaptive immunity. Current Opinion Immunol 19: 1–10.
11. Kuperman DA, Huang X, Koth LL, Chang GH, Dolganov GM, et al. (2002) Direct effects of interleukin-13 on epithelial cells cause airway hyperreactivity and mucus overproduction in asthma. Nature Med 8: 883–889.
12. Walker W, Healey GD and Hopkin JM (2009) RNA interference of STAT6 rapidly attenuates ongoing interleukin-13-mediated events in lung epithelial cells. Immunology 127: 256–266.
13. Healey GD, Zinnen S, Lockridge JA, Richards I, Evans N, et al. (2010) Identification of small interfering RNA targeting Signal Transducer and Activator of Transcription 6: Characterisation and selection of candidates for pre-clinical development. J RNAi Gene Silencing 25: 401–410.
14. Finkelman FD and Wills-Karp M (2000) Usefulness and optimization of mouse models of allergic airway disease. J Allergy Clin Immunol 121: 603–606.
15. Holmes AM, Solarri R and Holgate ST (2011) Animal models of asthma: value, limitations and opportunities for alternative approaches. Drug Discovery Today 16: 659–670.
16. Wenzel S and Holgate ST (2006) The mouse trap: it still yields few answers in asthma. Am J Respir Crit Care Med 174: 1173–1178.
17. Braunstahl GJ (2009) United airways concept. What does it teach us about systemic inflammation in airways disease? Proc Am Thorac Soc 6: 652–654.
18. Murugan A, Pysz-Ricard C and Callhouen VJ (2009) Biomarkers in asthma. Curr Opin Pulm Med 15: 12–18.
19. Brouzek JL, Bouquet J, Barra-Capugni CE, Bonini S, Canonica GW, et al. (2010) Allergic rhinitis and its impact on asthma (ARIA) guidelines. 2010 revision. J Allergy Clin Immunol 126: 466–476.
20. Togias A (2003) Rhinitis and asthma: evidence for respiratory system integration. J Allergy Clin Immunol 111: 1171–1183.
21. Healey GD, Evans N, Hopkin JM, Davies G and Walker W (2013) Evaluation of nasal epithelium sampling as a tool in the preclinical development of siRNA-based therapeutics for asthma. J Cell Mol Med 17: 356–364.
22. Scagiente B, Dupas B, Farra R, Grassi M, Pozzato G, et al. (2011) Improving siRNA bio-distribution and minimizing side effects. Curr Drug Metab 12: 11–23.
23. Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. Nucl Acids Res 29: 2002–2007.
24. Hågglers AR, Conradt RA and Burton PS (1990) Caco-2 cell monolayers as a model for drug transport across the intestinal mucosa. Pharm Res 7: 902–910.
25. Neurark MM, Burton PS and Borcard RT (1996) The use of surfactants to enhance the permeability of peptides through Caco-2 cells by inhibition of an apically polarized efflux system. Pharm, Res 13: 520–534.
26. Chen C, Rizodon DA, Brouwer AJ, Zhou Z, Lee DH, et al. (2005) Real-time quantification of microRNAs by stem-loop RT-PCR. Nucl Acids Res 33: el79.
27. Volpe DA (2011) Drug permeability and transporter assays in Caco-2 and MDCK cell lines. Future Med Chem 16: 2063–2077.
28. Håkema JR, Carey SA and Wagner JG (2006) The nose revisited: a brief review of the comparative structure, function, and toxicologic pathology of the nasal epithelium. Toxicologic Pathol 34: 252–269.
29. Bakhia N, Hashami K, G B and Kaakoush MH (2013) siRNA therapeutics in the treatment of diseases. Ther Deliv 4: 45–57.
30. Musacchio T and Torchilin VP (2013) siRNA delivery: from basics to therapeutic applications. Front Biosci 18: 58–79.
31. DeVincenzo J, Cehelsky JE, Alvarez R, Elbashir R, Elbashir S, et al. (2006) Evaluation of the safety, tolerability and pharmacokinetics of ALN-RSN01, a
novel RNAi antiviral therapeutic directed against respiratory syncytial virus (RSV). Antiviral Res 77: 225–231.

32. Zimmerer Z, Hulvey J, Simandi Z, Varalhaya E, Havelda Z, et al. (2013) A versatile method to design stem-loop primer-based quantitative PCR assays for detecting small regulatory RNA molecules. PLoS One 8: e53168.

33. Morrissey DV, Lockridge JA, Shaw L, Blanchard K, Jensen K, et al. (2005) Potent and persistent in vivo anti-HBV activity of chemically modified siRNAs. Nat Biotechnol 23: 1002–1007.

34. Rana TM (2007) Illuminating the silence: understanding the structure and function of small RNAs. Nat Rev Mol Cell Biol 8: 23–36.

35. Takashii Y, Nishikawa M and Takakura Y (2009) Nonviral vector-mediated RNA interference: its gene silencing characteristics and important factors to achieve RNAi-based gene therapy. Adv Drug Deliv Rev 61: 760–766.

36. Whitehead KA, Langer R and Anderson DG (2009) Knocking down barriers: advances in siRNA delivery. Nat Rev Drug Discovery 8: 129–138.

37. Grimm D (2009) Small silencing RNAs: state-of-the-art. Adv Drug Deliv Rev 61: 672–703.

38. Wang J, Lu Z, Wientjes MG and Au JL (2010) Delivery of siRNA therapeutics: barriers and carriers. AAPS J 12: 492–503.

39. Clark KL, Hughes SA, Balsa R, Coates J, Moores K, et al. (2013) Pharmacological Characterization of a Novel ENaC siRNA (GSK225745) With potential for the treatment of cystic fibrosis. Mol Ther Nucleic Acids 2: e65.

40. Merkel OM and Kissel T (2012) Nonviral pulmonary delivery of siRNA. Acc Chem Res 45: 961–970.

41. Moschos SA, Frick M, Taylor B, Turnpenny P, Graves H, et al. (2011) Uptake, efficacy, and systemic distribution of naked, inhaled short interfering RNA (siRNA) and locked nucleic acid (LNA) antisense. Mol Ther 19: 2163–2168.

42. Darcan-Nicolasen Y, Meınicke H, Fels G, Hegen O, Haberland A, et al. (2009) Small interfering RNA against transcription factor STAT6 inhibits allergic airway inflammation and hyperreactivity in mice. J Immunol 182: 7501–7508.

43. Lively TN, Kossen K, Balhorn A, Koya T, Zinnen S, et al. (2008) Effect of chemically modified IL-13 short interfering RNA on development of airway hyperresponsiveness in mice. J Allergy Clin Immunol 121: 88–94.

44. Dolovich MB and Dhand R (2011) Aerosol drug delivery: developments in device design and clinical use. Lancet 377: 1032–1045.

45. Bourdin A, Gras D, Vachier I and Chanze P (2009) Upper airway. 1. Allergic rhinitis and asthma: united disease through epithelial cells. Thorax 64: 999–1004.

46. Bousquet J, Vignola A.M. and Demoly P (2003) Links between rhinitis and asthma. Allergy 58: 691–706.

47. McCaskill J, Singhania R, Burgess M, Allavena R, Wu S, et al. (2013) Efficient biodistribution and gene silencing in the lung epithelium via intravenous liposomal delivery of siRNA. Mol Ther Nucleic Acids 2: e96.