RNAI INHIBITION OF ALPHA-ENAC EXPRESSION

TECHNICAL FIELD

The invention relates to the field of ENaC-mediated airway ion transport and compositions and methods for modulating alpha-ENaC expression, and more particularly to the down-regulation of alpha-ENaC by oligonucleotides via RNA interference which are administered locally to the lungs and nasal passage via inhalation/intranasal administration, or are administered systemically, e.g. by via intravenous injection.

BACKGROUND

RNA interference or "RNAi" is a term initially coined by Fire and co-workers to describe the observation that double-stranded RNA (dsRNA) can block gene expression when it is introduced into worms (Fire *et al.*, *Nature* 391:806-811, 1998). Short dsRNA directs gene-specific, post-transcriptional silencing in many organisms, including vertebrates, and has provided a new tool for studying gene function. This technology has been reviewed numerous times recently, see, for example Novina, C.D:, and Sharp, P., Nature 2004, 430:161, and Sandy, P., et al., Biotechniques 2005, 39:215, hereby incorporated by reference.

The mucosal surfaces at the interface between the environment and the body have evolved a number of protective mechanisms. A principal form of such innate defense is to cleanse these surfaces with liquid. Typically, the quantity of the liquid layer on a mucosal surface reflects the balance between epithelial liquid secretion, often reflecting anion (Cl^- and/or HCO_3^-) secretion coupled with water (and a cation counter-ion), and epithelial liquid absorption, often reflecting Na^+ absorption, coupled with water and counter anion (Cl^- and/or HCO_3^-). Many diseases of mucosal surfaces are caused by too little protective liquid on those mucosal surfaces created by an imbalance between secretion (too little) and absorption (relatively too much). The defective salt transport processes that characterize these mucosal dysfunctions reside in the epithelial layer of the mucosal surface. One approach to replenish the protective liquid layer on mucosal surfaces is to "re-balance" the system by blocking Na^+ channel mediated liquid absorption. The epithelial protein that mediates the rate-limiting step of Na^+ and liquid absorption is the epithelial Na^+ channel (ENaC). Alpha-ENaC is positioned on the apical surface of the epithelium, i.e. the mucosal surface-environmental interface. Inhibition of alpha-ENaC mediated Na^+ mediated liquid absorption may achieve therapeutic

utility. Therefore, there is a need for the development of effective therapies for the treatment and prevention of diseases or disorders in which alpha-ENaC is implicated, e.g. cystic fibrosis in humans and animals, and particularly for therapies with high efficiency. One prerequisite for high efficiency is that the active ingredient is not degraded too quickly in a physiological environment.

SUMMARY

The present invention provides specific compositions and methods that are useful in reducing alpha-ENaC levels in a subject, e.g., a mammal, such as a human, e.g. by inhaled, intranasal or intratracheal administration of such agents.

The present invention specifically provides iRNA agents consisting of, consisting essentially of or comprising at least 15 or more contiguous nucleotides for alpha-ENaC, and more particularly agents comprising 15 or more contiguous nucleotides from one of the sequences provided in Tables 1A-1D. The iRNA agent preferably comprises less than 30 nucleotides per strand, e.g., 21-23 nucleotides, such as those provided in Tables 1A-1D. The double stranded iRNA agent can either have blunt ends or more preferably have overhangs of 1-4 nucleotides from one or both 3' ends of the agent.

Further, the iRNA agent can either contain only naturally occuring ribonucleotide subunits, or can be synthesized so as to contain one or more modifications to the sugar, phosphate or base of one or more of the ribonucleotide subunits that is included in the agent. The iRNA agent can be further modified so as to be attached to a ligand that is selected to improve stability, distribution or cellular uptake of the agent, e.g. cholesterol. The iRNA agents can further be in isolated form or can be part of a pharmaceutical composition used for the methods described herein, particularly as a pharmaceutical composition formulated for delivery to the lungs or nasal passage or formulated for parental administration. The pharmaceutical compositions can contain one or more iRNA agents, and in some embodiments, will contain two or more iRNA agents, each one directed to a different segment the alpha-ENaC gene.

One aspect of the present invention relates to a double-stranded oligonucleotide comprising at least one non-natural nucleobase. In certain embodiments, the non-natural nucleobase is difluorotolyl, nitroindolyl, nitropyrrolyl, or nitroimidazolyl. In a preferred

embodiment, the non-natural nucleobase is difluorotolyl. In certain embodiments, only one of the two oligonucleotide strands comprising the double-stranded oligonucleotide contains a non-natural nucleobase. In certain embodiments, both of the oligonucleotide strands comprising the double-stranded oligonucleotide independently contain a non-natural nucleobase.

The present invention further provides methods for reducing the level of alpha-ENaC mRNA in a cell. Such methods comprise the step of administering one of the iRNA agents of the present invention to a subject as further described below. The present methods utilize the cellular mechanisms involved in RNA interference to selectively degrade the target RNA in a cell and are comprised of the step of contacting a cell with one of the iRNA agents of the present invention. Such methods can be performed directly on a cell or can be performed on a mammalian subject by administering to a subject one of the iRNA agents/pharmaceutical compositions of the present invention. Reduction of target RNA in a cell results in a reduction in the amount of encoded protein produced, and in an organism, results in reduction of epithelial potential difference, decreased fluid absorption and increased mucociliary clearance.

The methods and compositions of the invention, e.g., the methods and iRNA agent compositions can be used with any dosage and/or formulation described herein, as well as with any route of administration described herein.

The details of one or more embodiments of the invention are set forth in the accompanying drawings and the description below. Other features, objects, and advantages of the invention will be apparent from this description, the drawings, and from the claims. This application incorporates all cited references, patents, and patent applications by references in their entirety for all purposes.

In the Figures:

Figure 1:Restriction digest map of pXoon contsruct for cloned cynomolgous α-EnaC.

Figure 2: Protein and DNA sequence of cynomologous monkey alpha-EnaC.

Figure 3: Cloning of the predicted off- target and the on- target recognition sites into the AY535007 dual luciferase reporter construct. Fragments consist of 19nt of the predicted target site and 10 nt of flanking sequence at both the 5' and 3' ends.

DETAILED DESCRIPTION OF CERTAIN PREFERRED EMBODIMENTS OF THE INVENTION

For ease of exposition the term "nucleotide" or "ribonucleotide" is sometimes used herein in reference to one or more monomeric subunits of an RNA agent. It will be understood that the usage of the term "ribonucleotide" or "nucleotide" herein can, in the case of a modified RNA or nucleotide surrogate, also refer to a modified nucleotide, or surrogate replacement moiety, as further described below, at one or more positions.

An "RNA agent" as used herein, is an unmodified RNA, modified RNA, or nucleoside surrogate, each of which is described herein or is well known in the RNA synthetic art. While numerous modified RNAs and nucleoside surrogates are described, preferred examples include those which have greater resistance to nuclease degradation than do unmodified RNAs. Preferred examples include those that have a 2' sugar modification, a modification in a single strand overhang, preferably a 3' single strand overhang, or, particularly if single stranded, a 5'-modification which includes one or more phosphate groups or one or more analogs of a phosphate group.

An "iRNA agent" (abbreviation for "interfering RNA agent") as used herein, is an RNA agent, which can downregulate the expression of a target gene, *e.g.* ENaC gene SCNN1A. While not wishing to be bound by theory, an iRNA agent may act by one or more of a number of mechanisms, including post-transcriptional cleavage of a target mRNA sometimes referred to in the art as RNAi, or pre-transcriptional or pre-translational mechanisms.

A "ds iRNA agent" (abbreviation for "double stranded iRNA agent"), as used herein, is an iRNA agent which includes more than one, and preferably two, strands in which interstrand hybridization can form a region of duplex structure. A "strand" herein refers to a contigouous sequence of nucleotides (including non-naturally occurring or modified

nucleotides). The two or more strands may be, or each form a part of, separate molecules, or they may be covalently interconnected, *e.g.*, by a linker, *e.g.*, a polyethyleneglycol linker, to form one molecule. At least one strand can include a region which is sufficiently complementary to a target RNA. Such strand is termed the "antisense strand." A second strand of the dsRNA agent, which comprises a region complementary to the antisense strand, is termed the "sense strand." However, a ds iRNA agent can also be formed from a single RNA molecule which is at least partly self-complementary, forming, *e.g.*, a hairpin or panhandle structure, including a duplex region. The latter are herein referred to as short hairpin RNAs or shRNAs. In such case, the term "strand" refers to one of the regions of the RNA molecule that is complementary to another region of the same RNA molecule.

Although, in mammalian cells, long ds iRNA agents can induce the interferon response which is frequently deleterious, short ds iRNA agents do not trigger the interferon response, at least not to an extent that is deleterious to the cell and/or host (Manche *et al.*, *Mol. Cell. Biol.* 12:5238, 1992; Lee *et al., Virology* 199:491, 1994; Castelli *et al., J. Exp. Med.* 186:967, 1997; Zheng *et al., RNA* 10:1934, 2004; Heidel *et al., Nature Biotechnol. 22 1579*). The iRNA agents of the present invention include molecules which are sufficiently short that they do not trigger a deleterious non-specific interferon response in normal mammalian cells. Thus, the administration of a composition including an iRNA agent (*e.g.*, formulated as described herein) to a subject can be used to decrease expression of alpha-ENaC in the subject, while circumventing an interferon response. Molecules that are short enough that they do not trigger a deleterious interferon response are termed siRNA agents or siRNAs herein. "siRNA agent" or "siRNA" as used herein, refers to an iRNA agent, *e.g.*, a ds iRNA agent, that is sufficiently short that it does not induce a deleterious interferon response in a mammalian, and particularly a human, cell, *e.g.*, it has a duplexed region of less than 60 but preferably less than 50, 40, or 30 nucleotide pairs.

The isolated iRNA agents described herein, including ds iRNA agents and siRNA agents, can mediate the decreased expression of alpha-ENaC, *e.g.*, by RNA degradation. For convenience, such RNA is also referred to herein as the RNA to be silenced. Such a nucleic acid is also referred to as a "target RNA", sometimes "target RNA molecule" or sometimes "target gene".

As used herein, the phrase "mediates RNAi" refers to the ability of an agent to silence, in a sequence-specific manner, a target gene. "Silencing a target gene" means the process whereby a cell containing and/or expressing a certain product of the target gene when not in contact with the agent, will contain and/or express at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, or 90% less of such gene product when contacted with the agent, as compared to a similar cell which has not been contacted with the agent. Such product of the target gene can, for example, be a messenger RNA (mRNA), a protein, or a regulatory element.

As used herein, the term "complementary" is used to indicate a sufficient degree of complementarity such that stable and specific binding occurs between a compound of the invention and a target RNA molecule, *e.g.*, alpha-ENaC mRNA. Specific binding requires a sufficient degree of complementarity to avoid non-specific binding of the oligomeric compound to non-target sequences under conditions in which specific binding is desired, *i.e.*, under physiological conditions in the case of *in vivo* assays or therapeutic treatment, or in the case of *in vitro* assays, under conditions in which the assays are performed. The non-target sequences typically differ from the target sequences by at least 2, 3 or 4 nucleotides.

As used herein, an iRNA agent is "sufficiently complementary" to a target RNA, *e.g.*, a target mRNA (*e.g.*, alpha-ENaC mRNA) if the iRNA agent reduces the production of a protein encoded by the target RNA in a cell. The iRNA agent may also be "exactly complementary" to the target RNA, *e.g.*, the target RNA and the iRNA agent anneal, preferably to form a hybrid made exclusively of Watson-Crick basepairs in the region of exact complementarity. A "sufficiently complementary" iRNA agent can include an internal region (*e.g.*, of at least 10 nucleotides) that is exactly complementary to a target alpha-ENaC RNA. Moreover, in some embodiments, the iRNA agent specifically discriminates a single-nucleotide difference. In this case, the iRNA agent only mediates RNAi if exact complementarity is found in the region (*e.g.*, within 7 nucleotides of) the single-nucleotide difference. Preferred iRNA agents will be based on or consist of or comprise the sense and antisense sequences provided in Tables 1A-1D.

As used herein, "essentially identical" when used referring to a first nucleotide sequence in comparison to a second nucleotide sequence means that the first nucleotide sequence is identical to the second nucleotide sequence except for up to one, two or three nucleotide substitutions (*e.g.*, adenosine replaced by uracil). "Essentially retaining the ability to inhibit alpha-ENaC expression in cultured human cells," as used herein referring to an iRNA agent not identical to but derived from one of the iRNA agents of Tables 1A-1D by deletion, addition or substitution of nucleotides, means that the derived iRNA agent possesses an inhibitory activity not less than 20% of the inhibitory activity of the iRNA agent of Tables 1A-1D from which it was derived. For example, an iRNA agent derived from an iRNA agent of Tables 1A-1D which lowers the amount of alpha-ENaC mRNA present in cultured human cells by 70% may itself lower the amount of mRNA present in cultured human cells by at least 50% in order to be considered as essentially retaining the ability to inhibit alpha-ENaC replication in cultured human cells. Optionally, an iRNA agent of the invention may lower the amount of alpha-ENaC mRNA present in cultured human cells by at least 50%.

As used herein, a "subject" refers to a mammalian organism undergoing treatment for a disorder mediated by alpha-ENaC. The subject can be any mammal, such as a cow, horse, mouse, rat, dog, pig, goat, or a primate. In the preferred embodiment, the subject is a human.

Design and Selection of iRNA agents

As used herein, "disorders associated with alpha-ENaC expression" refers to any biological or pathological state that (1) is mediated at least in part by the presence of alpha-ENaC and (2) whose outcome can be affected by reducing the level of the alpha-ENaC present. Specific disorders associated with alpha-ENaC expression are noted below.

The present invention is based on the design, synthesis and generation of iRNA agents that target alpha-ENaC and the demonstration of silencing of the alpha-ENaC gene *in vitro* in cultured cells after incubation with an iRNA agent, and the resulting protective effect towards alpha-ENaC mediated disorders.

An iRNA agent can be rationally designed based on sequence information and desired characteristics. For example, an iRNA agent can be designed according to the relative melting temperature of the candidate duplex. Generally, the duplex should have a lower melting temperature at the 5' end of the antisense strand than at the 3' end of the antisense strand.

The present invention provides compositions containing siRNA(s) and/or shRNA(s) targeted to one or more alpha-ENaC transcripts.

For any particular gene target that is selected, the design of siRNAs or shRNAs for use in accordance with the present invention will preferably follow certain guidelines. Also, in many cases, the agent that is delivered to a cell according to the present invention may undergo one or more processing steps before becoming an active suppressing agent (see below for further discussion); in such cases, those of ordinary skill in the art will appreciate that the relevant agent will preferably be designed to include sequences that may be necessary for its processing.

Diseases mediated by dysfunction of the epithelial sodium channel, include diseases associated with the regulation of fluid volumes across epithelial membranes. For example, the volume of airway surface liquid is a key regulator of mucociliary clearance and the maintenance of lung health. The blockade of the epithelial sodium channel will promote fluid accumulation on the mucosal side of the airway epithelium thereby promoting mucus clearance and preventing the accumulation of mucus and sputum in respiratory tissues (including lung airways). Such diseases include respiratory diseases, such as cystic fibrosis, primary ciliary dyskinesia, chronic bronchitis, chronic obstructive pulmonary disease (COPD), asthma, respiratory tract infections (acute and chronic; viral and bacterial) and lung carcinoma. Diseases mediated by blockade of the epithelial sodium channel also include diseases other than respiratory diseases that are associated with abnormal fluid regulation across an epithelium, perhaps involving abnormal physiology of the protective surface liquids on their surface, e.g., xerostomia (dry mouth) or keratoconjunctivitis sire (dry eye). Furthermore, blockade of the epithelial sodium channel in the kidney could be used to promote diuresis and thereby induce a hypotensive effect.

Treatment in accordance with the invention may be symptomatic or prophylactic.

Asthma includes both intrinsic (non-allergic) asthma and extrinsic (allergic) asthma, mild asthma, moderate asthma, severe asthma, bronchitic asthma, exercise-induced asthma, occupational asthma and asthma induced following bacterial infection. Treatment of asthma is also to be understood as embracing treatment of subjects, e.g., of less than 4 or 5 years of age, exhibiting wheezing symptoms and diagnosed or diagnosable as "wheezy infants", an established patient category of major medical concern and now often identified as incipient or

early-phase asthmatics. (For convenience this particular asthmatic condition is referred to as "wheezy-infant syndrome".)

Prophylactic efficacy in the treatment of asthma will be evidenced by reduced frequency or severity of symptomatic attack, e.g., of acute asthmatic or bronchoconstrictor attack, improvement in lung function or improved airways hyperreactivity. It may further be evidenced by reduced requirement for other, symptomatic therapy, i.e., therapy for or intended to restrict or abort symptomatic attack when it occurs, e.g., anti-inflammatory (e.g., corticosteroid) or bronchodilatory. Prophylactic benefit in asthma may, in particular, be apparent in subjects prone to "morning dipping". "Morning dipping" is a recognized asthmatic syndrome, common to a substantial percentage of asthmatics and characterized by asthma attack, e.g., between the hours of about 4-6 am, i.e., at a time normally substantially distant from any previously administered symptomatic asthma therapy.

Chronic obstructive pulmonary disease includes chronic bronchitis or dyspnea associated therewith, emphysema, as well as exacerbation of airways hyperreactivity consequent to other drug therapy, in particular, other inhaled drug therapy. The invention is also applicable to the treatment of bronchitis of whatever type or genesis including, e.g., acute, arachidic, catarrhal, croupus, chronic or phthinoid bronchitis.

Based on the results shown herein, the present invention provides iRNA agents that reduce alpha-ENaC expression in cultured cells and in a subject, e.g. a mammalian, for example a human. Tables 1A-1D provide exemplary iRNA agents targeting alpha-ENaC, based on the standard nomenclature abbreviations given in Table A.

Table 1A, Seq Id No.s 305-608, Table 1B and Table 1D, Seq Id No.s 1519-1644 list siRNAs that do not comprise nucleotide modifications except for one phosphorothioate linkage between the 3'-terminal and the penultimate thymidines. The remaining Seq Ids in Tables 1A-1D lists siRNAs wherein all nucleotides comprising pyrimidine bases are 2'-*O*-methyl-modified nucleotides in the sense strand, and all uridines in a sequence context of 5'-ua-3' as well as all cytidines in a sequence context of or 5'-ca-3' are 2'-*O*-methyl-modified nucleotides in the antisense strand.

Based on these results, the invention specifically provides an iRNA agent that includes a sense strand having at least 15 contiguous nucleotides of the sense strand

sequences of the agents provided in Tables 1A-1D, and an antisense strand having at least 15 contiguous nucleotides of the antisense sequences of the agents provided in Tables 1A-1D.

The iRNA agents shown in Tables 1A-1D are composed of two strands of 19 nucleotides in length which are complementary or identical to the target sequence, plus a 3'-TT overhang. The present invention provides agents that comprise at least 15, or at least 16, 17, or 18, or 19 contiguous nucleotides from these sequences. However, while these lengths may potentially be optimal, the iRNA agents are not meant to be limited to these lengths. The skilled person is well aware that shorter or longer iRNA agents may be similarly effective, since, within certain length ranges, the efficacy is rather a function of the nucleotide sequence than strand length. For example, Yang, *et al., PNAS* 99:9942-9947 (2002), demonstrated similar efficacies for iRNA agents of lengths between 21 and 30 base pairs. Others have shown effective silencing of genes by iRNA agents down to a length of approx. 15 base pairs (Byrom, *et al.,* "Inducing RNAi with siRNA Cocktails Generated by RNase III" *Tech Notes* 10(1), Ambion, Inc., Austin, TX).

Therefore, it is possible and contemplated by the instant invention to select from the sequences provided in Tables 1A-1D a partial sequence of between 15 to 19 nucleotides for the generation of an iRNA agent derived from one of the sequences provided in Tables 1A-1D. Alternatively, one may add one or several nucleotides to one of the sequences provided in Tables 1A-1D, or an agent comprising 15 contiguous nucleotides from one of these agents, preferably, but not necessarily, in such a fashion that the added nucleotides are complementary to the respective sequence of the target gene, *e.g.*, alpha-ENaC. For example, the first 15 nucleotides from one of the agents can be combined with the 8 nucleotides found 5' to these sequence in alpha-ENaC mRNA to obtain an agent with 23 nucleotides in the sense and antisense strands. All such derived iRNA agents are included in the iRNA agents of the present invention, provided they essentially retain the ability to inhibit alpha-ENaC replication in cultured human cells.

The antisense strand of an iRNA agent should be equal to or at least, 14, 15, 16, 17, 18, 19, 25, 29, 40, or 50 nucleotides in length. It should be equal to or less than 60, 50, 40, or 30, nucleotides in length. Preferred ranges are 15-30, 17 to 25, 19 to 23, and 19 to 21 nucleotides in length.

The sense strand of an iRNA agent should be equal to or at least 14, 15, 16 17, 18, 19, 25, 29, 40, or 50 nucleotides in length. It should be equal to or less than 60, 50, 40, or 30 nucleotides in length. Preferred ranges are 15-30, 17 to 25, 19 to 23, and 19 to 21 nucleotides in length.

The double stranded portion of an iRNA agent should be equal to or at least, 15, 16 17, 18, 19, 20, 21, 22, 23, 24, 25, 29, 40, or 50 nucleotide pairs in length. It should be equal to or less than 60, 50, 40, or 30 nucleotides pairs in length. Preferred ranges are 15-30, 17 to 25, 19 to 23, and 19 to 21 nucleotides pairs in length.

Generally, the iRNA agents of the instant invention include a region of sufficient complementarity to the alpha-ENaC mRNA, and are of sufficient length in terms of nucleotides, that the iRNA agent, or a fragment thereof, can mediate down regulation of the alpha-ENaC gene. It is not necessary that there be perfect complementarity between the iRNA agent and the target gene, but the correspondence must be sufficient to enable the iRNA agent, or a cleavage product thereof, to direct sequence specific silencing, *e.g.*, by RNAi cleavage of an alpha-ENaC mRNA.

Therefore, the iRNA agents of the instant invention include agents comprising a sense strand and antisense strand each comprising a sequence of at least 16, 17 or 18 nucleotides which is essentially identical, as defined below, to one of the sequences of Tables 1A-1D, except that not more than 1, 2 or 3 nucleotides per strand, respectively, have been substituted by other nucleotides (*e.g.* adenosine replaced by uracil), while essentially retaining the ability to inhibit alpha-ENaC expression in cultured human cells. These agents will therefore possess at least 15 nucleotides identical to one of the sequences of Tables 1A-1D, but 1, 2 or 3 base mismatches with respect to either the target alpha-ENaC sequence or between the sense and antisense strand are introduced. Mismatches to the target alpha-ENaC RNA sequence, particularly in the antisense strand, are most tolerated in the terminal regions and if present are preferably in a terminal region or regions, *e.g.*, within 6, 5, 4, or 3 nucleotides of a 5' and/or 3' terminus, most preferably within 6, 5, 4, or 3 nucleotides of the 5'-terminus of the sense strand or the 3'-terminus of the antisense strand. The sense strand need only be sufficiently complementary with the antisense strand to maintain the overall double stranded character of the molecule.

It is preferred that the sense and antisense strands be chosen such that the iRNA agent includes a single strand or unpaired region at one or both ends of the molecule. Thus, an iRNA agent contains sense and antisense strands, preferably paired to contain an overhang, *e.g.*, one or two 5' or 3' overhangs but preferably a 3' overhang of 2-3 nucleotides. Most embodiments will have a 3' overhang. Preferred siRNA agents will have single-stranded overhangs, preferably 3' overhangs, of 1 to 4, or preferably 2 or 3 nucleotides, in length, at one or both ends of the iRNA agent. The overhangs can be the result of one strand being longer than the other, or the result of two strands of the same length being staggered. The unpaired nucleotides forming the overhang can be ribonucleotides, or they can be deoxyribonucleotides, preferably thymidine. 5'-ends are preferably phosphorylated, or they may be unphosphorylated.

Preferred lengths for the duplexed region are between 15 and 30, most preferably 18, 19, 20, 21, 22, and 23 nucleotides in length, *e.g.*, in the siRNA agent range discussed above. siRNA agents can resemble in length and structure the natural Dicer processed products from long dsRNAs. Embodiments in which the two strands of the siRNA agent are linked, *e.g.*, covalently linked, are also included. Hairpin, or other single strand structures which provide the required double stranded region, and preferably a 3' overhang are also within the invention.

Evaluation of Candidate iRNA Agents

As noted above, the present invention provides a system for identifying siRNAs that are useful as inhibitors of alpha-ENaC. Since, as noted above, shRNAs are processed intracellularly to produce siRNAs having duplex portions with the same sequence as the stem structure of the shRNA, the system is equally useful for identifying shRNAs that are useful as inhibitors of alpha-ENaC. For purposes of description this section will refer to siRNAs, but the system also encompasses corresponding shRNAs. Specifically, the present invention demonstrates the successful preparation of siRNAs targeted to inhibit alpha-ENaC activity. The techniques and reagents described herein can readily be applied to design potential new siRNAs, targeted to other genes or gene regions, and tested for their activity in inhibiting alpha-ENaC as discussed herein.

In various embodiments of the invention potential alpha-ENaC inhibitors can be tested for suppression of endogenous alpha-ENaC expression by introducing candidate

siRNA(s) into cells (e.g., by exogenous administration or by introducing a vector or construct that directs endogenous synthesis of siRNA into the cell), or in laboratory animals by pulmonary or nasal administration. Alternately, potential alpha-ENaC inhibitors can be tested *in vitro* by transient co-transfection of candidate siRNA(s) together with an alpha-ENaC-expression plasmid . The ability of the candidate siRNA(s) to reduce target transcript levels and/or to inhibit or suppress one or more aspects or features of alpha-ENaC activity such as epithelial potential difference or airway surface fluid absorption is then assessed..

Cells or laboratory animals to which inventive siRNA compositions have been delivered (test cells/animals) may be compared with similar or comparable cells or laboratory animals that have not received the inventive composition (control cells/animals, e.g., cells/animals that have received either no siRNA or a control siRNA such as an siRNA targeted to a non-endogenous transcript such as green fluorescent protein (GFP)). The ion transport phenotype of the test cells/animals can be compared with the phenotype of control cells/animals, providing that the inventive siRNA share sequence cross-reactivity with the test cell type/species. Production of alpha-ENaC protein and short circuit current (*in vitro* or *ex vivo*) may be compared in the test cells/animals relative to the control cells/animals. Other indicia of alpha-ENaC activity, including *ex vivo* epithelial potential difference or *in vivo* mucocilliary clearance or whole body magnetic resonance imaging (MRI), can be similarly compared. Generally, test cells/animals and control cells/animals would be from the same species and, for cells, of similar or identical cell type. For example, cells from the same cell line could be compared. When the test cell is a primary cell, typically the control cell would also be a primary cell.

For example, the ability of a candidate siRNA to inhibit alpha-ENaC activity may conveniently be determined by (i) delivering the candidate siRNA to cells (ii) assessing the expression levels of alpha-ENaC mRNA relative to an endogenously expressed control gene (iii) comparing the amiloride-sensitive current in an *in vitro* cell model produced in the presence of the siRNA with the amount produced in the absence of the siRNA. This latter assay may be used to test siRNAs that target any target transcript that may influence alpha-ENaC activity indirectly and is not limited to siRNAs that target the transcripts that encode the ENaC channel subunits.

The ability of a candidate siRNA to reduce the level of the target transcript may be assessed by measuring the amount of the target transcript using, for example, Northern blots,

nuclease protection assays, probe hybridization, reverse transcription (RT)-PCR, real-time RT-PCR, microarray analysis, etc. The ability of a candidate siRNA to inhibit production of a polypeptide encoded by the target transcript (either at the transcriptional or post-transcriptional level) may be measured using a variety of antibody-based approaches including, but not limited to, Western blots, immunoassays, ELISA, flow cytometry, protein microarrays, etc. In general, any method of measuring the amount of either the target transcript or a polypeptide encoded by the target transcript may be used.

In general, certain preferred alpha-ENaC iRNA inhibitors reduce the target transcript level at least about 2 fold, preferably at least about 4 fold, more preferably at least about 8 fold, at least about 16 fold, at least about 64 fold or to an even greater degree relative to the level that would be present in the absence of the inhibitor (e.g., in a comparable control cell lacking the inhibitor). In general, certain preferred alpha-ENaC iRNA inhibitors inhibit ENaC channel activity, so that the activity is lower in a cell containing the inhibitor than in a control cell not containing the inhibitor by at least about 2 fold, preferably at least about 4 fold, more preferably at least about 8 fold, at least about 16 fold, at least about 64 fold, at least about 100 fold, at least about 200 fold, or to an even greater degree.

Certain preferred alpha-ENaC iRNA inhibitors inhibit ENaC channel activity for at least 12 hours, at least 24 hours, at least 36 hours, at least 48 hours, at least 60 hours, at least 72 hours, at least 96 hours, at least 120 hours, at least 144 hours or at least 168 hours following administration of the siRNA and infection of the cells. Certain preferred alpha-ENaC inhibitors prevent (i.e., reduce to undetectable levels) or significantly reduce alpha-ENaC activity for at least 24 hours, at least 36 hours, at least 48 hours, or at least 60 hours following administration of the siRNA. According to various embodiments of the invention a significant reduction in alpha-ENaC activity is a reduction to less than approximately 90% of the level that would occur in the absence of the siRNA, a reduction to less than approximately 75% of the level that would occur in the absence of the siRNA, a reduction to less than approximately 50% of the level that would occur in the absence of the siRNA, a reduction to less than approximately 25% of the level that would occur in the absence of the siRNA, or a reduction to less than approximately 10% of the level that would occur in the absence of the siRNA. Reduction in alpha-ENaC activity may be measured using any suitable method including, but not limited to, short circuit current measurement of amiloride

sensitivity *in vitro*, epithelial potential difference *ex vivo* or *in vivo* mucocilliary clearance or whole body/lung MRI.

Stability testing, modification, and retesting of iRNA agents

A candidate iRNA agent can be evaluated with respect to stability, *e.g.*, its susceptibility to cleavage by an endonuclease or exonuclease, such as when the iRNA agent is introduced into the body of a subject. Methods can be employed to identify sites that are susceptible to modification, particularly cleavage, *e.g.*, cleavage by a component found in the body of a subject. Such methods may include the isolation and identification of most abundant fragments formed by degradation of the candidate iRNA agent after its incubation with isolated biological media *in vitro*, e.g. serum, plasma, sputum, cerebrospinal fluid, or cell or tissue homogenates, or after contacting a subject with the candidate iRNA agent *in vivo*, thereby identifying sites prone to cleavage. Such methods are, for example, without limitation, in International Patent Application Publication No. WO2005115481, filed on May 27, 2005.

When sites susceptible to cleavage are identified, a further iRNA agent can be designed and/or synthesized wherein the potential cleavage site is made resistant to cleavage, *e.g.* by introduction of a 2'-modification on the site of cleavage, *e.g.* a 2'-O-methyl group. This further iRNA agent can be retested for stability, and this process may be iterated until an iRNA agent is found exhibiting the desired stability.

In Vivo Testing

An iRNA agent identified as being capable of inhibiting alpha-ENaC gene expression can be tested for functionality *in vivo* in an animal model (*e.g.*, in a mammal, such as in mouse, rat, guinea-pig or primate). For example, the iRNA agent can be administered to an animal, and the iRNA agent evaluated with respect to its biodistribution, stability, and its ability to inhibit alpha-ENaC expression or modulate a biological or pathological process mediated at least in part by alpha-ENaC.

The iRNA agent can be administered directly to the target tissue, such as by injection, or the iRNA agent can be administered to the animal model in the same manner that it would be administered to a human. Preferably, the iRNA agent is delivered to the subject's airways, such as by intranasal, inhaled or intratracheal administration.

The iRNA agent can also be evaluated for its intracellular distribution. The evaluation can include determining whether the iRNA agent was taken up into the cell. The evaluation can also include determining the stability (*e.g.*, the half-life) of the iRNA agent. Evaluation of an iRNA agent *in vivo* can be facilitated by use of an iRNA agent conjugated to a traceable marker (*e.g.*, a fluorescent marker such as fluorescein; a radioactive label, such as ^{35}S, ^{32}P, ^{33}P, or ^{3}H; gold particles; or antigen particles for immunohistochemistry).

The iRNA agent can be evaluated with respect to its ability to down regulate alpha-alpha-ENaC expression. Levels of alpha-ENaC gene expression *in vivo* can be measured, for example, by *in situ* hybridization, or by the isolation of RNA from tissue prior to and following exposure to the iRNA agent. Where the animal needs to be sacrificed in order to harvest the tissue, an untreated control animal will serve for comparison. alpha-ENaC RNA can be detected by any desired method, including but not limited to RT-PCR, northern blot, branched-DNA assay, or RNAase protection assay. Alternatively, or additionally, alpha-ENaC gene expression can be monitored by performing western blot analysis or immunostaining on tissue extracts treated with the iRNA agent.

Potential alpha-ENaC inhibitors can be tested using any variety of animal models that have been developed. Compositions comprising candidate siRNA(s), constructs or vectors capable of directing synthesis of such siRNAs within a host cell, or cells engineered or manipulated to contain candidate siRNAs may be administered to an animal. The ability of the composition to suppress alpha-ENaC expression and/or to modify ENaC-dependent phenotypes and/or lessen their severity relative to animals that have not received the potential alpha-ENaC inhibitor is assessed. Such models include, but are not limited to, murine, rat, guinea pig, sheep and non-human primate models for ENaC-dependent phenotypes, all of which are known in the art and are used for testing the efficacy of potential alpha-ENaC therapeutics.

Utilising the systems invented for identifying candidate therapeutic siRNA agents, suitable therapeutic agents are selected from Duplex identifiers ND-8302, ND-8332, ND-8348, ND-8356, ND-8357, ND-8373, ND-8381, ND-8396, ND-8450 and ND-8453, more suitably selected from ND-8356, ND-8357 and ND-8396.

iRNA Chemistry

Described herein are isolated iRNA agents, *e.g.*, ds RNA agents that mediate RNAi to inhibit expression of the alpha-ENaC gene.

RNA agents discussed herein include otherwise unmodified RNA as well as RNA which has been modified, *e.g.*, to improve efficacy, and polymers of nucleoside surrogates. Unmodified RNA refers to a molecule in which the components of the nucleic acid, namely sugars, bases, and phosphate moieties, are the same or essentially the same as that which occur in nature, preferably as occur naturally in the human body. The art has referred to rare or unusual, but naturally occurring, RNAs as modified RNAs, see, *e.g.*, Limbach *et al.* *Nucleic Acids Res.* 22: 2183-2196, 1994. Such rare or unusual RNAs, often termed modified RNAs (apparently because they are typically the result of a post-transcriptional modification) are within the term unmodified RNA, as used herein. Modified RNA as used herein refers to a molecule in which one or more of the components of the nucleic acid, namely sugars, bases, and phosphate moieties, are different from that which occurs in nature, preferably different from that which occurs in the human body. While they are referred to as modified "RNAs," they will of course, because of the modification, include molecules which are not RNAs. Nucleoside surrogates are molecules in which the ribophosphate backbone is replaced with a non-ribophosphate construct that allows the bases to the presented in the correct spatial relationship such that hybridization is substantially similar to what is seen with a ribophosphate backbone, *e.g.*, non-charged mimics of the ribophosphate backbone. Examples of the above are discussed herein.

Modifications described herein can be incorporated into any double-stranded RNA and RNA-like molecule described herein, *e.g.*, an iRNA agent. It may be desirable to modify one or both of the antisense and sense strands of an iRNA agent. As nucleic acids are polymers of subunits or monomers, many of the modifications described below occur at a position which is repeated within a nucleic acid, *e.g.*, a modification of a base, or a phosphate moiety, or the non-linking oxygen of a phosphate moiety. In some cases the modification will occur at all of the subject positions in the nucleic acid but in many, and in fact in most, cases it will not. By way of example, a modification may only occur at a 3' or 5' terminal position, may only occur in a terminal region, *e.g.* at a position on a terminal nucleotide or in the last 2, 3, 4, 5, or 10 nucleotides of a strand. A modification may occur in a double strand

region, a single strand region, or in both. *E.g.*, a phosphorothioate modification at a non-linking O position may only occur at one or both termini, may only occur in a terminal regions, *e.g.*, at a position on a terminal nucleotide or in the last 2, 3, 4, 5, or 10 nucleotides of a strand, or may occur in double strand and single strand regions, particularly at termini. Similarly, a modification may occur on the sense strand, antisense strand, or both. In some cases, the sense and antisense strand will have the same modifications or the same class of modifications, but in other cases the sense and antisense strand will have different modifications, *e.g.*, in some cases it may be desirable to modify only one strand, *e.g.* the sense strand.

Two prime objectives for the introduction of modifications into iRNA agents is their stabilization towards degradation in biological environments and the improvement of pharmacological properties, *e.g.* pharmacodynamic properties, which are further discussed below. Other suitable modifications to a sugar, base, or backbone of an iRNA agent are described in PCT Application No. PCT/US2004/01193, filed January 16, 2004. An iRNA agent can include a non-naturally occurring base, such as the bases described in PCT Application No. PCT/US2004/011822, filed April 16, 2004. An iRNA agent can include a non-naturally occurring sugar, such as a non-carbohydrate cyclic carrier molecule. Exemplary features of non-naturally occurring sugars for use in iRNA agents are described in PCT Application No. PCT/US2004/11829, filed April 16, 2003.

An iRNA agent can include an internucleotide linkage (*e.g.*, the chiral phosphorothioate linkage) useful for increasing nuclease resistance. In addition, or in the alternative, an iRNA agent can include a ribose mimic for increased nuclease resistance. Exemplary internucleotide linkages and ribose mimics for increased nuclease resistance are described in PCT Application No. PCT/US2004/07070, filed on March 8, 2004.

An iRNA agent can include ligand-conjugated monomer subunits and monomers for oligonucleotide synthesis. Exemplary monomers are described in U.S. Application No. 10/916,185, filed on August 10, 2004.

An iRNA agent can have a ZXY structure, such as is described in PCT Application No. PCT/US2004/07070, filed on March 8, 2004.

An iRNA agent can be complexed with an amphipathic moiety. Exemplary amphipathic moieties for use with iRNA agents are described in PCT Application No. PCT/US2004/07070, filed on March 8, 2004.

In another embodiment, the iRNA agent can be complexed to a delivery agent that features a modular complex. The complex can include a carrier agent linked to one or more of (preferably two or more, more preferably all three of): (a) a condensing agent (*e.g.*, an agent capable of attracting, *e.g.*, binding, a nucleic acid, *e.g.*, through ionic or electrostatic interactions); (b) a fusogenic agent (*e.g.*, an agent capable of fusing and/or being transported through a cell membrane); and (c) a targeting group, *e.g.*, a cell or tissue targeting agent, *e.g.*, a lectin, glycoprotein, lipid or protein, *e.g.*, an antibody, that binds to a specified cell type. iRNA agents complexed to a delivery agent are described in PCT Application No. PCT/US2004/07070, filed on March 8, 2004.

An iRNA agent can have non-canonical pairings, such as between the sense and antisense sequences of the iRNA duplex. Exemplary features of non-canonical iRNA agents are described in PCT Application No. PCT/US2004/07070, filed on March 8, 2004.

Enhanced nuclease resistance

An iRNA agent, *e.g.*, an iRNA agent that targets alpha-ENaC, can have enhanced resistance to nucleases.

One way to increase resistance is to identify cleavage sites and modify such sites to inhibit cleavage, as described in U.S. Application No. 60/559,917, filed on May 4, 2004. For example, the dinucleotides 5'-ua-3', 5'-ca-3', 5'-ug-3', 5'-uu-3', or 5'-cc-3' can serve as cleavage sites. In certain embodiments, all the pyrimidines of an iRNA agent carry a 2'-modification in either the sense strand, the antisense strand, or both strands, and the iRNA agent therefore has enhanced resistance to endonucleases. Enhanced nuclease resistance can also be achieved by modifying the 5' nucleotide, resulting, for example, in at least one 5'-uridine-adenine-3' (5'-ua-3') dinucleotide wherein the uridine is a 2'-modified nucleotide; at least one 5'-cytidine-adenine-3' (5'-ca-3') dinucleotide, wherein the 5'-cytidine is a 2'-modified nucleotide; at least one 5'-uridine-guanine-3' (5'-ug-3') dinucleotide, wherein the 5'-uridine is a 2'-modified nucleotide; at least one 5'-uridine-uridine-3' (5'-uu-3') dinucleotide, wherein the 5'-uridine is a 2'-modified nucleotide; or at least one 5'-cytidine-cytidine-3' (5'-cc-3') dinucleotide, wherein the 5'-cytidine is a 2'-modified nucleotide, as

described in International Application No. PCT/US2005/018931, filed on May 27, 2005. The iRNA agent can include at least 2, at least 3, at least 4 or at least 5 of such dinucleotides. In a particularly preferred embodiment, the 5' nucleotide in all occurrences of the sequence motifs 5'-ua-3' and 5'-ca-3' in either the sense strand, the antisense strand, or both strands is a modified nucleotide. Preferably, the 5' nucleotide in all occurrences of the sequence motifs 5'-ua-3', 5'-ca-3' and 5'-ug-3' in either the sense strand, the antisense strand, or both strands is a modified nucleotide. More preferably, all pyrimidine nucleotides in the sense strand are modified nucleotides, and the 5' nucleotide in all occurrences of the sequence motifs 5'-ua-3' and 5'-ca-3' in the antisense strand are modified nucleotides, or where the antisense strand does comprise neither of a 5'-ua-3' and a 5'-ca-3' motif, in all occurrences of the sequence motif 5'-ug-3'.

Preferably, the 2'-modified nucleotides include, for example, a 2'-modified ribose unit, *e.g.*, the 2'-hydroxyl group (OH) can be modified or replaced with a number of different "oxy" or "deoxy" substituents.

Examples of "oxy"-2' hydroxyl group modifications include alkoxy or aryloxy (OR, *e.g.*, R = H, alkyl, cycloalkyl, aryl, aralkyl, heteroaryl or sugar); polyethyleneglycols (PEG), $O(CH_2CH_2O)_nCH_2CH_2OR$; "locked" nucleic acids (LNA) in which the 2' hydroxyl is connected, *e.g.*, by a methylene bridge, to the 4' carbon of the same ribose sugar; O-AMINE and aminoalkoxy, $O(CH_2)_n$AMINE, (*e.g.*, AMINE = NH_2; alkylamino, dialkylamino, heterocyclyl amino, arylamino, diaryl amino, heteroaryl amino, or diheteroaryl amino, ethylene diamine, polyamino). It is noteworthy that oligonucleotides containing only the methoxyethyl group (MOE), ($OCH_2CH_2OCH_3$, a PEG derivative), exhibit nuclease stabilities comparable to those modified with the robust phosphorothioate modification.

"Deoxy" modifications include hydrogen (*i.e.* deoxyribose sugars, which are of particular relevance to the overhang portions of partially ds RNA); halo (*e.g.*, fluoro); amino (*e.g.* NH_2; alkylamino, dialkylamino, heterocyclyl, arylamino, diaryl amino, heteroaryl amino, diheteroaryl amino, or amino acid); $NH(CH_2CH_2NH)_nCH_2CH_2$-AMINE (AMINE = NH_2; alkylamino, dialkylamino, heterocyclyl amino, arylamino, diaryl amino, heteroaryl amino,or diheteroaryl amino), -NHC(O)R (R = alkyl, cycloalkyl, aryl, aralkyl, heteroaryl or sugar), cyano; mercapto; alkyl-thio-alkyl; thioalkoxy; and alkyl, cycloalkyl, aryl, alkenyl and alkynyl, which may be optionally substituted with *e.g.*, an amino functionality.

Preferred substitutents are 2'-methoxyethyl, 2'-OCH_3, 2'-O-allyl, 2'-C- allyl, and 2'-fluoro.

The inclusion of furanose sugars in the oligonucleotide backbone can also decrease endonucleolytic cleavage. An iRNA agent can be further modified by including a 3' cationic group, or by inverting the nucleoside at the 3'-terminus with a 3'-3' linkage. In another alternative, the 3'-terminus can be blocked with an aminoalkyl group, *e.g.*, a 3' C5-aminoalkyl dT. Other 3' conjugates can inhibit 3'-5' exonucleolytic cleavage. While not being bound by theory, a 3' conjugate, such as naproxen or ibuprofen, may inhibit exonucleolytic cleavage by sterically blocking the exonuclease from binding to the 3'-end of oligonucleotide. Even small alkyl chains, aryl groups, or heterocyclic conjugates or modified sugars (D-ribose, deoxyribose, glucose etc.) can block 3'-5'-exonucleases.

Nucleolytic cleavage can also be inhibited by the introduction of phosphate linker modifications, *e.g.*, phosphorothioate linkages. Thus, preferred iRNA agents include nucleotide dimers enriched or pure for a particular chiral form of a modified phosphate group containing a heteroatom at a nonbridging position normally occupied by oxygen. The heteroatom can be S, Se, Nr_2, or Br_3. When the heteroatom is S, enriched or chirally pure Sp linkage is preferred. Enriched means at least 70, 80, 90, 95, or 99% of the preferred form. Modified phosphate linkages are particularly efficient in inhibiting exonucleolytic cleavage when introduced near the 5'- or 3'-terminal positions, and preferably the 5'-terminal positions, of an iRNA agent.

5' conjugates can also inhibit 5'-3' exonucleolytic cleavage. While not being bound by theory, a 5' conjugate, such as naproxen or ibuprofen, may inhibit exonucleolytic cleavage by sterically blocking the exonuclease from binding to the 5'-end of oligonucleotide. Even small alkyl chains, aryl groups, or heterocyclic conjugates or modified sugars (D-ribose, deoxyribose, glucose etc.) can block 3'-5'-exonucleases.

An iRNA agent can have increased resistance to nucleases when a duplexed iRNA agent includes a single-stranded nucleotide overhang on at least one end. In preferred embodiments, the nucleotide overhang includes 1 to 4, preferably 2 to 3, unpaired nucleotides. In a preferred embodiment, the unpaired nucleotide of the single-stranded overhang that is directly adjacent to the terminal nucleotide pair contains a purine base, and the terminal nucleotide pair is a G-C pair, or at least two of the last four complementary

nucleotide pairs are G-C pairs. In further embodiments, the nucleotide overhang may have 1 or 2 unpaired nucleotides, and in an exemplary embodiment the nucleotide overhang is 5'-gc-3'. In preferred embodiments, the nucleotide overhang is on the 3'-end of the antisense strand. In one embodiment, the iRNA agent includes the motif 5'-cgc-3' on the 3'-end of the antisense strand, such that a 2-nt overhang 5'-gc-3' is formed.

Thus, an iRNA agent can include modifications so as to inhibit degradation, *e.g.*, by nucleases, *e.g.*, endonucleases or exonucleases, found in the body of a subject. These monomers are referred to herein as NRMs, or Nuclease Resistance promoting Monomers, the corresponding modifications as NRM modifications. In many cases these modifications will modulate other properties of the iRNA agent as well, *e.g.*, the ability to interact with a protein, *e.g.*, a transport protein, *e.g.*, serum albumin, or a member of the RISC, or the ability of the first and second sequences to form a duplex with one another or to form a duplex with another sequence, *e.g.*, a target molecule.

One or more different NRM modifications can be introduced into an iRNA agent or into a sequence of an iRNA agent. An NRM modification can be used more than once in a sequence or in an iRNA agent.

NRM modifications include some which can be placed only at the terminus and others which can go at any position. Some NRM modifications can inhibit hybridization so it is preferable to use them only in terminal regions, and preferable to not use them at the cleavage site or in the cleavage region of a sequence which targets a subject sequence or gene, particularly on the antisense strand. They can be used anywhere in a sense strand, provided that sufficient hybridization between the two strands of the ds iRNA agent is maintained. In some embodiments it is desirable to put the NRM at the cleavage site or in the cleavage region of a sense strand, as it can minimize off-target silencing.

In most cases, NRM modifications will be distributed differently depending on whether they are comprised on a sense or antisense strand. If on an antisense strand, modifications which interfere with or inhibit endonuclease cleavage should not be inserted in the region which is subject to RISC mediated cleavage, *e.g.*, the cleavage site or the cleavage region (As described in Elbashir *et al.*, 2001, Genes and Dev. 15: 188, hereby incorporated by reference). Cleavage of the target occurs about in the middle of a 20 or 21 nt antisense strand, or about 10 or 11 nucleotides upstream of the first nucleotide on the target mRNA

which is complementary to the antisense strand. As used herein cleavage site refers to the nucleotides on either side of the cleavage site, on the target or on the iRNA agent strand which hybridizes to it. Cleavage region means the nucleotides within 1, 2, or 3 nucleotides of the cleavagee site, in either direction.

Such modifications can be introduced into the terminal regions, *e.g.*, at the terminal position or with 2, 3, 4, or 5 positions of the terminus, of a sense or antisense strand.

Tethered Ligands

The properties of an iRNA agent, including its pharmacological properties, can be influenced and tailored, for example, by the introduction of ligands, *e.g.* tethered ligands. In addition, pharmacological properties of an iRNA agent can be improved by incorporating a ligand in a formulation of the iRNA agent when the iRNA agent either has or does have a tethered ligand.

A wide variety of entities, *e.g.*, ligands, can be tethered to an iRNA agent or used as formluation conjugate or additive, *e.g.*, to the carrier of a ligand-conjugated monomer subunit. Examples are described below in the context of a ligand-conjugated monomer subunit but that is only preferred, entities can be coupled at other points to an iRNA agent.

Preferred moieties are ligands, which are coupled, preferably covalently, either directly or indirectly, via an intervening tether to the carrier. In preferred embodiments, the ligand is attached to the carrier *via* an intervening tether. The ligand or tethered ligand may be present on the ligand-conjugated monomer when the ligand-conjugated monomer is incorporated into the growing strand. In some embodiments, the ligand may be incorporated into a "precursor" ligand-conjugated monomer subunit after a "precursor" ligand-conjugated monomer subunit has been incorporated into the growing strand. For example, a monomer having, *e.g.*, an amino-terminated tether, *e.g.*, TAP-$(CH_2)_nNH_2$ may be incorporated into a growing sense or antisense strand. In a subsequent operation, i.e., after incorporation of the precursor monomer subunit into the strand, a ligand having an electrophilic group, *e.g.*, a pentafluorophenyl ester or aldehyde group, can subsequently be attached to the precursor ligand-conjugated monomer by coupling the electrophilic group of the ligand with the terminal nucleophilic group of the precursor ligand-conjugated monomer subunit tether.

In preferred embodiments, a ligand alters the distribution, targeting or lifetime of an iRNA agent into which it is incorporated. In preferred embodiments a ligand provides an enhanced affinity for a selected target, *e.g.*, molecule, cell or cell type, compartment, *e.g.*, a cellular or organ compartment, tissue, organ or region of the body, as, *e.g.*, compared to a species absent such a ligand.

Preferred ligands can improve transport, hybridization, and specificity properties and may also improve nuclease resistance of the resultant natural or modified oligoribonucleotide, or a polymeric molecule comprising any combination of monomers described herein and/or natural or modified ribonucleotides.

Ligands in general can include therapeutic modifiers, *e.g.*, for enhancing uptake; diagnostic compounds or reporter groups *e.g.*, for monitoring distribution; cross-linking agents; nuclease-resistance conferring moieties; and natural or unusual nucleobases. General examples include lipophilic molecules, lipids, lectins, steroids (*e.g.*,uvaol, hecigenin, diosgenin), terpenes (*e.g.*, triterpenes, *e.g.*, sarsasapogenin, Friedelin, epifriedelanol derivatized lithocholic acid), vitamins, carbohydrates (*e.g.*, a dextran, pullulan, chitin, chitosan, synthetic (eg Oligo Lactate 15-mer) and natural (eg low and medium molecular weight) polymers, inulin, cyclodextrin or hyaluronic acid), proteins, protein binding agents, integrin targeting molecules, polycationics, peptides, polyamines, and peptide mimics. Other examples include folic acid or epithelial cell receptor ligands, such as transferin.

The ligand may be a naturally occurring or recombinant or synthetic molecule, such as a synthetic polymer, *e.g.*, a synthetic polyamino acid. Examples of polyamino acids include polylysine (PLL), poly L-aspartic acid, poly L-glutamic acid, styrene-maleic acid anhydride copolymer, poly(L-lactide-co-glycolied) copolymer, divinyl ether-maleic anhydride copolymer, N-(2-hydroxypropyl)methacrylamide copolymer (HMPA), polyethylene glycol (PEG), polyvinyl alcohol (PVA), polyurethane, poly(2-ethylacrylic acid), N-isopropylacrylamide polymers, or polyphosphazine. Example of polyamines include: polyethylenimine, polylysine (PLL), spermine, spermidine, polyamine, pseudopeptide-polyamine, peptidomimetic polyamine, dendrimer polyamine, arginine, amidine, protamine, cationic moieties, *e.g.*, cationic lipid, cationic porphyrin, quaternary salt of a polyamine, or an alpha helical peptide.

Ligands can also include targeting groups, *e.g.*, a cell or tissue targeting agent, *e.g.*, a thyrotropin, melanotropin, surfactant protein A, mucin carbohydrate, a glycosylated polyaminoacid, transferrin, bisphosphonate, polyglutamate, polyaspartate, or an Arg-Gly-Asp (RGD) peptide or RGD peptide mimetic.

Ligands can be proteins, *e.g.*, glycoproteins, lipoproteins, *e.g.* low density lipoprotein (LDL), or albumins, *e.g.* human serum albumin (HSA), or peptides, *e.g.*, molecules having a specific affinity for a co-ligand, or antibodies *e.g.*, an antibody, that binds to a specified cell type such as a cancer cell, endothelial cell, or bone cell. Ligands may also include hormones and hormone receptors. They can also include non-peptidic species, such as cofactors, multivalent lactose, multivalent galactose, N-acetyl-galactosamine, N-acetyl-glucosamine, multivalent mannose, or multivalent fucose.

The ligand can be a substance, *e.g*, a drug, which can increase the uptake of the iRNA agent into the cell, for example, by disrupting the cell's cytoskeleton, *e.g.*, by disrupting the cell's microtubules, microfilaments, and/or intermediate filaments. The drug can be, for example, taxon, vincristine, vinblastine, cytochalasin, nocodazole, japlakinolide, latrunculin A, phalloidin, swinholide A, indanocine, myoservin, tetracyclin.

In one aspect, the ligand is a lipid or lipid-based molecule. Such a lipid or lipid-based molecule preferably binds a serum protein, *e.g.*, human serum albumin (HSA). An HSA binding ligand allows for distribution of the conjugate to a target tissue, *e.g.*, liver tissue, including parenchymal cells of the liver. Other molecules that can bind HSA can also be used as ligands. For example, neproxin or aspirin can be used. A lipid or lipid-based ligand can (a) increase resistance to degradation of the conjugate, (b) increase targeting or transport into a target cell or cell membrane, and/or (c) can be used to adjust binding to a serum protein, *e.g.*, HSA.

A lipid based ligand can be used to modulate, *e.g.*, control the binding of the conjugate to a target tissue. For example, a lipid or lipid-based ligand that binds to HSA more strongly will be less likely to be targeted to the kidney and therefore less likely to be cleared from the body.

In a preferred embodiment, the lipid based ligand binds HSA. Preferably, it binds HSA with a sufficient affinity such that the conjugate will be preferably distributed to a non-

kidney tissue. However, it is preferred that the affinity not be so strong that the HSA-ligand binding cannot be reversed.

In another aspect, the ligand is a moiety, *e.g.*, a vitamin or nutrient, which is taken up by a target cell, *e.g.*, a proliferating cell. These are particularly useful for treating disorders characterized by unwanted cell proliferation, *e.g.*, of the malignant or non-malignant type, *e.g.*, cancer cells. Exemplary vitamins include vitamin A, E, and K. Other exemplary vitamins include the B vitamins, *e.g.*, folic acid, B12, riboflavin, biotin, pyridoxal or other vitamins or nutrients taken up by cancer cells.

In another aspect, the ligand is a cell-permeation agent, preferably a helical cell-permeation agent. Preferably, the agent is amphipathic. An exemplary agent is a peptide such as tat or antennapedia. If the agent is a peptide, it can be modified, including a peptidylmimetic, invertomers, non-peptide or pseudo-peptide linkages, and use of D-amino acids. The helical agent is preferably an alpha-helical agent, which preferably has a lipophilic and a lipophobic phase. The cell permeation agent can be linked covalently to the iRNA agent or be part of an iRNA-peptide complex.

5'-Phosphate modifications

In preferred embodiments, iRNA agents are 5' phosphorylated or include a phosphoryl analog at the 5' prime terminus. 5'-phosphate modifications of the antisense strand include those which are compatible with RISC-mediated gene silencing. Suitable modifications include: 5'-monophosphate ((HO)2(O)P-O-5'); 5'-diphosphate ((HO)2(O)P-O-P(HO)(O)-O-5'); 5'-triphosphate ((HO)2(O)P-O-(HO)(O)P-O-P(HO)(O)-O-5'); 5'-guanosine cap (7-methylated or non-methylated) (7m-G-O-5'-(HO)(O)P-O-(HO)(O)P-O-P(HO)(O)-O-5'); 5'-adenosine cap (Appp), and any modified or unmodified nucleotide cap structure (N-O-5'-(HO)(O)P-O-(HO)(O)P-O-P(HO)(O)-O-5'); 5'-monothiophosphate (phosphorothioate; (HO)2(S)P-O-5'); 5'-monodithiophosphate (phosphorodithioate; (HO)(HS)(S)P-O-5'), 5'-phosphorothiolate ((HO)2(O)P-S-5'); any additional combination of oxygen/sulfur replaced monophosphate, diphosphate and triphosphates (*e.g.* 5'-alpha-thiotriphosphate, 5'-gamma-thiotriphosphate, etc.), 5'-phosphoramidates ((HO)2(O)P-NH-5', (HO)(NH2)(O)P-O-5'), 5'-alkylphosphonates (R=alkyl=methyl, ethyl, isopropyl, propyl, etc., *e.g.* RP(OH)(O)-O-5'-, (OH)2(O)P-5'-CH2-), 5'-alkyletherphosphonates (R=alkylether=methoxymethyl (MeOCH2-), ethoxymethyl, etc., *e.g.* RP(OH)(O)-O-5'-).

The sense strand can be modified in order to inactivate the sense strand and prevent formation of an active RISC, thereby potentially reducing off-target effects. This can be accomplished by a modification which prevents 5'-phosphorylation of the sense strand, *e.g.*, by modification with a 5'-O-methyl ribonucleotide (see Nykänen *et al.*, (2001) ATP requirements and small interfering RNA structure in the RNA interference pathway. Cell 107, 309-321.) Other modifications which prevent phosphorylation can also be used, *e.g.*, simply substituting the 5'-OH by H rather than O-Me. Alternatively, a large bulky group may be added to the 5'-phosphate turning it into a phosphodiester linkage.

Non-Natural Nucleobases

Nitropyrrolyl and nitroindolyl are non-natural nucleobases that are members of a class of compounds known as universal bases. Universal bases are those compounds that can replace any of the four naturally occuring bases without substantially affecting the melting behavior or activity of the oligonucleotide duplex. In contrast to the stabilizing, hydrogen-bonding interactions associated with naturally occurring nucleobases, it is postulated that oligonucleotide duplexes containing 3-nitropyrrolyl nucleobases are stabilized solely by stacking interactions. The absence of significant hydrogen-bonding interactions with nitropyrrolyl nucleobases obviates the specificity for a specific complementary base. In addition, various reports confirm that 4-, 5- and 6-nitroindolyl display very little specificity for the four natural bases. Interestingly, an oligonucleotide duplex containing 5-nitroindolyl was more stable than the corresponding oligonucleotides containing 4-nitroindolyl and 6-nitroindolyl. Procedures for the preparation of 1-(2'-O-methyl-β-D-ribofuranosyl)-5-nitroindole are described in Gaubert, G.; Wengel, J. *Tetrahedron Letters* **2004**, *45*, 5629. Other universal bases amenable to the present invention include hypoxanthinyl, isoinosinyl, 2-aza-inosinyl, 7-deaza-inosinyl, nitroimidazolyl, nitropyrazolyl, nitrobenzimidazolyl, nitroindazolyl, aminoindolyl, pyrrolopyrimidinyl, and structural derivatives thereof. For a more detailed discussion, including synthetic procedures, of nitropyrrolyl, nitroindolyl, and other universal bases mentioned above see Vallone et al., Nucleic Acids Research, 27(17):3589-3596 (1999); Loakes et al., J. Mol. Bio., 270:426-436 (1997); Loakes et al., Nucleic Acids Research, 22(20):4039-4043 (1994); Oliver et al., Organic Letters, Vol. 3(13):1977-1980 (2001); Amosova et al., Nucleic Acids Research, 25(10):1930-1934 (1997); Loakes et al., Nucleic Acids Research, 29(12):2437-2447 (2001); Bergstrom et al., J. Am.

Chem. Soc., 117:1201-1209 (1995); Franchetti et al., Biorg. Med. Chem. Lett. 11:67-69 (2001); and Nair et al., Nucelosides, Nucleotides & Nucleic Acids, 20(4-7):735-738 (2001).

Difluorotolyl is a non-natural nucleobase that functions as a universal base. Difluorotolyl is an isostere of the natural nucleobase thymine. But unlike thymine, difluorotolyl shows no appreciable selectivity for any of the natural bases. Other aromatic compounds that function as universal bases and are amenable to the present invention are 4-fluoro-6-methylbenzimidazole and 4-methylbenzimidazole. In addition, the relatively hydrophobic isocarbostyrilyl derivatives 3-methyl isocarbostyrilyl, 5-methyl isocarbostyrilyl, and 3-methyl-7-propynyl isocarbostyrilyl are universal bases which cause only slight destabilization of oligonucleotide duplexes compared to the oligonucleotide sequence containing only natural bases. Other non-natural nucleobases contemplated in the present invention include 7-azaindolyl, 6-methyl-7-azaindolyl, imidizopyridinyl, 9-methyl-imidizopyridinyl, pyrrolopyrizinyl, isocarbostyrilyl, 7-propynyl isocarbostyrilyl, propynyl-7-azaindolyl, 2,4,5-trimethylphenyl, 4-methylindolyl, 4,6-dimethylindolyl, phenyl, napthalenyl, anthracenyl, phenanthracenyl, pyrenyl, stilbenyl, tetracenyl, pentacenyl, and structural derivates thereof. For a more detailed discussion, including synthetic procedures, of difluorotolyl, 4-fluoro-6-methylbenzimidazole, 4-methylbenzimidazole, and other non-natural bases mentioned above, see: Schweitzer et al., J. Org. Chem., 59:7238-7242 (1994); Berger et al., Nucleic Acids Research, 28(15):2911-2914 (2000); Moran et al., J. Am. Chem. Soc., 119:2056-2057 (1997); Morales et al., J. Am. Chem. Soc., 121:2323-2324 (1999); Guckian et al., J. Am. Chem. Soc., 118:8182-8183 (1996); Morales et al., J. Am. Chem. Soc., 122(6):1001-1007 (2000); McMinn et al., J. Am. Chem. Soc., 121:11585-11586 (1999); Guckian et al., J. Org. Chem., 63:9652-9656 (1998); Moran et al., Proc. Natl. Acad. Sci., 94:10506-10511 (1997); Das et al., J. Chem. Soc., Perkin Trans., 1:197-206 (2002); Shibata et al., J. Chem. Soc., Perkin Trans., 1:1605-1611 (2001); Wu et al., J. Am. Chem. Soc., 122(32):7621-7632 (2000); O'Neill et al., J. Org. Chem., 67:5869-5875 (2002); Chaudhuri et al., J. Am. Chem. Soc., 117:10434-10442 (1995); and U.S. Patent No. 6,218,108.

Transport of iRNA agents into cells

Not wishing to be bound by any theory, the chemical similarity between cholesterol-conjugated iRNA agents and certain constituents of lipoproteins (*e.g.* cholesterol, cholesteryl esters, phospholipids) may lead to the association of iRNA agents with lipoproteins (*e.g.* LDL, HDL) in blood and/or the interaction of the iRNA agent with cellular components

having an affinity for cholesterol, *e.g.* components of the cholesterol transport pathway. Lipoproteins as well as their constituents are taken up and processed by cells by various active and passive transport mechanisms, for example, without limitation, endocytosis of LDL-receptor bound LDL, endocytosis of oxidized or otherwise modified LDLs through interaction with Scavenger receptor A, Scavenger receptor B1-mediated uptake of HDL cholesterol in the liver, pinocytosis, or transport of cholesterol across membranes by ABC (ATP-binding cassette) transporter proteins, *e.g.* ABC-A1, ABC-G1 or ABC-G4. Hence, cholesterol-conjugated iRNA agents could enjoy facilitated uptake by cells possessing such transport mechanisms, *e.g.* cells of the liver. As such, the present invention provides evidence and general methods for targeting iRNA agents to cells expressing certain cell surface components, *e.g.* receptors, by conjugating a natural ligand for such component (*e.g.* cholesterol) to the iRNA agent, or by conjugating a chemical moiety (*e.g.* cholesterol) to the iRNA agent which associates with or binds to a natural ligand for the component (*e.g.* LDL, HDL).

Other Embodiments

An iRNA agent, can be produced in a cell *in vivo*, *e.g.*, from exogenous DNA templates that are delivered into the cell. For example, the DNA templates can be inserted into vectors and used as gene therapy vectors. Gene therapy vectors can be delivered to a subject by, for example, intravenous injection, local administration (U.S. Pat. No. 5,328,470), or by stereotactic injection (see, *e.g.*, Chen *et al. Proc. Natl. Acad. Sci. USA* 91:3054-3057, 1994). The pharmaceutical preparation of the gene therapy vector can include the gene therapy vector in an acceptable diluent, or can comprise a slow release matrix in which the gene delivery vehicle is imbedded. The DNA templates, for example, can include two transcription units, one that produces a transcript that includes the top strand of an iRNA agent and one that produces a transcript that includes the bottom strand of an iRNA agent. When the templates are transcribed, the iRNA agent is produced, and processed into siRNA agent fragments that mediate gene silencing.

Formulation

The present invention also includes pharmaceutical compositions and formulations which include the dsRNA compounds of the invention. The pharmaceutical compositions of the present invention may be administered in a number of ways depending upon whether

local or systemic treatment is desired and upon the area to be treated. Administration may be topical, pulmonary, e.g., by inhalation or insufflation of powders or aerosols, including by nebulizer; intratracheal, intranasal, epidermal and transdermal, oral or parenteral. Parenteral administration includes intravenous, intraarterial, subcutaneous, intraperitoneal or intramuscular injection or infusion; or intracranial, e.g., intrathecal or intraventricular, administration.

Pharmaceutical compositions and formulations for topical administration may include transdermal patches, ointments, lotions, creams, gels, drops, suppositories, sprays, liquids and powders. Conventional pharmaceutical carriers, aqueous, powder or oily bases, thickeners and the like may be necessary or desirable. Coated condoms, gloves and the like may also be useful. Preferred topical formulations include those in which the dsRNAs of the invention are in admixture with a topical delivery agent such as lipids, liposomes, fatty acids, fatty acid esters, steroids, chelating agents and surfactants. Preferred lipids and liposomes include neutral (e.g. dioleoylphosphatidyl ethanolamine = DOPE, dimyristoylphosphatidyl choline = DMPC, distearolyphosphatidyl choline) negative (e.g. dimyristoylphosphatidyl glycerol = DMPG) and cationic (e.g. dioleoyltetramethylaminopropyl = DOTAP and dioleoylphosphatidyl ethanolamine = DOTMA), e.g. (+/-)-N-(3-aminopropyl)-N,N-dimethyl-2,3-bis (dodecyloxy)-1-propanaminium bromide = GAP-DLRIE). DsRNAs of the invention may be encapsulated within liposomes or may form complexes thereto, in particular to cationic liposomes. Alternatively, dsRNAs may be complexed to lipids, in particular to cationic lipids. Preferred fatty acids and esters include but are not limited arachidonic acid, oleic acid, eicosanoic acid, lauric acid, caprylic acid, capric acid, myristic acid, palmitic acid, stearic acid, linoleic acid, linolenic acid, dicaprate, tricaprate, monoolein, dilaurin, glyceryl 1-monocaprate, 1-dodecylazacycloheptan-2-one, an acylcarnitine, an acylcholine, or a C_{1-10} alkyl ester (e.g. isopropylmyristate IPM), monoglyceride, diglyceride or pharmaceutically acceptable salt thereof. Topical formulations are described in detail in U.S. patent application Ser. No. 09/315,298 filed on May 20, 1999 which is incorporated herein by reference in its entirety.

Compositions and formulations for oral administration include powders or granules, microparticulates, nanoparticulates, suspensions or solutions in water or non-aqueous media, capsules, gel capsules, sachets, tablets or minitablets. Thickeners, flavoring agents, diluents, emulsifiers, dispersing aids or binders may be desirable. Preferred oral formulations are those

in which dsRNAs of the invention are administered in conjunction with one or more penetration enhancers, surfactants, and chelators. Preferred surfactants include fatty acids and/or esters or salts thereof, bile acids and/or salts thereof. Preferred bile acids/salts include chenodeoxycholic acid (CDCA) and ursodeoxychenodeoxycholic acid (UDCA), cholic acid, dehydrocholic acid, deoxycholic acid, glucholic acid, glycholic acid, glycodeoxycholic acid, taurocholic acid, taurodeoxycholic acid, sodium tauro-24,25-dihydro-fusidate and sodium glycodihydrofusidate. Preferred fatty acids include arachidonic acid, undecanoic acid, oleic acid, lauric acid, caprylic acid, capric acid, myristic acid, palmitic acid, stearic acid, linoleic acid, linolenic acid, dicaprate, tricaprate, monoolein, dilaurin, glyceryl 1-monocaprate, 1-dodecylazacycloheptan-2-one, an acylcarnitine, an acylcholine, or a monoglyceride, a diglyceride or a pharmaceutically acceptable salt thereof (e.g. sodium). Also preferred are combinations of penetration enhancers, for example, fatty acids/salts in combination with bile acids/salts. A particularly preferred combination is the sodium salt of lauric acid, capric acid and UDCA. Further penetration enhancers include polyoxyethylene-9-lauryl ether, polyoxyethylene-20-cetyl ether. DsRNAs of the invention may be delivered orally, in granular form including sprayed dried particles, or complexed to form micro or nanoparticles. DsRNA complexing agents include poly-amino acids; polyimines; polyacrylates; polyalkylacrylates, polyoxethanes, polyalkylcyanoacrylates; cationized gelatins, albumins, starches, acrylates, polyethyleneglycols (PEG) and starches; polyalkylcyanoacrylates; DEAE-derivatized polyimines, pollulans, celluloses and starches. Particularly preferred complexing agents include chitosan, N-trimethylchitosan, poly-L-lysine, polyhistidine, polyornithine, polyspermines, protamine, polyvinylpyridine, polythiodiethylaminomethylethylene P(TDAE), polyaminostyrene (e.g. p-amino), poly(methylcyanoacrylate), poly(ethylcyanoacrylate), poly(butylcyanoacrylate), poly(isobutylcyanoacrylate), poly(isohexylcynaoacrylate), DEAE-methacrylate, DEAE-hexylacrylate, DEAE-acrylamide, DEAE-albumin and DEAE-dextran, polymethylacrylate, polyhexylacrylate, poly(D,L-lactic acid), poly(DL-lactic-co-glycolic acid (PLGA), alginate, and polyethyleneglycol (PEG). Oral formulations for dsRNAs and their preparation are described in detail in U.S. application. Ser. No. 08/886,829 (filed Jul. 1, 1997), Ser. No. 09/108,673 (filed Jul. 1, 1998), Ser. No. 09/256,515 (filed Feb. 23, 1999), Ser. No. 09/082,624 (filed May 21, 1998) and Ser. No. 09/315,298 (filed May 20, 1999), each of which is incorporated herein by reference in their entirety.

Compositions and formulations for parenteral, intrathecal or intraventricular administration may include sterile aqueous solutions which may also contain buffers, diluents and other suitable additives such as, but not limited to, penetration enhancers, carrier compounds and other pharmaceutically acceptable carriers or excipients.

Pharmaceutical compositions of the present invention include, but are not limited to, solutions, emulsions, and liposome-containing formulations. These compositions may be generated from a variety of components that include, but are not limited to, preformed liquids, self-emulsifying solids and self-emulsifying semisolids.

The pharmaceutical formulations of the present invention, which may conveniently be presented in unit dosage form, may be prepared according to conventional techniques well known in the pharmaceutical industry. Such techniques include the step of bringing into association the active ingredients with the pharmaceutical carrier(s) or excipient(s). In general, the formulations are prepared by uniformly and intimately bringing into association the active ingredients with liquid carriers or finely divided solid carriers or both, and then, if necessary, shaping the product.

The compositions of the present invention may be formulated into any of many possible dosage forms such as, but not limited to, tablets, capsules, gel capsules, liquid syrups, soft gels, suppositories, and enemas. The compositions of the present invention may also be formulated as suspensions in aqueous, non-aqueous or mixed media. Aqueous suspensions may further contain substances which increase the viscosity of the suspension including, for example, sodium carboxymethylcellulose, sorbitol and/or dextran. The suspension may also contain stabilizers.

In one embodiment of the present invention the pharmaceutical compositions may be formulated and used as foams. Pharmaceutical foams include formulations such as, but not limited to, emulsions, microemulsions, creams, jellies and liposomes. While basically similar in nature these formulations vary in the components and the consistency of the final product. The preparation of such compositions and formulations is generally known to those skilled in the pharmaceutical and formulation arts and may be applied to the formulation of the compositions of the present invention.

Emulsions

The compositions of the present invention may be prepared and formulated as emulsions. Emulsions are typically heterogenous systems of one liquid dispersed in another in the form of droplets usually exceeding 0.1 μm in diameter (Idson, in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199; Rosoff, in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., Volume 1, p. 245; Block in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 2, p. 335; Higuchi et al., in Remington's Pharmaceutical Sciences, Mack Publishing Co., Easton, Pa., 1985, p. 301). Emulsions are often biphasic systems comprising two immiscible liquid phases intimately mixed and dispersed with each other. In general, emulsions may be of either the water-in-oil (w/o) or the oil-in-water (o/w) variety. When an aqueous phase is finely divided into and dispersed as minute droplets into a bulk oily phase, the resulting composition is called a water-in-oil (w/o) emulsion. Alternatively, when an oily phase is finely divided into and dispersed as minute droplets into a bulk aqueous phase, the resulting composition is called an oil-in-water (o/w) emulsion. Emulsions may contain additional components in addition to the dispersed phases, and the active drug which may be present as a solution in either the aqueous phase, oily phase or itself as a separate phase. Pharmaceutical excipients such as emulsifiers, stabilizers, dyes, and anti-oxidants may also be present in emulsions as needed. Pharmaceutical emulsions may also be multiple emulsions that are comprised of more than two phases such as, for example, in the case of oil-in-water-in-oil (o/w/o) and water-in-oil-in-water (w/o/w) emulsions. Such complex formulations often provide certain advantages that simple binary emulsions do not. Multiple emulsions in which individual oil droplets of an o/w emulsion enclose small water droplets constitute a w/o/w emulsion. Likewise a system of oil droplets enclosed in globules of water stabilized in an oily continuous phase provides an o/w/o emulsion.

Emulsions are characterized by little or no thermodynamic stability. Often, the dispersed or discontinuous phase of the emulsion is well dispersed into the external or continuous phase and maintained in this form through the means of emulsifiers or the viscosity of the formulation. Either of the phases of the emulsion may be a semisolid or a solid, as is the case of emulsion-style ointment bases and creams. Other means of stabilizing emulsions entail the use of emulsifiers that may be incorporated into either phase of the

emulsion. Emulsifiers may broadly be classified into four categories: synthetic surfactants, naturally occurring emulsifiers, absorption bases, and finely dispersed solids (Idson, in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199).

Synthetic surfactants, also known as surface active agents, have found wide applicability in the formulation of emulsions and have been reviewed in the literature (Rieger, in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 285; Idson, in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), Marcel Dekker, Inc., New York, N.Y., 1988, volume 1, p. 199). Surfactants are typically amphiphilic and comprise a hydrophilic and a hydrophobic portion. The ratio of the hydrophilic to the hydrophobic nature of the surfactant has been termed the hydrophile/lipophile balance (HLB) and is a valuable tool in categorizing and selecting surfactants in the preparation of formulations. Surfactants may be classified into different classes based on the nature of the hydrophilic group: nonionic, anionic, cationic and amphoteric (Rieger, in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 285).

Naturally occurring emulsifiers used in emulsion formulations include lanolin, beeswax, phosphatides, lecithin and acacia. Absorption bases possess hydrophilic properties such that they can soak up water to form w/o emulsions yet retain their semisolid consistencies, such as anhydrous lanolin and hydrophilic petrolatum. Finely divided solids have also been used as good emulsifiers especially in combination with surfactants and in viscous preparations. These include polar inorganic solids, such as heavy metal hydroxides, nonswelling clays such as bentonite, attapulgite, hectorite, kaolin, montmorillonite, colloidal aluminum silicate and colloidal magnesium aluminum silicate, pigments and nonpolar solids such as carbon or glyceryl tristearate.

A large variety of non-emulsifying materials are also included in emulsion formulations and contribute to the properties of emulsions. These include fats, oils, waxes, fatty acids, fatty alcohols, fatty esters, humectants, hydrophilic colloids, preservatives and antioxidants (Block, in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 335; Idson, in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199).

Hydrophilic colloids or hydrocolloids include naturally occurring gums and synthetic polymers such as polysaccharides (for example, acacia, agar, alginic acid, carrageenan, guar gum, karaya gum, and tragacanth), cellulose derivatives (for example, carboxymethylcellulose and carboxypropylcellulose), and synthetic polymers (for example, carbomers, cellulose ethers, and carboxyvinyl polymers). These disperse or swell in water to form colloidal solutions that stabilize emulsions by forming strong interfacial films around the dispersed-phase droplets and by increasing the viscosity of the external phase.

Since emulsions often contain a number of ingredients such as carbohydrates, proteins, sterols and phosphatides that may readily support the growth of microbes, these formulations often incorporate preservatives. Commonly used preservatives included in emulsion formulations include methyl paraben, propyl paraben, quaternary ammonium salts, benzalkonium chloride, esters of p-hydroxybenzoic acid, and boric acid. Antioxidants are also commonly added to emulsion formulations to prevent deterioration of the formulation. Antioxidants used may be free radical scavengers such as tocopherols, alkyl gallates, butylated hydroxyanisole, butylated hydroxytoluene, or reducing agents such as ascorbic acid and sodium metabisulfite, and antioxidant synergists such as citric acid, tartaric acid, and lecithin.

The application of emulsion formulations via dermatological, oral and parenteral routes and methods for their manufacture have been reviewed in the literature (Idson, in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199). Emulsion formulations for oral delivery have been very widely used because of ease of formulation, as well as efficacy from an absorption and bioavailability standpoint (Rosoff, in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 245; Idson, in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199). Mineral-oil base laxatives, oil-soluble vitamins and high fat nutritive preparations are among the materials that have commonly been administered orally as o/w emulsions.

In one embodiment of the present invention, the compositions of dsRNAs and nucleic acids are formulated as microemulsions. A microemulsion may be defined as a system of water, oil and amphiphile which is a single optically isotropic and thermodynamically stable liquid solution (Rosoff, in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker

(Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 245). Typically microemulsions are systems that are prepared by first dispersing an oil in an aqueous surfactant solution and then adding a sufficient amount of a fourth component, generally an intermediate chain-length alcohol to form a transparent system. Therefore, microemulsions have also been described as thermodynamically stable, isotropically clear dispersions of two immiscible liquids that are stabilized by interfacial films of surface-active molecules (Leung and Shah, in: Controlled Release of Drugs: Polymers and Aggregate Systems, Rosoff, M., Ed., 1989, VCH Publishers, New York, pages 185-215). Microemulsions commonly are prepared via a combination of three to five components that include oil, water, surfactant, cosurfactant and electrolyte. Whether the microemulsion is of the water-in-oil (w/o) or an oil-in-water (o/w) type is dependent on the properties of the oil and surfactant used and on the structure and geometric packing of the polar heads and hydrocarbon tails of the surfactant molecules (Schott, in Remington's Pharmaceutical Sciences, Mack Publishing Co., Easton, Pa., 1985, p. 271).

The phenomenological approach utilizing phase diagrams has been extensively studied and has yielded a comprehensive knowledge, to one skilled in the art, of how to formulate microemulsions (Rosoff, in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 245; Block, in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 335). Compared to conventional emulsions, microemulsions offer the advantage of solubilizing water-insoluble drugs in a formulation of thermodynamically stable droplets that are formed spontaneously.

Surfactants used in the preparation of microemulsions include, but are not limited to, ionic surfactants, non-ionic surfactants, Brij 96, polyoxyethylene oleyl ethers, polyglycerol fatty acid esters, tetraglycerol monolaurate (ML310), tetraglycerol monooleate (MO310), hexaglycerol monooleate (PO310), hexaglycerol pentaoleate (PO500), decaglycerol monocaprate (MCA750), decaglycerol monooleate (MO750), decaglycerol sequioleate (SO750), decaglycerol decaoleate (DAO750), alone or in combination with cosurfactants. The cosurfactant, usually a short-chain alcohol such as ethanol, 1-propanol, and 1-butanol, serves to increase the interfacial fluidity by penetrating into the surfactant film and consequently creating a disordered film because of the void space generated among surfactant molecules. Microemulsions may, however, be prepared without the use of cosurfactants and

alcohol-free self-emulsifying microemulsion systems are known in the art. The aqueous phase may typically be, but is not limited to, water, an aqueous solution of the drug, glycerol, PEG300, PEG400, polyglycerols, propylene glycols, and derivatives of ethylene glycol. The oil phase may include, but is not limited to, materials such as Captex 300, Captex 355, Capmul MCM, fatty acid esters, medium chain (C_8-C_{12}) mono, di, and tri-glycerides, polyoxyethylated glyceryl fatty acid esters, fatty alcohols, polyglycolized glycerides, saturated polyglycolized C_8-C_{10} glycerides, vegetable oils and silicone oil.

Microemulsions are particularly of interest from the standpoint of drug solubilization and the enhanced absorption of drugs. Lipid based microemulsions (both o/w and w/o) have been proposed to enhance the oral bioavailability of drugs, including peptides (Constantinides et al., Pharmaceutical Research, 1994, 11, 1385-1390; Ritschel, Meth. Find. Exp. Clin. Pharmacol., 1993, 13, 205). Microemulsions afford advantages of improved drug solubilization, protection of drug from enzymatic hydrolysis, possible enhancement of drug absorption due to surfactant-induced alterations in membrane fluidity and permeability, ease of preparation, ease of oral administration over solid dosage forms, improved clinical potency, and decreased toxicity (Constantinides et al., Pharmaceutical Research, 1994, 11, 1385; Ho et al., J. Pharm. Sci., 1996, 85, 138-143). Often microemulsions may form spontaneously when their components are brought together at ambient temperature. This may be particularly advantageous when formulating thermolabile drugs, peptides or dsRNAs. Microemulsions have also been effective in the transdermal delivery of active components in both cosmetic and pharmaceutical applications. It is expected that the microemulsion compositions and formulations of the present invention will facilitate the increased systemic absorption of dsRNAs and nucleic acids from the gastrointestinal tract, as well as improve the local cellular uptake of dsRNAs and nucleic acids within the gastrointestinal tract, vagina, buccal cavity and other areas of administration.

Microemulsions of the present invention may also contain additional components and additives such as sorbitan monostearate (Grill 3), Labrasol, and penetration enhancers to improve the properties of the formulation and to enhance the absorption of the dsRNAs and nucleic acids of the present invention. Penetration enhancers used in the microemulsions of the present invention may be classified as belonging to one of five broad categories surfactants, fatty acids, bile salts, chelating agents, and non-chelating non-

surfactants (Lee et al., Critical Reviews in Therapeutic Drug Carrier Systems, 1991, p. 92). Each of these classes has been discussed above.

Liposomes

There are many organized surfactant structures besides microemulsions that have been studied and used for the formulation of drugs. These include monolayers, micelles, bilayers and vesicles. Vesicles, such as liposomes, have attracted great interest because of their specificity and the duration of action they offer from the standpoint of drug delivery. As used in the present invention, the term "liposome" means a vesicle composed of amphiphilic lipids arranged in a spherical bilayer or bilayers.

Liposomes are unilamellar or multilamellar vesicles which have a membrane formed from a lipophilic material and an aqueous interior. The aqueous portion contains the composition to be delivered. Cationic liposomes possess the advantage of being able to fuse to the cell wall. Non-cationic liposomes, although not able to fuse as efficiently with the cell membrane, are taken up by macrophages in vivo.

In order to cross intact mammalian skin, lipid vesicles must pass through a series of fine pores, each with a diameter less than 50 nm, under the influence of a suitable transdermal gradient. Therefore, it is desirable to use a liposome which is highly deformable and able to pass through such fine pores.

Further advantages of liposomes include; liposomes obtained from natural phospholipids are biocompatible and biodegradable; liposomes can incorporate a wide range of water and lipid soluble drugs; liposomes can protect encapsulated drugs in their internal compartments from metabolism and degradation (Rosoff, in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 245). Important considerations in the preparation of liposome formulations are the lipid surface charge, vesicle size and the aqueous volume of the liposomes.

Liposomes are useful for the transfer and delivery of active ingredients to the site of action. Because the liposomal membrane is structurally similar to biological membranes, when liposomes are applied to a tissue, the liposomes start to merge with the cellular membranes and as the merging of the liposome and cell progresses, the liposomal contents are emptied into the cell where the active agent may act.

Liposomal formulations have been the focus of extensive investigation as the mode of delivery for many drugs. There is growing evidence that for topical administration, liposomes present several advantages over other formulations. Such advantages include reduced side-effects related to high systemic absorption of the administered drug, increased accumulation of the administered drug at the desired target, and the ability to administer a wide variety of drugs, both hydrophilic and hydrophobic, into the skin.

Several reports have detailed the ability of liposomes to deliver agents including high-molecular weight DNA into the skin. Compounds including analgesics, antibodies, hormones and high-molecular weight DNAs have been administered to the skin. The majority of applications resulted in the targeting of the upper epidermis

Liposomes fall into two broad classes. Cationic liposomes are positively charged liposomes which interact with the negatively charged DNA molecules to form a stable complex. The positively charged DNA/liposome complex binds to the negatively charged cell surface and is internalized in an endosome. Due to the acidic pH within the endosome, the liposomes are ruptured, releasing their contents into the cell cytoplasm (Wang et al., Biochem. Biophys. Res. Commun., 1987, 147, 980-985).

Liposomes which are pH-sensitive or negatively-charged, entrap DNA rather than complex with it. Since both the DNA and the lipid are similarly charged, repulsion rather than complex formation occurs. Nevertheless, some DNA is entrapped within the aqueous interior of these liposomes. pH-sensitive liposomes have been used to deliver DNA encoding the thymidine kinase gene to cell monolayers in culture. Expression of the exogenous gene was detected in the target cells (Zhou et al., Journal of Controlled Release, 1992, 19, 269-274).

One major type of liposomal composition includes phospholipids other than naturally derived phosphatidylcholine. Neutral liposome compositions, for example, can be formed from dimyristoyl phosphatidylcholine (DMPC) or dipalmitoyl phosphatidylcholine (DPPC). Anionic liposome compositions generally are formed from dimyristoyl phosphatidylglycerol, while anionic fusogenic liposomes are formed primarily from dioleoyl phosphatidylethanolamine (DOPE). Another type of liposomal composition is formed from phosphatidylcholine (PC) such as, for example, soybean PC, and egg PC. Another type is formed from mixtures of phospholipid and/or phosphatidylcholine and/or cholesterol.

Several studies have assessed the topical delivery of liposomal drug formulations to the skin. Application of liposomes containing interferon to guinea pig skin resulted in a reduction of skin herpes sores while delivery of interferon via other means (e.g. as a solution or as an emulsion) were ineffective (Weiner et al., Journal of Drug Targeting, 1992, 2, 405-410). Further, an additional study tested the efficacy of interferon administered as part of a liposomal formulation to the administration of interferon using an aqueous system, and concluded that the liposomal formulation was superior to aqueous administration (du Plessis et al., Antiviral Research, 1992, 18, 259-265).

Non-ionic liposomal systems have also been examined to determine their utility in the delivery of drugs to the skin, in particular systems comprising non-ionic surfactant and cholesterol. Non-ionic liposomal formulations comprising Novasome.TM. I (glyceryl dilaurate/cholesterol/polyoxyethylene-10-stearyl ether) and Novasome.TM. II (glyceryl distearate/cholesterol/polyoxyethylene-10-stearyl ether) were used to deliver cyclosporin-A into the dermis of mouse skin. Results indicated that such non-ionic liposomal systems were effective in facilitating the deposition of cyclosporin-A into different layers of the skin (Hu et al. S.T.P.Pharma. Sci., 1994, 4, 6, 466).

Liposomes also include "sterically stabilized" liposomes, a term which, as used herein, refers to liposomes comprising one or more specialized lipids that, when incorporated into liposomes, result in enhanced circulation lifetimes relative to liposomes lacking such specialized lipids. Examples of sterically stabilized liposomes are those in which part of the vesicle-forming lipid portion of the liposome (A) comprises one or more glycolipids, such as monosialoganglioside G_m1, or (B) is derivatized with one or more hydrophilic polymers, such as a polyethylene glycol (PEG) moiety. While not wishing to be bound by any particular theory, it is thought in the art that, at least for sterically stabilized liposomes containing gangliosides, sphingomyelin, or PEG-derivatized lipids, the enhanced circulation half-life of these sterically stabilized liposomes derives from a reduced uptake into cells of the reticuloendothelial system (RES) (Allen et al., FEBS Letters, 1987, 223, 42; Wu et al., Cancer Research, 1993, 53, 3765).

Various liposomes comprising one or more glycolipids are known in the art. Papahadjopoulos et al. (Ann. N.Y. Acad. Sci., 1987, 507, 64) reported the ability of monosialoganglioside G_m1, galactocerebroside sulfate and phosphatidylinositol to improve blood half-lives of liposomes. These findings were expounded upon by Gabizon et al. (Proc.

Natl. Acad. Sci. U.S.A., 1988, 85, 6949). U.S. Pat. No. 4,837,028 and WO 88/04924, both to Allen et al., disclose liposomes comprising (1) sphingomyelin and (2) the ganglioside G_m1 or a galactocerebroside sulfate ester. U.S. Pat. No. 5,543,152 (Webb et al.) discloses liposomes comprising sphingomyelin. Liposomes comprising 1,2-sn-dimyristoylphosphat-idylcholine are disclosed in WO 97/13499 (Lim et al).

Many liposomes comprising lipids derivatized with one or more hydrophilic polymers, and methods of preparation thereof, are known in the art. Sunamoto et al. (Bull. Chem. Soc. Jpn., 1980, 53, 2778) described liposomes comprising a nonionic detergent, $2C_{1215G}$, that contains a PEG moiety. Illum et al. (FEBS Lett., 1984, 167, 79) noted that hydrophilic coating of polystyrene particles with polymeric glycols results in significantly enhanced blood half-lives. Synthetic phospholipids modified by the attachment of carboxylic groups of polyalkylene glycols (e.g., PEG) are described by Sears (U.S. Pat. Nos. 4,426,330 and 4,534,899). Klibanov et al. (FEBS Lett., 1990, 268, 235) described experiments demonstrating that liposomes comprising phosphatidylethanolamine (PE) derivatized with PEG or PEG stearate have significant increases in blood circulation half-lives. Blume et al. (Biochimica et Biophysica Acta, 1990, 1029, 91) extended such observations to other PEG-derivatized phospholipids, e.g., DSPE-PEG, formed from the combination of distearoylphosphatidylethanolamine (DSPE) and PEG. Liposomes having covalently bound PEG moieties on their external surface are described in European Patent No. EP 0 445 131 B1 and WO 90/04384 to Fisher. Liposome compositions containing 1-20 mole percent of PE derivatized with PEG, and methods of use thereof, are described by Woodle et al. (U.S. Pat. Nos. 5,013,556 and 5,356,633) and Martin et al. (U.S. Pat. No. 5,213,804 and European Patent No. EP 0 496 813 B1). Liposomes comprising a number of other lipid-polymer conjugates are disclosed in WO 91/05545 and U.S. Pat. No. 5,225,212 (both to Martin et al.) and in WO 94/20073 (Zalipsky et al.) Liposomes comprising PEG-modified ceramide lipids are described in WO 96/10391 (Choi et al). U.S. Pat. No. 5,540,935 (Miyazaki et al.) and U.S. Pat. No. 5,556,948 (Tagawa et al.) describe PEG-containing liposomes that can be further derivatized with functional moieties on their surfaces.

A limited number of liposomes comprising nucleic acids are known in the art. WO 96/40062 to Thierry et al. discloses methods for encapsulating high molecular weight nucleic acids in liposomes. U.S. Pat. No. 5,264,221 to Tagawa et al. discloses protein-bonded liposomes and asserts that the contents of such liposomes may include dsRNA. U.S. Pat. No.

5,665,710 to Rahman et al. describes certain methods of encapsulating oligodeoxynucleotides in liposomes. WO 97/04787 to Love et al. discloses liposomes comprising dsRNAs targeted to the raf gene.

Transfersomes are yet another type of liposomes, and are highly deformable lipid aggregates which are attractive candidates for drug delivery vehicles. Transfersomes may be described as lipid droplets which are so highly deformable that they are easily able to penetrate through pores which are smaller than the droplet. Transfersomes are adaptable to the environment in which they are used, e.g. they are self-optimizing (adaptive to the shape of pores in the skin), self-repairing, frequently reach their targets without fragmenting, and often self-loading. To make transfersomes it is possible to add surface edge-activators, usually surfactants, to a standard liposomal composition. Transfersomes have been used to deliver serum albumin to the skin. The transfersome-mediated delivery of serum albumin has been shown to be as effective as subcutaneous injection of a solution containing serum albumin.

Surfactants find wide application in formulations such as emulsions (including microemulsions) and liposomes. The most common way of classifying and ranking the properties of the many different types of surfactants, both natural and synthetic, is by the use of the hydrophile/lipophile balance (HLB). The nature of the hydrophilic group (also known as the "head") provides the most useful means for categorizing the different surfactants used in formulations (Rieger, in Pharmaceutical Dosage Forms, Marcel Dekker, Inc., New York, N.Y., 1988, p. 285).

If the surfactant molecule is not ionized, it is classified as a nonionic surfactant. Nonionic surfactants find wide application in pharmaceutical and cosmetic products and are usable over a wide range of pH values. In general their HLB values range from 2 to about 18 depending on their structure. Nonionic surfactants include nonionic esters such as ethylene glycol esters, propylene glycol esters, glyceryl esters, polyglyceryl esters, sorbitan esters, sucrose esters, and ethoxylated esters. Nonionic alkanolamides and ethers such as fatty alcohol ethoxylates, propoxylated alcohols, and ethoxylated/propoxylated block polymers are also included in this class. The polyoxyethylene surfactants are the most popular members of the nonionic surfactant class.

If the surfactant molecule carries a negative charge when it is dissolved or dispersed in water, the surfactant is classified as anionic. Anionic surfactants include carboxylates such

as soaps, acyl lactylates, acyl amides of amino acids, esters of sulfuric acid such as alkyl sulfates and ethoxylated alkyl sulfates, sulfonates such as alkyl benzene sulfonates, acyl isethionates, acyl taurates and sulfosuccinates, and phosphates. The most important members of the anionic surfactant class are the alkyl sulfates and the soaps.

If the surfactant molecule carries a positive charge when it is dissolved or dispersed in water, the surfactant is classified as cationic. Cationic surfactants include quaternary ammonium salts and ethoxylated amines. The quaternary ammonium salts are the most used members of this class.

If the surfactant molecule has the ability to carry either a positive or negative charge, the surfactant is classified as amphoteric. Amphoteric surfactants include acrylic acid derivatives, substituted alkylamides, N-alkylbetaines and phosphatides.

The use of surfactants in drug products, formulations and in emulsions has been reviewed (Rieger, in Pharmaceutical Dosage Forms, Marcel Dekker, Inc., New York, N.Y., 1988, p. 285).

Penetration Enhancers

In one embodiment, the present invention employs various penetration enhancers to effect the efficient delivery of nucleic acids, particularly dsRNAs, to the skin of animals. Most drugs are present in solution in both ionized and nonionized forms. However, usually only lipid soluble or lipophilic drugs readily cross cell membranes. It has been discovered that even non-lipophilic drugs may cross cell membranes if the membrane to be crossed is treated with a penetration enhancer. In addition to aiding the diffusion of non-lipophilic drugs across cell membranes, penetration enhancers also enhance the permeability of lipophilic drugs.

Penetration enhancers may be classified as belonging to one of five broad categories, i.e., surfactants, fatty acids, bile salts, chelating agents, and non-chelating non-surfactants (Lee et al., Critical Reviews in Therapeutic Drug Carrier Systems, 1991, p.92). Each of the above mentioned classes of penetration enhancers are described below in greater detail.

Surfactants: In connection with the present invention, surfactants (or "surface-active agents") are chemical entities which, when dissolved in an aqueous solution, reduce the surface tension of the solution or the interfacial tension between the aqueous solution and

another liquid, with the result that absorption of dsRNAs through the mucosa is enhanced. In addition to bile salts and fatty acids, these penetration enhancers include, for example, sodium lauryl sulfate, polyoxyethylene-9-lauryl ether and polyoxyethylene-20-cetyl ether) (Lee et al., Critical Reviews in Therapeutic Drug Carrier Systems, 1991, p.92); and perfluorochemical emulsions, such as FC-43 (Takahashi et al., J. Pharm. Pharmacol., 1988, 40, 252).

Fatty acids: Various fatty acids and their derivatives which act as penetration enhancers include, for example, oleic acid, lauric acid, capric acid (n-decanoic acid), myristic acid, palmitic acid, stearic acid, linoleic acid, linolenic acid, dicaprate, tricaprate, monoolein (1-monooleoyl-rac-glycerol), dilaurin, caprylic acid, arachidonic acid, glycerol 1-monocaprate, 1-dodecylazacycloheptan-2-one, acylcarnitines, acylcholines, C_1 - C_{10} alkyl esters thereof (e.g., methyl, isopropyl and t-butyl), and mono- and di-glycerides thereof (i.e., oleate, laurate, caprate, myristate, palmitate, stearate, linoleate, etc.) (Lee et al., Critical Reviews in Therapeutic Drug Carryier Systems, 1991, p.92; Muranishi, Critical Reviews in Therapeutic Drug Carrier Systems, 1990, 7, 1-33; El Hariri et al., J. Pharm. Pharmacol., 1992, 44, 651-654).

Bile salts: The physiological role of bile includes the facilitation of dispersion and absorption of lipids and fat-soluble vitamins (Brunton, Chapter 38 in: Goodman & Gilman's The Pharmacological Basis of Therapeutics, 9th Ed., Hardman et al. Eds., McGraw-Hill, New York, 1996, pp. 934-935). Various natural bile salts, and their synthetic derivatives, act as penetration enhancers. Thus the term "bile salts" includes any of the naturally occurring components of bile as well as any of their synthetic derivatives. The bile salts of the invention include, for example, cholic acid (or its pharmaceutically acceptable sodium salt, sodium cholate), dehydrocholic acid (sodium dehydrocholate), deoxycholic acid (sodium deoxycholate), glucholic acid (sodium glucholate), glycholic acid (sodium glycocholate), glycodeoxycholic acid (sodium glycodeoxycholate), taurocholic acid (sodium taurocholate), taurodeoxycholic acid (sodium taurodeoxycholate), chenodeoxycholic acid (sodium chenodeoxycholate), ursodeoxycholic acid (UDCA), sodium tauro-24,25-dihydro-fusidate (STDHF), sodium glycodihydrofusidate and polyoxyethylene-9-lauryl ether (POE) (Lee et al., Critical Reviews in Therapeutic Drug Carrier Systems, 1991, page 92; Swinyard, Chapter 39 In: Remington's Pharmaceutical Sciences, 18th Ed., Gennaro, ed., Mack Publishing Co., Easton, Pa., 1990, pages 782-783; Muranishi, Critical Reviews in Therapeutic Drug Carrier

Systems, 1990, 7, 1-33; Yamamoto et al., J. Pharm. Exp. Ther., 1992, 263, 25; Yamashita et al., J. Pharm. Sci., 1990, 79, 579-583).

Chelating Agents: Chelating agents, as used in connection with the present invention, can be defined as compounds that remove metallic ions from solution by forming complexes therewith, with the result that absorption of dsRNAs through the mucosa is enhanced. With regards to their use as penetration enhancers in the present invention, chelating agents have the added advantage of also serving as DNase inhibitors, as most characterized DNA nucleases require a divalent metal ion for catalysis and are thus inhibited by chelating agents (Jarrett, J. Chromatogr., 1993, 618, 315-339). Chelating agents of the invention include but are not limited to disodium ethylenediaminetetraacetate (EDTA), citric acid, salicylates (e.g., sodium salicylate, 5-methoxysalicylate and homovanilate), N-acyl derivatives of collagen, laureth-9 and N-amino acyl derivatives of beta-diketones (enamines)(Lee et al., Critical Reviews in Therapeutic Drug Carrier Systems, 1991, page 92; Muranishi, Critical Reviews in Therapeutic Drug Carrier Systems, 1990, 7, 1-33; Buur et al., J. Control Rel., 1990, 14, 43-51).

Non-chelating non-surfactants: As used herein, non-chelating non-surfactant penetration enhancing compounds can be defined as compounds that demonstrate insignificant activity as chelating agents or as surfactants but that nonetheless enhance absorption of dsRNAs through the alimentary mucosa (Muranishi, Critical Reviews in Therapeutic Drug Carrier Systems, 1990, 7, 1-33). This class of penetration enhancers include, for example, unsaturated cyclic ureas, 1-alkyl- and 1-alkenylazacyclo-alkanone derivatives (Lee et al., Critical Reviews in Therapeutic Drug Carrier Systems, 1991, page 92); and non-steroidal anti-inflammatory agents such as diclofenac sodium, indomethacin and phenylbutazone (Yamashita et al., J. Pharm. Pharmacol., 1987, 39, 621-626).

Agents that enhance uptake of dsRNAs at the cellular level may also be added to the pharmaceutical and other compositions of the present invention. For example, cationic lipids, such as lipofectin (Junichi et al, U.S. Pat. No. 5,705,188), cationic glycerol derivatives, and polycationic molecules, such as polylysine (Lollo et al., PCT Application WO 97/30731) and other peptides, are also known to enhance the cellular uptake of dsRNAs.

Other agents may be utilized to enhance the penetration of the administered nucleic acids, including glycols such as ethylene glycol and propylene glycol, pyrrols such as 2-pyrrol, azones, and terpenes such as limonene and menthone.

Carriers

Certain compositions of the present invention also incorporate carrier compounds in the formulation. As used herein, "carrier compound" or "carrier" can refer to a nucleic acid, or analog thereof, which is inert (i.e., does not possess biological activity per se) but is recognized as a nucleic acid by *in vivo* processes that reduce the bioavailability of a nucleic acid having biological activity by, for example, degrading the biologically active nucleic acid or promoting its removal from circulation. The coadministration of a nucleic acid and a carrier compound, typically with an excess of the latter substance, can result in a substantial reduction of the amount of nucleic acid recovered in the liver, kidney or other extracirculatory reservoirs, presumably due to competition between the carrier compound and the nucleic acid for a common receptor. For example, the recovery of a partially phosphorothioate dsRNA in hepatic tissue can be reduced when it is coadministered with polyinosinic acid, dextran sulfate, polycytidic acid or 4-acetamido-4'isothiocyano-stilbene-2,2'-disulfonic acid (Miyao et al., Antisense Res. Dev., 1995, 5, 115-121; Takakura et al., Antisense & Nucl. Acid Drug Dev., 1996, 6, 177-183.

Excipients

In contrast to a carrier compound, a "pharmaceutical carrier" or "excipient" is a pharmaceutically acceptable solvent, suspending agent or any other pharmacologically inert vehicle for delivering one or more nucleic acids to an animal. The excipient may be liquid or solid and is selected, with the planned manner of administration in mind, so as to provide for the desired bulk, consistency, etc., when combined with a nucleic acid and the other components of a given pharmaceutical composition. Typical pharmaceutical carriers include, but are not limited to, binding agents (e.g., pregelatinized maize starch, polyvinylpyrrolidone or hydroxypropyl methylcellulose, etc.); fillers (e.g., lactose and other sugars, microcrystalline cellulose, pectin, gelatin, calcium sulfate, ethyl cellulose, polyacrylates or calcium hydrogen phosphate, etc.); lubricants (e.g., magnesium stearate, talc, silica, colloidal silicon dioxide, stearic acid, metallic stearates, hydrogenated vegetable oils, corn starch,

polyethylene glycols, sodium benzoate, sodium acetate, etc.); disintegrants (e.g., starch, sodium starch glycolate, etc.); and wetting agents (e.g., sodium lauryl sulphate, etc).

Pharmaceutically acceptable organic or inorganic excipient suitable for non-parenteral administration which do not deleteriously react with nucleic acids can also be used to formulate the compositions of the present invention. Suitable pharmaceutically acceptable carriers include, but are not limited to, water, salt solutions, alcohols, polyethylene glycols, gelatin, lactose, amylose, magnesium stearate, talc, silicic acid, viscous paraffin, hydroxymethylcellulose, polyvinylpyrrolidone and the like.

Formulations for topical administration of nucleic acids may include sterile and non-sterile aqueous solutions, non-aqueous solutions in common solvents such as alcohols, or solutions of the nucleic acids in liquid or solid oil bases. The solutions may also contain buffers, diluents and other suitable additives. Pharmaceutically acceptable organic or inorganic excipients suitable for non-parenteral administration which do not deleteriously react with nucleic acids can be used.

Suitable pharmaceutically acceptable excipients include, but are not limited to, water, salt solutions, alcohol, polyethylene glycols, gelatin, lactose, amylose, magnesium stearate, talc, silicic acid, viscous paraffin, hydroxymethylcellulose, polyvinylpyrrolidone and the like.

Pharmaceutical compositions for the delivery to the respiratory tract

Another aspect of the invention provides for the delivery of iRNA agents to the respiratory tract, particularly for the treatment of cystic fibrosis. The respiratory tract includes the upper airways, including the oropharynx and larynx, followed by the lower airways, which include the trachea followed by bifurcations into the bronchi and bronchioli. The upper and lower airways are called the conductive airways. The terminal bronchioli then divide into respiratory bronchioli which then lead to the ultimate respiratory zone, the alveoli, or deep lung. The epithelium of the conductive airways is the primary target of inhaled therapeutic aerosols for delivery of iRNA agents such as alpha-ENaC iRNA agents.

Pulmonary delivery compositions can be delivered by inhalation by the patient of a dispersion so that the composition, preferably the iRNA agent, within the dispersion can reach the lung where it can, for example, be readily absorbed through the alveolar region

directly into blood circulation. Pulmonary delivery can be effective both for systemic delivery and for localized delivery to treat diseases of the lungs.

Pulmonary delivery can be achieved by different approaches, including the use of nebulized, aerosolized, micellular and dry powder-based formulations; administration by inhalation may be oral and/or nasal. Delivery can be achieved with liquid nebulizers, aerosol-based inhalers, and dry powder dispersion devices. Metered-dose devices are preferred. One of the benefits of using an atomizer or inhaler is that the potential for contamination is minimized because the devices are self contained. Dry powder dispersion devices, for example, deliver drugs that may be readily formulated as dry powders. An iRNA composition may be stably stored as lyophilized or spray-dried powders by itself or in combination with suitable powder carriers. The delivery of a composition for inhalation can be mediated by a dosing timing element which can include a timer, a dose counter, time measuring device, or a time indicator which when incorporated into the device enables dose tracking, compliance monitoring, and/or dose triggering to a patient during administration of the aerosol medicament.

Examples of pharmaceutical devices for aerosol delivery include metered dose inhalers (MDIs), dry powder inhalers (DPIs), and air-jet nebulizers. Exemplary delivery systems by inhalation which can be readily adapted for delivery of the subject iRNA agents are described in, for example, U.S. Pat. Nos. 5,756,353; 5,858,784; and PCT applications WO98/31346; WO98/10796; WO00/27359; WO01/54664; WO02/060412. Other aerosol formulations that may be used for delivering the iRNA agents are described in U.S. Pat. Nos. 6,294,153; 6,344,194; 6,071,497, and PCT applications WO02/066078; WO02/053190; WO01/60420; WO00/66206. Further, methods for delivering iRNA agents can be adapted from those used in delivering other oligonucleotides (e.g., an antisense oligonucleotide) by inhalation, such as described in Templin et al., Antisense Nucleic Acid Drug Dev, 2000, 10:359-68; Sandrasagra et al., Expert Opin Biol Ther, 2001, 1:979-83; Sandrasagra et al., Antisense Nucleic Acid Drug Dev, 2002, 12:177-81.

The delivery of the inventive agents may also involve the administration of so called "pro-drugs", i.e. formulations or chemical modifications of a therapeutic substance that require some form of processing or transport by systems innate to the subject organism to release the therapeutic substance, preferably at the site where its action is desired; this latter embodiment may be used in conjunction with delivery of the respiratory tract, but also

together with other embodiments of the present invention. For example, the human lungs can remove or rapidly degrade hydrolytically cleavable deposited aerosols over periods ranging from minutes to hours. In the upper airways, ciliated epithelia contribute to the "mucociliary excalator" by which particles are swept from the airways toward the mouth. Pavia, D., "Lung Mucociliary Clearance," in Aerosols and the Lung: Clinical and Experimental Aspects, Clarke, S. W. and Pavia, D., Eds., Butterworths, London, 1984. In the deep lungs, alveolar macrophages are capable of phagocytosing particles soon after their deposition. Warheit et al. Microscopy Res. Tech., 26: 412-422 (1993); and Brain, J. D., "Physiology and Pathophysiology of Pulmonary Macrophages," in The Reticuloendothelial System, S. M. Reichard and J. Filkins, Eds., Plenum, New. York., pp. 315-327, 1985.

In preferred embodiments, particularly where systemic dosing with the iRNA agent is desired, the aerosoled iRNA agents are formulated as microparticles. Microparticles having a diameter of between 0.5 and ten microns can penetrate the lungs, passing through most of the natural barriers. A diameter of less than ten microns is required to bypass the throat; a diameter of 0.5 microns or greater is required to avoid being exhaled.

Other Components

Compositions of the present invention may additionally contain other adjunct components conventionally found in pharmaceutical compositions, at their art-established usage levels. Thus, for example, the compositions may contain additional, compatible, pharmaceutically-active materials such as, for example, antipruritics, astringents, local anesthetics or anti-inflammatory agents, or may contain additional materials useful in physically formulating various dosage forms of the compositions of the present invention, such as dyes, flavoring agents, preservatives, antioxidants, opacifiers, thickening agents and stabilizers. However, such materials, when added, should not unduly interfere with the biological activities of the components of the compositions of the present invention. The formulations can be sterilized and, if desired, mixed with auxiliary agents, e.g., lubricants, preservatives, stabilizers, wetting agents, emulsifiers, salts for influencing osmotic pressure, buffers, colorings, flavorings and/or aromatic substances and the like which do not deleteriously interact with the nucleic acid(s) of the formulation.

Aqueous suspensions may contain substances which increase the viscosity of the suspension including, for example, sodium carboxymethylcellulose, sorbitol and/or dextran. The suspension may also contain stabilizers.

Certain embodiments of the invention provide pharmaceutical combinations and compositions containing (a) one or more dsRNA agents and (b) one or more other therapeutic agents which function by a non-RNA interference mechanism.

Accordingly, the invention includes a combination of an iRNA of the present invention with an anti-inflammatory, bronchodilatory, antihistamine, anti-tussive, antibiotic or DNase drug substance, said epithelial sodium channel blocker and said drug substance being in the same or different pharmaceutical composition.

Suitable antibiotics include macrolide antibiotics, e.g., tobramycin (TOBI™).

Suitable DNase drug substances include dornase alfa (Pulmozyme™), a highly-purified solution of recombinant human deoxyribonuclease I (rhDNase), which selectively cleaves DNA. Dornase alfa is used to treat cystic fibrosis.

Other useful combinations of epithelial sodium channel blockers with anti-inflammatory drugs are those with antagonists of chemokine receptors, e.g., CCR-1, CCR-2, CCR-3, CCR-4, CCR-5, CCR-6, CCR-7, CCR-8, CCR-9 and CCR10, CXCR1, CXCR2, CXCR3, CXCR4, CXCR5, particularly CCR-5 antagonists, such as Schering-Plough antagonists SC-351125, SCH-55700 and SCH-D; Takeda antagonists, such as *N*-[[4-[[[6,7-dihydro-2-(4-methyl-phenyl)-5*H*-benzo-cyclohepten-8-yl]carbonyl]amino]phenyl]-methyl]tetrahydro-*N,N*-dimethyl-2*H*-pyran-4-amin-ium chloride (TAK-770); and CCR-5 antagonists described in USP 6,166,037 (particularly claims 18 and 19), WO 00/66558 (particularly claim 8), WO 00/66559 (particularly claim 9), WO 04/018425 and WO 04/026873.

Suitable anti-inflammatory drugs include steroids, in particular, glucocorticosteroids, such as budesonide, beclamethasone dipropionate, fluticasone propionate, ciclesonide or mometasone furoate, or steroids described in WO 02/88167, WO 02/12266, WO 02/100879, WO 02/00679 (especially those of Examples 3, 11, 14, 17, 19, 26, 34, 37, 39, 51, 60, 67, 72, 73, 90, 99 and 101), WO 03/35668, WO 03/48181, WO 03/62259, WO 03/64445, WO 03/72592, WO 04/39827 and WO 04/66920; non-steroidal glucocorticoid receptor

agonists, such as those described in DE 10261874, WO 00/00531, WO 02/10143, WO 03/82280, WO 03/82787, WO 03/86294, WO 03/104195, WO 03/101932, WO 04/05229, WO 04/18429, WO 04/19935 and WO 04/26248; LTD4 antagonists, such as montelukast and zafirlukast; PDE4 inhibitors, such as cilomilast (Ariflo® GlaxoSmithKline), Roflumilast (Byk Gulden),V-11294A (Napp), BAY19-8004 (Bayer), SCH-351591 (Schering-Plough), Arofylline (Almirall Prodesfarma), PD189659 / PD168787 (Parke-Davis), AWD-12-281 (Asta Medica), CDC-801 (Celgene), SelCID(TM) CC-10004 (Celgene), VM554/UM565 (Vernalis), T-440 (Tanabe), KW-4490 (Kyowa Hakko Kogyo), and those disclosed in WO 92/19594, WO 93/19749, WO 93/19750, WO 93/19751, WO 98/18796, WO 99/16766, WO 01/13953, WO 03/104204, WO 03/104205, WO 03/39544, WO 04/000814, WO 04/000839, WO 04/005258, WO 04/018450, WO 04/018451, WO 04/018457, WO 04/018465, WO 04/018431, WO 04/018449, WO 04/018450, WO 04/018451, WO 04/018457, WO 04/018465, WO 04/019944, WO 04/019945, WO 04/045607 and WO 04/037805; adenosine A2B receptor antagonists such as those described in WO 02/42298; and beta-2 adrenoceptor agonists, such as albuterol (salbutamol), metaproterenol, terbutaline, salmeterol fenoterol, procaterol, and especially, formoterol, carmoterol and pharmaceutically acceptable salts thereof, and compounds (in free or salt or solvate form) of formula (I) of WO 0075114, which document is incorporated herein by reference, preferably compounds of the Examples thereof, especially indacaterol and pharmaceutically acceptable salts thereof, as well as compounds (in free or salt or solvate form) of formula (I) of WO 04/16601, and also compounds of EP 1440966, JP 05025045, WO 93/18007, WO 99/64035, USP 2002/0055651, WO 01/42193, WO 01/83462, WO 02/66422, WO 02/70490, WO 02/76933, WO 03/24439, WO 03/42160, WO 03/42164, WO 03/72539, WO 03/91204, WO 03/99764, WO 04/16578, WO 04/22547, WO 04/32921, WO 04/33412, WO 04/37768, WO 04/37773, WO 04/37807, WO 04/39762, WO 04/39766, WO 04/45618, WO 04/46083, WO 04/80964, WO 04/108765 and WO 04/108676.

Suitable bronchodilatory drugs include anticholinergic or antimuscarinic agents, in particular, ipratropium bromide, oxitropium bromide, tiotropium salts and CHF 4226 (Chiesi), and glycopyrrolate, but also those described in EP 424021, USP 3,714,357, USP 5,171,744, WO 01/04118, WO 02/00652, WO 02/51841, WO 02/53564, WO 03/00840, WO 03/33495, WO 03/53966, WO 03/87094, WO 04/018422 and WO 04/05285.

Suitable dual anti-inflammatory and bronchodilatory drugs include dual beta-2 adrenoceptor agonist/muscarinic antagonists such as those disclosed in USP 2004/0167167, WO 04/74246 and WO 04/74812.

Suitable antihistamine drug substances include cetirizine hydrochloride, acetaminophen, clemastine fumarate, promethazine, loratidine, desloratidine, diphenhydramine and fexofenadine hydrochloride, activastine, astemizole, azelastine, ebastine, epinastine, mizolastine and tefenadine, as well as those disclosed in JP 2004107299, WO 03/099807 and WO 04/026841.

Other useful combinations of agents of the invention with anti-inflammatory drugs are those with antagonists of chemokine receptors, e.g., CCR-1, CCR-2, CCR-3, CCR-4, CCR-5, CCR-6, CCR-7, CCR-8, CCR-9 and CCR10, CXCR1, CXCR2, CXCR3, CXCR4, CXCR5, particularly CCR-5 antagonists, such as Schering-Plough antagonists SC-351125, SCH-55700 and SCH-D; Takeda antagonists, such as *N*-[[4-[[[6,7-dihydro-2-(4-methylphenyl)-5*H*-benzo-cyclohepten-8-yl]carbonyl]amino]phenyl]-methyl]tetrahydro-*N,N*-dimethyl-2*H*-pyran-4-amin-ium chloride (TAK-770), and CCR-5 antagonists described in USP 6,166,037 (particularly claims 18 and 19), WO 00/66558 (particularly claim 8), WO 00/66559 (particularly claim 9), WO 04/018425 and WO 04/026873.

Other useful additional therapeutic agents may also be selected from the group consisting of cytokine binding molecules, particularly antibodies of other cytokines, in particular a combination with an anti-IL4 antibody, such as described in PCT/EP2005/00836, an anti-IgE antibody, such as Xolair®, an anti-IL31 antibody, an anti-IL31R antibody, an anti-TSLP antibody, an anti-TSLP receptor antibody, an anti-endoglin antibody, an anti-IL1b antibody or an anti-IL13 antibody, such as described in WO05/007699.

Two or more combined compounds may be used together in a single formulation, separately, concomitantly or sequentially.

Toxicity and therapeutic efficacy of such compounds can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, e.g., for determining the LD50 (the dose lethal to 50% of the population) and the ED50 (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD50/ED50. Compounds which exhibit high therapeutic indices are preferred.

The data obtained from cell culture assays and animal studies can be used in formulating a range of dosage for use in humans. The dosage of compositions of the invention lies generally within a range of circulating concentrations that include the ED50 with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. For any compound used in the method of the invention, the therapeutically effective dose can be estimated initially from cell culture assays. A dose may be formulated in animal models to achieve a circulating plasma concentration range of the compound or, when appropriate, of the polypeptide product of a target sequence (e.g., achieving a decreased concentration of the polypeptide) that includes the IC50 (i.e., the concentration of the test compound which achieves a half-maximal inhibition of symptoms) as determined in cell culture. Such information can be used to more accurately determine useful doses in humans. Levels in plasma may be measured, for example, by high performance liquid chromatography.

In addition to their administration individually or as a plurality, as discussed above, the dsRNAs of the invention can be administered in combination with other known agents effective in treatment of ENaC related disorders. In any event, the administering physician can adjust the amount and timing of dsRNA administration on the basis of results observed using standard measures of efficacy known in the art or described herein.

Treatment Methods and Routes of Delivery

A composition that includes an iRNA agent, *e.g.*, an iRNA agent that targets alpha-ENaC, can be delivered to a subject by a variety of routes to achieve either local delivery to the site of action or systemic delivery to the subject. Exemplary routes include direct local administration to the site of treatment, such as the lungs and nasal passage as well as intravenous, nasal, oral, and ocular delivery. The preferred means of administering the iRNA agents of the present invention is through direct admisitration to the lungs and nasal passage as a liquid, aerosol or nebulized solution.

Formulations for inhalation or parenteral administration are well known in the art. Such formulation may include sterile aqueous solutions which may also contain buffers, diluents and other suitable additives. For intravenous use, the total concentration of solutes should be controlled to render the preparation isotonic.

The active compounds disclosed herein are preferably administered to the lung(s) or nasal passage of a subject by any suitable means. Active compounds may be administered by administering an aerosol suspension of respirable particles comprised of the active compound or active compounds, which the subject inhales. The active compound can be aerosolized in a variety of forms, such as, but not limited to, dry powder inhalants, metered dose inhalants, or liquid/liquid suspensions. The respirable particles may be liquid or solid. The particles may optionally contain other therapeutic ingredients such as amiloride, benzamil or phenamil, with the selected compound included in an amount effective to inhibit the reabsorption of water from airway mucous secretions, as described in U.S. Pat. No. 4,501,729.

The particulate pharmaceutical composition may optionally be combined with a carrier to aid in dispersion or transport. A suitable carrier such as a sugar (i.e., lactose, sucrose, trehalose, mannitol) may be blended with the active compound or compounds in any suitable ratio (e.g., a 1 to 1 ratio by weight).

Particles comprised of the active compound for practicing the present invention should include particles of respirable size, that is, particles of a size sufficiently small to pass through the mouth or nose and larynx upon inhalation and into the bronchi and alveoli of the lungs. In general, particles ranging from about 1 to 10 microns in size (more particularly, less than about 5 microns in size) are respirable. Particles of non-respirable size which are included in the aerosol tend to deposit in the throat and be swallowed, and the quantity of non-respirable particles in the aerosol is preferably minimized. For nasal administration, a particle size in the range of 10-500 uM is preferred to ensure retention in the nasal cavity.

Liquid pharmaceutical compositions of active compound for producing an aerosol may be prepared by combining the active compound with a suitable vehicle, such as sterile pyrogen free water. The hypertonic saline solutions used to carry out the present invention are preferably sterile, pyrogen-free solutions, comprising from one to fifteen percent (by weight) of the physiologically acceptable salt, and more preferably from three to seven percent by weight of the physiologically acceptable salt.

Aerosols of liquid particles comprising the active compound may be produced by any suitable means, such as with a pressure-driven jet nebulizer or an ultrasonic nebulizer. See, e.g., U.S. Pat. No. 4,501,729. Nebulizers are commercially available devices which transform solutions or suspensions of the active ingredient into a therapeutic aerosol mist either by

means of acceleration of compressed gas, typically air or oxygen, through a narrow venturi orifice or by means of ultrasonic agitation.

Suitable formulations for use in nebulizers consist of the active ingredient in a liquid carrier, the active ingredient comprising up to 40% w/w of the formulation, but preferably less than 20% w/w. The carrier is typically water (and most preferably sterile, pyrogen-free water) or a dilute aqueous alcoholic solution, preferably made isotonic, but may be hypertonic with body fluids by the addition of, for example, sodium chloride. Optional additives include preservatives if the formulation is not made sterile, for example, methyl hydroxybenzoate, antioxidants, flavoring agents, volatile oils, buffering agents and surfactants.

Aerosols of solid particles comprising the active compound may likewise be produced with any solid particulate therapeutic aerosol generator. Aerosol generators for administering solid particulate therapeutics to a subject produce particles which are respirable and generate a volume of aerosol containing a predetermined metered dose of a therapeutic at a rate suitable for human administration. One illustrative type of solid particulate aerosol generator is an insufflator. Suitable formulations for administration by insufflation include finely comminuted powders which may be delivered by means of an insufflator or taken into the nasal cavity in the manner of a snuff. In the insufflator, the powder (e.g., a metered dose thereof effective to carry out the treatments described herein) is contained in capsules or cartridges, typically made of gelatin or plastic, which are either pierced or opened in situ and the powder delivered by air drawn through the device upon inhalation or by means of a manually-operated pump. The powder employed in the insufflator consists either solely of the active ingredient or of a powder blend comprising the active ingredient, a suitable powder diluent, such as lactose, and an optional surfactant. The active ingredient typically comprises from 0.1 to 100 w/w of the formulation.

A second type of illustrative aerosol generator comprises a metered dose inhaler. Metered dose inhalers are pressurized aerosol dispensers, typically containing a suspension or solution formulation of the active ingredient in a liquefied propellant. During use these devices discharge the formulation through a valve adapted to deliver a metered volume, typically from 10 to 200 ul, to produce a fine particle spray containing the active ingredient. Suitable propellants include certain chlorofluorocarbon compounds, for example, dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane and mixtures

thereof. The formulation may additionally contain one or more co-solvents, for example, ethanol, surfactants, such as oleic acid or sorbitan trioleate, antioxidant and suitable flavoring agents.

An iRNA agent can be incorporated into pharmaceutical compositions suitable for administration. For example, compositions can include one or more species of an iRNA agent and a pharmaceutically acceptable carrier. As used herein the language "pharmaceutically acceptable carrier" is intended to include any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like, compatible with pharmaceutical administration. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active compound, use thereof in the compositions is contemplated. Supplementary active compounds can also be incorporated into the compositions.

Administration can be provided by the subject or by another person, *e.g.*, a caregiver. A caregiver can be any entity involved with providing care to the human: for example, a hospital, hospice, doctor's office, outpatient clinic; a healthcare worker such as a doctor, nurse, or other practitioner; or a spouse or guardian, such as a parent. The medication can be provided in measured doses or in a dispenser which delivers a metered dose.

The term "therapeutically effective amount" is the amount present in the composition that is needed to provide the desired level of drug in the subject to be treated to give the anticipated physiological response.

The term "physiologically effective amount" is that amount delivered to a subject to give the desired palliative or curative effect.

The term "pharmaceutically acceptable carrier" means that the carrier can be taken into the lungs with no significant adverse toxicological effects on the lungs.

The term "co-administration" refers to administering to a subject two or more agents, and in particular two or more iRNA agents. The agents can be contained in a single pharmaceutical composition and be administered at the same time, or the agents can be contained in separate formulation and administered serially to a subject. So long as the two

agents can be detected in the subject at the same time, the two agents are said to be co-administered.

The types of pharmaceutical excipients that are useful as carrier include stabilizers such as human serum albumin (HSA), bulking agents such as carbohydrates, amino acids and polypeptides; pH adjusters or buffers; salts such as sodium chloride; and the like. These carriers may be in a crystalline or amorphous form or may be a mixture of the two.

Bulking agents that are particularly valuable include compatible carbohydrates, polypeptides, amino acids or combinations thereof. Suitable carbohydrates include monosaccharides such as galactose, D-mannose, sorbose, and the like; disaccharides, such as lactose, trehalose, and the like; cyclodextrins, such as 2-hydroxypropyl-.beta.-cyclodextrin; and polysaccharides, such as raffinose, maltodextrins, dextrans, and the like; alditols, such as mannitol, xylitol, and the like. A preferred group of carbohydrates includes lactose, threhalose, raffinose maltodextrins, and mannitol. Suitable polypeptides include aspartame. Amino acids include alanine and glycine, with glycine being preferred.

Suitable pH adjusters or buffers include organic salts prepared from organic acids and bases, such as sodium citrate, sodium ascorbate, and the like; sodium citrate is preferred.

Dosage

An iRNA agent can be administered at a unit dose less than about 75mg per kg of bodyweight, or less than about 70, 60, 50, 40, 30, 20, 10, 5, 2, 1, 0.5, 0.1, 0.05, 0.01, 0.005, 0.001, or 0.0005 mg per kg of bodyweight, and less than 200 nmol of iRNA agent (*e.g.*, about 4.4×10^{16} copies) per kg of bodyweight, or less than 1500, 750, 300, 150, 75, 15, 7.5, 1.5, 0.75, 0.15, 0.075, 0.015, 0.0075, 0.0015, 0.00075, 0.00015 nmol of iRNA agent per kg of bodyweight. The unit dose, for example, can be administered by injection (*e.g.*, intravenous or intramuscular, intrathecally, or directly into an organ), an inhaled dose, or a topical application.

The dosage can be an amount effective to treat or prevent a disease or disorder. It can be given prophylactically or as the primary or a part of a treatment protocol.

In one embodiment, the unit dose is administered less frequently than once a day, *e.g.*, less than every 2, 4, 8 or 30 days. In another embodiment, the unit dose is not administered

with a frequency (*e.g.*, not a regular frequency). For example, the unit dose may be administered a single time. Because iRNA agent mediated silencing can persist for several days after administering the iRNA agent composition, in many instances, it is possible to administer the composition with a frequency of less than once per day, or, for some instances, only once for the entire therapeutic regimen.

In one embodiment, a subject is administered an initial dose, and one or more maintenance doses of an iRNA agent, *e.g.*, a double-stranded iRNA agent, or siRNA agent, (*e.g.*, a precursor, *e.g.*, a larger iRNA agent which can be processed into an siRNA agent, or a DNA which encodes an iRNA agent, *e.g.*, a double-stranded iRNA agent, or siRNA agent, or precursor thereof). The maintenance dose or doses are generally lower than the initial dose, *e.g.*, one-half less of the initial dose. A maintenance regimen can include treating the subject with a dose or doses ranging from 0.01 to 75 mg/kg of body weight per day, *e.g.*, 70, 60, 50, 40, 30, 20, 10, 5, 2, 1, 0.5, 0.1, 0.05, 0.01, 0.005, 0.001, or 0.0005 mg per kg of body weight per day. The maintenance doses are preferably administered no more than once every 5, 10, or 30 days. Further, the treatment regimen may last for a period of time which will vary depending upon the nature of the particular disease, its severity and the overall condition of the patient. In preferred embodiments the dosage may be delivered no more than once per day, *e.g.*, no more than once per 24, 36, 48, or more hours, *e.g.*, no more than once every 5 or 8 days. Following treatment, the patient can be monitored for changes in his condition and for alleviation of the symptoms of the disease state. The dosage of the compound may either be increased in the event the patient does not respond significantly to current dosage levels, or the dose may be decreased if an alleviation of the symptoms of the disease state is observed, if the disease state has been ablated, or if undesired side-effects are observed.

The effective dose can be administered in a single dose or in two or more doses, as desired or considered appropriate under the specific circumstances. If desired to facilitate repeated or frequent infusions, implantation of a delivery device, *e.g.*, a pump, semi-permanent stent (*e.g.*, intravenous, intraperitoneal, intracisternal or intracapsular), or reservoir may be advisable.

Following successful treatment, it may be desirable to have the patient undergo maintenance therapy to prevent the recurrence of the disease state, wherein the compound of the invention is administered in maintenance doses, ranging from 0.001 g to 100 g per kg of body weight (see US 6,107,094).

The concentration of the iRNA agent composition is an amount sufficient to be effective in treating or preventing a disorder or to regulate a physiological condition in humans. The concentration or amount of iRNA agent administered will depend on the parameters determined for the agent and the method of administration, *e.g.* nasal, buccal, or pulmonary. For example, nasal formulations tend to require much lower concentrations of some ingredients in order to avoid irritation or burning of the nasal passages. It is sometimes desirable to dilute an oral formulation up to 10-100 times in order to provide a suitable nasal formulation.

Certain factors may influence the dosage required to effectively treat a subject, including but not limited to the severity of the disease or disorder, previous treatments, the general health and/or age of the subject, and other diseases present. It will also be appreciated that the effective dosage of an iRNA agent such as an siRNA used for treatment may increase or decrease over the course of a particular treatment. Changes in dosage may result and become apparent from the results of diagnostic assays. For example, the subject can be monitored after administering an iRNA agent composition. Based on information from the monitoring, an additional amount of the iRNA agent composition can be administered.

Dosing is dependent on severity and responsiveness of the disease condition to be treated, with the course of treatment lasting from several days to several months, or until a cure is effected or a diminution of disease state is achieved. Optimal dosing schedules can be calculated from measurements of drug accumulation in the body of the patient. Persons of ordinary skill can easily determine optimum dosages, dosing methodologies and repetition rates. Optimum dosages may vary depending on the relative potency of individual compounds, and can generally be estimated based on EC50s found to be effective in *in vitro* and *in vivo* animal models as described above.

iRNA agents of the present invention as described herein may be useful in the treatment and (where appropriate) in the prevention of any one of the following diseases/disorders;

Cystic fibrosis, Liddles syndrome, renal insufficiency, hypertension, electrolyte imbalances.

In particular in some embodiments, iRNA agents of the invention may be used to treat and/or prevent adverse clinical manifestations of these diseases/disorders.

The invention is further illustrated by the following examples, which should not be construed as further limiting.

EXAMPLES

Source of reagents

Where the source of a reagent is not specifically given herein, such reagent may be obtained from any supplier of reagents for molecular biology at a quality/purity standard for application in molecular biology.

Example 1: Selection of sequences

In order to identify therapeutic siRNAs to downmodulate expression of the alpha subunit of the epithelial sodium channel ENaC (α-ENaC), screening sets were defined based on a bioinformatic analysis. The key drivers for the design of the screening set were predicted specificity of the siRNAs against the transcriptome of the relevant species. For the identification of alpha-ENaC siRNAs and an efficient delivery system a three pronged approach was used: Rat was selected as the test species to address silencing efficacy *in vivo* after intratracheal delivery, guinea pig was selected as the disease model organism to demonstrate that alpha-ENaC mRNA reduction results in a measurable functional effect. The therapeutic siRNA molecule has to target human alpha-ENaC as well as the alpha-ENaC sequence of at least one toxicology-relevant species, in this case, rhesus monkey.

Initial analysis of the relevant alpha-ENaC mRNA sequence revealed few sequences can be identified that fulfil the specificity requirements and at the same time target alpha-ENaC mRNA in all relevant species. Therefore it was decided to design independent screening sets for the therapeutic siRNA and for the surrogate molecules to be tested in the relevant disease model (Tables 1A,1B,1C and 1D).

All siRNAs recognize the human alpha-ENaC sequence, as a human cell culture system was selected for determination of *in vitro* activity (H441, see below). Therefore all siRNAs can be used to target human alpha-ENaC mRNA in a therapeutic setting.

The therapeutic screening sets were designed to contain only siRNA sequences that are fully complementary to the human and rhesus monkey alpha-ENaC sequences.

Design and *in silico* selection of siRNAs targeting alpha-ENaC (SCNN1A)

siRNA design was carried out to identify siRNAs for the four previously defined sets (see above)

a) "Initial screening set"
b) "Extended screening set"
c) "*In vivo* surrogate set for rat"
d) "*In vivo* surrogate set for guinea pig"

Initial screening set

The aim for *in silico* selection of an initial screening set was to identify siRNAs specifically targeting human alpha-ENaC, as well as its rhesus monkey ortholog. The human target mRNA (NM_001038.4) was downloaded from NCBI resource (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?CMD=search&DB=nucleotide) during the complete siRNA selection procedure. In order to identify the alpha-ENaC rhesus (*Macaca mulatta*) ortholog, the human sequence was used in a blastn search at Baylor College of Medicine (http://www.hgsc.bcm.tmc.edu/blast/?organism=Mmulatta) against Mmulatta contigs as of 2004 10 01. All hit regions were extracted and assembled by the CAP assembly tool to generate a first assembly sequence. Further, a BLAST search was performed with the human sequence at UCSC (http://genome.ucsc.edu/cgi-bin/hgBlat?command=start&org=Rhesus&db=rheMac2&hgsid=84859356) against Rhesus freeze 12 March 2005. The scaffold hit 84554 was downloaded and used together with the first assembly sequence by CAP to generate the final consensus sequence for rhesus alpha-ENaC.

Following extraction of all overlapping 19mer sequences out of the human mRNA, conserved 19mers were identified that had identical sequences in the assembled rhesus consensus sequence. Those 19mer sequences were defined as the pool of human-rhesus cross-reactive siRNA (sense) sequences, represented by 1185 19mers.

The corresponding antisense sequences were generated and tested for specificity in human. For this, their predicted potential for interacting with irrelevant target mRNAs (off-

target potential) was taken as parameter. Sequences with low off-target potential were defined as preferable and predicted to be more specific.

For further selection, candidate siRNAs were ranked according to their predicted potential for interacting with other host sequences (here, without limitation, human) . siRNAs with low off-target potential are assumed to be more specific *in vivo*. For predicting siRNA-specific off-target potential, the following assumptions were made:

1) off-target potential of a strand can be deduced from the number and distribution of mismatches to an off-target
2) the most relevant off-target, that is the gene predicted to have the highest probability to be silenced due to tolerance of mismatches, determines the off-target potential of the strand
3) positions 2 to 9 (counting 5' to 3') of a strand (seed region) may contribute more to off-target potential than rest of sequence (that is non-seed and cleavage site region) (Haley, B., and Zamore, P.D., Nat Struct Mol Biol. 2004, 11:599).
4) positions 10 and 11 (counting 5' to 3') of a strand (cleavage site region) may contribute more to off-target potential than non-seed region (that is positions 12 to 18, counting 5' to 3')
5) positions 1 and 19 of each strand are not relevant for off-target interactions
6) off-target potential can be expressed by the off-target score of the most relevant off-target, calculated based on number and position of mismatches of the strand to the most homologous region in the off-target gene considering assumptions 3 to 5
7) assuming potential abortion of sense strand activity by internal modifications introduced, only off-target potential of antisense strand will be relevant

To identify potential off-target genes, 19mer antisense sequences were subjected to a homology search against publicly available human mRNA sequences, assumed to represent the human comprehensive transcriptome.

To this purpose, fastA (version 3.4) searches were performed with all 19mer sequences against a human RefSeq database (available version from ftp://ftp.ncbi.nih.gov/refseq/ on Nov., 18 2005). FastA search was executed with parameters-values-pairs –f 30 -g 30 in order to take into account the homology over the full length of the 19mer without any gaps. In addition, in order to ensure the listing of all relevant off-target hits in the fastA output file the parameter –E 15000 was used.

The search resulted in a list of potential off-targets for each input sequence listed by descending sequence homology over the complete 19mer.

To rank all potential off-targets according to assumptions 3 to 5, and by this identify the most relevant off-target gene and its off-target score, fastA output files were analyzed by a perl script.

The script extracted the following off-target properties for each 19mer input sequence and each off-target gene to calculate the off-target score:

- Number of mismatches in non-seed region
- Number of mismatches in seed region
- Number of mismatches in cleavage site region

The off-target score was calculated by considering assumptions 3 to 5 as follows:

Off-target score	=	number of seed mismatches * 10
	+	number of cleavage site mismatches * 1.2
	+	number of non-seed mismatches * 1

The most relevant off-target gene for each 19mer sequence was defined as the gene with the lowest off-target score. Accordingly, the lowest off-target score was defined as representative for the off-target potential of each siRNA, represented by the 19mer antisense sequence analyzed.

Calculated off-target potential was used as sorting parameter (descending by off-target score) in order to generate a ranking for all human-rhesus cross-reactive siRNA sequences.

An off-target score of 3 or more was defined as prerequisite for siRNA selection, whereas all sequences containing 4 or more G's in a row (poly-G sequences) were excluded, leading to selection of a total of 152 siRNAs targeting human and rhesus ENaC alpha (see Table 1a).

Extended screening set

The aim for *in silico* selection of the extended screening set was to identify all further siRNAs targeting human alpha-ENaC with sufficient specificity, that were excluded from the initial set due to missing cross-reactivity to rhesus. The remaining sequences from the pool of 19mers derived from human alpha-ENaC that have not been analyzed before were taken and

the corresponding antisense sequences were generated. The most relevant off-target gene and its corresponding off-target scores were calculated as described in section "Initial screening set".

For determining cross-reactivity to mouse and guinea pig (*Cavia porcellus*/cobya), alpha-ENaC sequences of these species were downloaded from NCBI nucleotide database [1] (accession numbers NM_011324.1 and AF071230 (full length)/DQ109811 (partial cds), respectively). The two guinea pig sequences were used to generate aguinea pig alpha-ENaC consensus sequence. Every human 19mer sequence was tested for presence in the mouse and guinea pig sequences. Positive sequences were assigned to the pool of human-mouse cross-reactive siRNA (sense) sequences, or human-guinea pig cross-reactive siRNA (sense) sequences. After exclusion of all poly-G sequences, sequences were selected with off-target scores of 3 or more as well as those with off-target scores of 2.2 or 2.4 and cross-reactivity to mouse, rhesus or guinea pig. The total number of siRNAs in the extended screening pool was 344 (see Table 1b).

In vivo rat surrogate set

The aim for *in silico* selection of the *in vivo* rat surrogate set was to identify all siRNAs targeting human and rat alpha-ENaC with sufficient specificity in rat. For identification of human-rat cross-reactive siRNAs, rat alpha-ENaC mRNA sequence was downloaded from NCBI nucleotide database (accession number, NM_031548.2), and all sequences out of the pool of human 19mers were tested for presence in the rat sequence, representing the pool of human-rat cross-reactive siRNA (sense) sequences.

The corresponding antisense sequences were generated and tested for specificity in rat. For this, the most relevant off-target gene in rat and its corresponding off-target scores were calculated as described in section "Initial screening set" using the rat mRNA set (RefSeq database) instead of the human transcripts. After exclusion of all poly-G sequences, a ranking was generated considering the rat off-target score in first priority and the human off-target score with second priority. Those 48 sequences from the top of the list were finally selected representing the *in vivo* rat surrogate set (see Table 1c).

In vivo guinea pig surrogate set

The aim for *in silico* selection of the *in vivo* guinea pig surrogate set was to identify all siRNAs targeting human and guinea pig alpha-ENaC that have not been selected in previous sets. The remaining siRNAs of the previously determined set of human-guinea pig cross-reactive siRNA (sense) sequences were ranked according to human off-target scores. The top 63 sequences (excluding poly-G sequences) were selected, representing the *in vivo* guinea pig surrogate set (see Table 1d).

Example 2: siRNA synthesis

Synthesis of nucleotides comprising natural bases

As the siRNAs from the screening sets are all potentially intended for *in vivo* administration, siRNAs were synthesised with a modification strategy that protects the siRNAs from degradation by endo- and exonucleases in a biological environment. In this strategy, the 3'-ends of both strands are protected from a 3'-> 5'-exonucleotitic activity by a phosphorothioate linkage between the two last nucleobases at the 3'-end. In order to inhibit endo-nucleolytic degradation of the siRNA all pyrimidines in the sense strand of the siRNA were replaced with the corresponding 2'-*O*-methyl-modified ribonucleotide. To reduce the number of modifications in the antisense strand, which is the more active strand and therefore more sensitive to modifications, we only modified the pyrimidines in the context of previously identified major nuclease cleavage sites with 2'-*O*-methyl groups. The major cleavage sites are the following two sequence motifs: 5'-UA-3' and 5'-CA-3'.

Since it has also been considered to use siRNAs in formulations that potentially protect the RNAs from the nucleolytic biological environment in the lung, the same set of siRNAs were also synthesized without any protection from endonucleolytic degradation.

Where the source of a reagent is not specifically given herein, such reagent may be obtained from any supplier of reagents for molecular biology at a quality/purity standard for application in molecular biology.

Single-stranded RNAs were produced by solid phase synthesis on a scale of 1 µmole using an Expedite 8909 synthesizer (Applied Biosystems, Applera Deutschland GmbH, Darmstadt, Germany) and controlled pore glass (CPG, 500Å, Proligo Biochemie GmbH, Hamburg, Germany) as solid support. RNA and RNA containing 2'-O-methyl nucleotides were generated by solid phase synthesis employing the corresponding phosphoramidites and

2'-O-methyl phosphoramidites, respectively (Proligo Biochemie GmbH, Hamburg, Germany). These building blocks were incorporated at selected sites within the sequence of the oligoribonucleotide chain using standard nucleoside phosphoramidite chemistry such as described in Current protocols in nucleic acid chemistry, Beaucage, S.L. et al. (Edrs.), John Wiley & Sons, Inc., New York, NY, USA. Phosphorothioate linkages were introduced by replacement of the iodine oxidizer solution with a solution of the Beaucage reagent (Chruachem Ltd, Glasgow, UK) in acetonitrile (1%). Further ancillary reagents were obtained from Mallinckrodt Baker (Griesheim, Germany).

Deprotection and purification of the crude oligoribonucleotides by anion exchange HPLC were carried out according to established procedures. Yields and concentrations were determined by UV absorption of a solution of the respective RNA at a wavelength of 260 nm using a spectral photometer (DU 640B, Beckman Coulter GmbH, Unterschleißheim, Germany). Double stranded RNA was generated by mixing an equimolar solution of complementary strands in annealing buffer (20 mM sodium phosphate, pH 6.8; 100 mM sodium chloride), heated in a water bath at 85 - 90°C for 3 minutes and cooled to room temperature over a period of 3 - 4 hours. The annealed RNA solution was diluted to a concentration of 50 µmole double stranded RNA/L and stored at –20 °C until use.

Example 3: siRNA testing *in vitro*

The ability of the iRNA agents to inhibit expression of alpha-ENaC was tested in human cell lines *in vitro*, or in rats *in vivo*. The iRNA agent is transfected into the cells, e.g., by transfection , allowed to act on the cells for a certain time, e.g., 24 hours, and levels of alpha-ENaC mRNA were determined by branched-DNA analysis. Alternatively, the iRNA agent is administered *in vivo* via the intratracheal route and the inhibition of alpha-ENaC mRNA expresion determined by branched-DNA analysis on the target organ.
Complementing these direct assays, we tested the inhibition of target gene expression by RNAi agents for alpha-ENaC mRNA recombinantly expressed in mammalian host cells.

Cell lines

H441 cells were obtained from the American Type Culture Collection (ATCC-Number: HTB-174, LCG Promochem GmbH, Wesel, Germany) and were grown in RPMI 1640, 10% fetal calf serum, 100u penicillin / 100 µg/mL streptomycin, 2 mM L-glutamine,

10 nM Hepes and 1 mM Sodium-Pyruvate (all from Biochrom AG, Berlin, Germany) at 37°C under a 5% CO_2/95% air atmosphere.

Primary human bronchial epithelial cells were obtained from Cambrex (Cat # CC-2540) and were routinely grown in BEGM media with singlequots (Cambrex Cat # CC-3170 minus tri-iodothreonine). For polarisation and growth at air liquid interface the cells were grown in a 1:1 mixture of BEGM:DMEM supplemented with 4.5 g/L D-Glucose (Gibco BRL Cat # 41965-039) and supplemented with singlequots (Cambrex Cat # CC-4175), as above but minus the tri-iodothreonine and GA1000 aliquots and in the presence of 50µg/mL Gentamycin (Gibco Brl Cat # 10131-015). As cells were maintained in serum-free media, trypsin neutralisation solution was used during passaging steps (Cambrex Cat # CC-5002). For polarisation and culture at air-liquid interface the cells were grown on semipermeable (0.4 micron) polycarbonate supports (Corning Costar Cat # 3407 #3460) and cultured throughout at 37°C under a 5% CO_2/95% air atmosphere.

Cos-1 African green monkey kidney cells (ATCC # CRL-1650) were grown in Dulbecco's MEM, 4.5 g/L glucose, 10% fetal bovine serum, 2 mM L-glutamine, 1.5 g/L sodium bicarbonate (Gibco BRL), 100u penicillin / 100 µg/mL streptomycin.

Example 3.1: *In vitro* screen for active alpha-ENaC siRNAs and IC50 determination in H441

One day prior to transfection, ENaC-alpha expression was induced in H441 cells (ATCC-Number: HTB-174, LCG Promochem GmbH, Wesel, Germany) by adding 100 nM of dexamthasone. Directly before transfection, cells were seeded at 1.5 x 10^4 cells / well on 96-well plates (Greiner Bio-One GmbH, Frickenhausen, Germany) in 75 µL of growth medium (RPMI 1640, 10% fetal calf serum, 100u penicillin / 100 µg/ml streptomycin, 2 mM L-glutamine, 10 nM Hepes and 1 mM Sodium-Pyruvate, all from Biochrom AG, Berlin, Germany). Transfections were performed in quadruplicates. For each well 0.5 µL Lipofectamine2000 (Invitrogen GmbH, Karlsruhe, Germany) were mixed with 12 µL Opti-MEM (Invitrogen) and incubated for 15 min at room temperature. For the siRNA concentration being 50 nM in the 100 µL transfection volume, 1 µL of a 5 µM siRNA were mixed with 11.5 µL Opti-MEM per well, combined with the Lipofectamine2000-Opti-MEM mixture and again incubated for 15 minutes at room temperature. siRNA-Lipofectamine2000-

complexes were applied completely (25 µL each per well) to the cells and cells were incubated for 24 h at 37°C and 5 % CO_2 in a humidified incubator (Heraeus GmbH, Hanau).

Cells were harvested by applying 50 µL of lysis mixture (content of the QuantiGene bDNA-kit from Genospectra, Fremont, USA) to each well containing 100 µL of growth medium and were lysed at 53°C for 30 min. Afterwards, 50 µL of the lysates were incubated with probesets specific to human ENaC-alpha and human GAPDH (sequence of probesets see below) and proceeded according to the manufacturer's protocol for QuantiGene. In the end chemoluminescence was measured in a Victor2-Light (Perkin Elmer, Wiesbaden, Germany) as RLUs (relative light units) and values obtained with the hENaC probeset were normalized to the respective GAPDH values for each well. Values obtained with siRNAs directed against ENaC-alpha were related to the value obtained with an unspecific siRNA (directed against HCV) which was set to 100%. The percentage residual expression of alpha-ENaC for siRNA examples is shown in Tables 1A-1D.

Effective siRNAs from the screen were further characterized by dose response curves. Transfections of dose response curves were performed at the following concentrations: 100 nM, 16.7 nM, 2.8 nM, 0.46 nM, 77 picoM, 12.8 picoM, 2.1 picoM, 0.35 picoM, 59.5 fM, 9.9 fM and mock (no siRNA) and diluted with Opti-MEM to a final concentration of 12.5 µl according to the above protocol. Data analysis was performed using Microsoft Excel add-in software XL-fit 4.2 (IDBS, Guildford, Surrey, UK) and applying the sigmoidal model number 606.

Probesets:

human alpha-ENaC:

FPL Name	Members	Function	Sequence	SEQ.I.D.NO:
hENAC001	.235.255.CE	CE	gtctgtccagggtttccttccTTTTTctcttggaaagaaagt	1645
hENAC002	.274.293.CE	CE	actgccattcttggtgcagtTTTTTctcttggaaagaaagt	1646
hENAC003	.344.367.CE	CE	ctctcctggaagcaggagtgaataTTTTTctcttggaaagaaagt	1647
hENAC004	.391.411.CE	CE	gccgcggatagaagatgtaggTTTTTctcttggaaagaaagt	1648
hENAC005	.501.521.CE	CE	gcacttggtgaaacagcccagTTTTTctcttggaaagaaagt	1649
hENAC006	.539.560.CE	CE	agcagagagctggtagctggtcTTTTTctcttggaaagaaagt	1650
hENAC007	.256.273.LE	LE	cgccataatcgccccccaaTTTTTaggcataggacccgtgtct	1651
hENAC008	.368.390.LE	LE	cacagccacactccttgatcatgTTTTTaggcataggacccgtgtct	1652
hENAC009	.412.431.LE	LE	acagtactccacgttctgggTTTTTaggcataggacccgtgtct	1653
hENAC010	.455.477.LE	LE	ggagcttatagtagcagtaccccTTTTTaggcataggacccgtgtct	1654
hENAC011	.522.538.LE	LE	acgctgcatggcttccgTTTTTaggcataggacccgtgtct	1655
hENAC012	.561.580.LE	LE	gagggccatcgtgagtaaccTTTTTaggcataggacccgtgtct	1656
hENAC013	.214.234.BL	BL	Tcatgctgatggaggtctcca	1657
hENAC014	.294.318.BL	BL	Ggtaaaggttctcaacaggaacatc	1658
hENAC015	.319.343.BL	BL	Cacacctgctgtgtgtactttgaag	1659
hENAC016	.432.454.BL	BL	Caggaactgtgctttctgtagtc	1660
hENAC017	.478.500.BL	BL	Gtggtctgaggagaagtcaacct	1661
hENAC018	.581.599.BL	BL	Ccattcctgggatgtcacc	1662

human GAPDH:

FPL Name	Members	Function	Sequence	SEQ.I.D.
hGAP001	AF261085.252.271.CE	CE	gaatttgccatgggtggaatTTTTTctcttggaaagaaagt	1663
hGAP002	AF261085.333.352.CE	CE	ggagggatctcgctcctggaTTTTTctcttggaaagaaagt	1664
hGAP003	AF261085.413.431.CE	CE	ccccagccttctccatggtTTTTTctcttggaaagaaagt	1665
hGAP004	AF261085.432.450.CE	CE	gctccccctgcaaatgagTTTTTctcttggaaagaaagt	1666
hGAP005	AF261085.272.289.LE	LE	agccttgacggtgccatgTTTTTaggcataggacccgtgtct	1667
hGAP006	AF261085.290.310.LE	LE	gatgacaagcttcccgttctcTTTTTaggcataggacccgtgtct	1668
hGAP007	AF261085.311.332.LE	LE	agatggtgatgggatttccattTTTTTaggcataggacccgtgtct	1669
hGAP008	AF261085.353.372.LE	LE	gcatcgccccacttgattttTTTTTaggcataggacccgtgtct	1670
hGAP009	AF261085.373.391.LE	LE	cacgacgtactcagcgccaTTTTTaggcataggacccgtgtct	1671
hGAP010	AF261085.451.472.LE	LE	ggcagagatgatgacccttttgTTTTTaggcataggacccgtgtct	1672
hGAP011	AF261085.392.412.BL	BL	Ggtgaagacgccagtggactc	1673

The IC_{50}s for siRNA examples is shown in Table 2A and 2B.

Example 3.2: Transient alpha-ENaC knockdown in a primary human bronchial epithelial model:

Human bronchial epithelial cells (donor reference 4F1499) were plated in 24-well plates at $1x10^5$ cells per well in 0.5 mL growth medium one day before transfection. The cells were 70% confluent on the day of siRNA transfection.

Each siRNA was resuspended at 100nM in 1mL of Optimem I (Invitrogen) and in a separate tube, Lipofectamine 2000 (Invitrogen) was diluted to 6 μL/mL in Optimem, giving an amount sufficient for transfection of four replicates in a 24-well plate. After 5 minutes at room temperature, the mixtures were combined to give the desired final concentration of 50nM siRNA and 3 μL/mL Lipofectamine 2000. The transfection mixture was incubated for a further 20 minutes at room temperature and 420 μL of the siRNA/reagent complex was

added to each well as dictated by the experimental design. Plates were gently rocked to ensure complete mixing and then incubated at 37°C in an incubator at 5% CO_2/95% air for 4 hours. Subsequently, the transfection mixture was aspirated and the cells were returned to normal culture conditions for a further 20 hours.

Cell lysates were prepared for branched-DNA analysis. A 2:1 medium:lysis buffer (Panomics) mixture was prepared and cells were lysed in 200 µL at 53°C for 30 minutes.After a visual check for complete lysis, the cell lysates were stored at -80 °C for subsequent analysis. Branched-DNA analysis was performed as described above, with alpha-ENaC expression normalized against GAPDH. The branched DNA analysis protocol used differs from that above only in that 20 µL of sample was applied to each well in this case.

Table 2C shows the alpha-ENaC expression in primary HBEC for siRNA examples.

Example 3.3: *In vitro* inhibition of exogenously expressed cloned cynomolgous alpha-ENaC gene expression for selected RNAi agents in Cos-1 cells

Cloning of the cynomolgous alpha-ENaC sequence

Primer sequences for amplification of 5′- UTR and CDS (nucleotides shown in brackets correspond to the *Macaca mulatta* (Rhesus monkey) α-ENaC cDNA sequence):

P745: 5′- CTCCATGTTCTGCGGCCGCGGATAGAAG-3′ (nt 1427) (SEQ.I.D.NO:1674)

P733: 5′- CCGGCCGGCGGGCGGGCT-3′ (nt 1) (SEQ.I.D.NO:1675)

P734: 5- CTCCCCAGCCCGGCCGCT-3′ (nt 17) (SEQ.I.D.NO:1676)

P735: 5′- GGCCGCTGCACCTGTAGGG-3′ (nt 28) (SEQ.I.D.NO:1677)

Primer sequences for amplification of CDS and 3′- UTR:

P737: 5′- ATGGAGTACTGTGACTACAGG-3′ (nt 1422) (SEQ.I.D.NO:1678)

P740: 5′- TTGAGCATCTGCCTACTTG-3′ (nt 3113) (SEQ.I.D.NO:1679)

Primer sequences for amplification of internal part of CDS:

P713: 5′- 5'-ATGGATGATGGTGGCTTTAACTTGCGG-3' (nt 1182) (SEQ.I.D.NO:1709)

P715: 5′- 5'-TCAGGGCCCCCCCAGAGG-3' (nt 2108) (SEQ.I.D.NO:1680)

Cynomolgus (*Macaca fascicularis*) lung total RNA (#R1534152-Cy-BC) was purchased from BioCat (Germany). Synthesis of cDNA was performed using the SuperScript III First Strand Synthesis System (Invitrogen). Synthesis of cDNA was performed using either random hexamers or oligo dT primers. In addition, cynomolgus lung first strand cDNA was also purchased from BioCat /#C1534160-Cy-BC). For PCR amplification, the Advantage 2 PCR kit (# K1910-1, Clontech) was used. Amplification of the 5′-UTR and parts of the CDS was performed using P745 and a equimolar mixture of P733, P734 and P735. For PCR amplification of the CDS and 3′-UTR, primers P737 and P740 were used. The primers P713 and P715 were used for amplification of parts of the CDS.

All PCR products were analysed by agarose gel electrophoresis and then cloned into the pCR2.1 vector using the TOPO TA cloning kit (Invitrogen) in TOP10 bacteria. Clones were then picked and DNA was isolated using the Qiagen Miniprep kit. After restriction enzyme digest with *EcoRI* and analysis by agarose gel electrophoresis, DNA from correct clones were subjected to sequencing.

The sequences were then aligned with the α-ENaC cDNA sequence of Rhesus monkey, and sequences of the individual clones were aligned with each other. The full-length cynomolgus alpha-ENaC cDNA was then cloned by digestion of the 5′-part (5′-UTR and CDS, clone 55) with *EcoR I* and *Not I*, digestion of the middle part of the CDS by *Not I* and *BstE II* (clone 15), and the 3′- part (CDS and 3′- UTR) by *BstE II* and *EcoR V* (clone 80). The digested DNA fragments were then subcloned into pcDNA3.1, digested with *EcoR I* and *EcoR V*. The full-length cynomolgus alpha-ENaC cDNA in pcDNA3.1 was then subjected to full-length sequencing (Ingenetix, Vienna, Austria). The cynomolgus alpha-ENaC cDNA sequence corresponds to nt 28 - 3113 of the Rhesus alpha-ENaC cDNA sequence. Finally the cynomolgus alpha-ENaC cDNA was then excised from pcDNA3.1-cynomolgus alpha-ENaC by digestion with *BamH I* and *EcoR V* and subcloned into the vector pXOON. The plasmid map is illustrated in Figure 1. Figure 2 depicts the protein (SEQ.I.D.NO:1681) and DNA (SEQ.I.D.NO:1682) sequence of cynomolgous alpha-ENaC.

Transfections:

COS-1 cells were seeded at $6x10^4$ cells/well on 24 well plates each in 0.5 mL of growth medium. One day after seeding the cells were co-transfected with the pXOON cynomolgous alpha-ENaC expression plasmid and the indicated siRNA. For each well, 4ng of alpha-ENaC expression plasmid and 600ng carrier plasmid (pNFAT-luc) were co-

transfected with the relevant siRNA (final concentration 45nM) using X-treme gene transfection reagent (Roche) at 3.75 µL/well in a total volume of 720µL/well Opti-MEM (Invitrogen) as described below.

Transfections were performed in triplicate for each sample. Plasmid/siRNA mastermixes (each for 3.5 wells) were prepared as follows: 14 ng alpha-ENaC expression plasmid, 2.1 µg carrier plasmid and 112 pmoles of relevant siRNA in a total volume 210 µL (Opti-MEM). A lipid mastermix was prepared for the whole transfection (105 µL lipid plus 1575 µL Opti-MEM for eight triplicate transfection samples). Plasmid/siRNA and lipid were mixed in equal volume to give a total volume of 420 µL transfection mix per triplicate sample (3.5x). Following a 20 minute incubation at room temperature, 120 µL of the relevant transfection mix was added to each well of cells in a final transfection volume of 720 µL (Opti-MEM). Cells were transfected for 24 hours at 37°C and 5 % CO_2 in a humidified incubator (Heraeus GmbH, Hanau, Germany) and harvested for branched-DNA analysis.

Cell lysates were prepared for branched DNA analysis. A 2:1 medium:lysis buffer (Panomics) mixture was prepared and cells were lysed in 200 µL at 53°C for 30 minutes.After a visual check for complete lysis, the cell lysates were stored at -80 °C for subsequent analysis. Branched-DNA analysis was performed as described above, with cyno alpha-ENaC expression normalized against eGFP from the expression plasmid. The branched-DNA analysis protocol used differs from that above only in that 20 µL of sample was applied to each well in this case.

Probesets:

cynomolgous alpha-ENaC:

FPL Name	Function	Sequence	SEQ.I.D.NO:
cyENa001	CE	cgccgtgggctgctgggTTTTTctcttggaaagaaagt	1683
cyENa002	CE	ggtaggagcggtggaactcTTTTTctcttggaaagaaagt	1684
cyENa003	CE	cagaagaactcgaagagctctcTTTTTctcttggaaagaaagt	1685
cyENa004	CE	cccagaaggccgtcttcatTTTTTctcttggaaagaaagt	1686

cyENa005	LE	ggtgcagagccagagcactgTTTTTctcttggaaagaaagt	1687
cyENa006	LE	gtgccgcaggttctgggTTTTTaggcataggacccgtgtct	1688
cyENa007	LE	gatcagggcctcctcctcTTTTTaggcataggacccgtgtct	1689
cyENa008	LE	ccgtggatggtggtattgttgTTTTTaggcataggacccgtgtct	1690
cyENa009	LE	gcggttgtgctgggagcTTTTTaggcataggacccgtgtct	1691
cyENa0010	LE	ttgccagtacatcatgccaaaTTTTTaggcataggacccgtgtct	1692
cyENa0011	BL	acaccaggcggatggcg	1693

eGFP:

FPL Name	Function	Sequence	SEQ.I.D.NO:
EGFP001	CE	ggcacgggcagcttgcTTTTTctcttggaaagaaagt	1694
EGFP002	CE	ggtagcggctgaagcactgTTTTTctcttggaaagaaagt	1695
EGFP003	CE	cctggacgtagccttcgggTTTTTctcttggaaagaaagt	1696
EGFP004	CE	ccttgaagaagatggtgcgctTTTTTctcttggaaagaaagt	1697
EGFP005	LE	cgaacttcacctcggcgcTTTTTctcttggaaagaaagt	1698
EGFP006	LE	ccttcagctcgatgcggtTTTTTctcttggaaagaaagt	1699
EGFP007	LE	gtcacgagggtgggccagTTTTTaggcataggacccgtgtct	1700
EGFP008	LE	cacgccgtaggtcagggtgTTTTTaggcataggacccgtgtct	1701
EGFP009	LE	gtgctgcttcatgtggtcggTTTTTaggcataggacccgtgtct	1702
EGFP0010	LE	tcaccagggtgtcgccctTTTTTaggcataggacccgtgtct	1703
EGFP0011	BL	cggtggtgcagatgaacttca	1704
EGFP0012	BL	catggcggacttgaagaagtc	1705
EGFP0013	BL	cgtcctccttgaagtcgatgc	1706

Table 2C shows the alpha-ENaC expression in cynomologous species for siRNA examples.

Example 3.4 Screening for Interferon-α induction

To evaluate the ability of siRNA to stimulate interferon-α (IFNα) release, siRNA was incubated with freshly purified peripheral blood mononuclear cells (PBMCs) *in vitro* for 24 hours. The siRNA was added either directly to PBMCs, or first complexed with a lipid transfection agent (GenePorter 2 or Lipofectamine 2000 or DOTAP transfection agent) and subsequently incubated with PBMCs. As positive controls for IFNα induction, unmodified control sequences DI_A_2216 and DI_A_5167 were included.

DI_A_2216: is a single-stranded antisense DNA molecule
5'- dGsdGsdGdGdGdAdCdGdAdTdCdGdTdCdGsdGsdGsdGsdGsdG -3'
(SEQ.I.D.NO:1707)

DI_A_5167 is a cholesterol-conjugated siRNA
5'- GUCAUCACACUGAAUACCAAU-s-chol - 3'
3'- CsAsCAGUAGUGUGACUUAUGGUUA-5' (SEQ.I.D.NO:1708)

After 24 hours, the IFNα was measured by ELISA. The basal IFNα level was determined for untreated cells and was always very close to a water-only control. The addition of transfection agent alone gave no or little increase of IFNα levels. Known stimulatory oligonucleotides were added to cells, either directly or in the presence of transfectant, and the expected increases of IFNα were observed. This setup allows to determine the stimulation of IFNα in human PBMC by siRNA (or other oligonucleotides).

Isolation of Human PBMCs: A concentrated fraction of leukocytes (buffy coat) was obtained from the Blood Bank Suhl, Institute for Transfusion Medicine, Germany. These cells were negative for a variety of pathogens, including HIV, HCV, and others. The buffy coat was diluted 1:1 with PBS, added to a tube containing Ficoll, and centrifuged for 20 minutes at 2200 rpm to allow fractionation. This was followed by removal of the turbid layer of white blood cells and transferred to a tube with fresh PBS and Ficoll, which was centrifuged for 15 minutes at 2200 rpm. The turbid layer of white blood cells was again removed, transferred to RPMI 1640 culture medium and centrifuged for 5 minutes at 1,200

rpm to pellet the white blood cells. The cells were resuspended in RPMI, pelleted as above, and resuspended in media with 10% FCS to 1 x 10^6/mL.

Interferon-α Measurement: Cells in culture were combined with either 500 nM (or 1μM) oligonucleotide in Optimem or 133 nM oligonucleotide in GP2 or Lipofectamine2000 or DOTAP transfection agent for 24 hours at 37°C. Interferon-α was measured with Bender Med Systems (Vienna, Austria) instant ELISA kit according to the manufacturer's instructions.

Selection of lead therapeutic sequences was based on a level of IFNα induction of less than 15% of the positive control.

Example 3.5 Determination of siRNA stability in sputum of CF patients

Sputum samples were collected by Dr. Ahmet Uluer, Children's Hospital Boston. After collection, sputum samples were treated with antibiotic and were UV-irradiated to reduce bacterial content. To determine siRNA stability in sputum samples, siRNAs were incubated in 30 μL sputum at a concentration of 5 μM at 37°C for the indicated times. The reaction was terminated by addition of proteinase K and samples were incubated at 42°C for another 20 minutes. A 40-mer RNA molecule made of L-nucleotides ("Spiegelmer") resistant to nuclease degradation was added and served as calibration standard. Samples were filtered through a 0.2 μm membrane to remove remaining debris. Samples were analyzed by denaturing ion exchange HPLC on a DNAPac PA 200 column (Dionex) at pH 11.0 using a gradient of sodium perchlorate for elution. siRNAs and degradation products were quantified by determination of the area under the peak for each sample. Concentration was normalized to the concentration in the un-incubated samples.

The selection of the lead therapeutic sequences (ND8356, ND8357 and ND8396) was based on an observed *in vitro* stability in CF sputum with a half-life greater than 60 minutes.

Example 3.6 Cross-checking of lead therapeutic sequences against known polymorphisms in human SCNN1A gene

To exclude known polymorphisms from the target sites of lead candidates, lead siRNA sequences were checked against the NCBI single nucleotide polymorphism (SNP) database (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?CMD=search&DB=snp). Of the10

known exon polymorphisms in the human SCNN1A gene, none were shown to be present in the target sites of any of the 10 most potent lead therapeutic candidates.

Example 3.7: In vitro profiling of top 5 predicted off-target sequences

A list of alignments for each sequence was sorted by homology over the 19-mer region. Off-targets were scored based on the number and position of the mismatches in accordance with the criteria described in example 1. The top 5 off-target sequences were identified for each lead therapeutic sequences (ND8356, ND8357 and ND8396). On- and off-target sequences were individually cloned into a dual luciferase reporter system. Each cloned fragment encompassed the target 19 nucleotides in addition to 10 nucleotides flanking region, both 5' and 3' of the target sequence. The fragments were cloned into a multiple cloning site 3' to the Renilla luciferase sequence, under the control of an SV40 promoter. The activity of each siRNA against both on- and off-target sequences was determined by the relative fluorescence of the target Renilla luciferase to the Firefly luciferase, the latter being independently controlled by the HSV-TK promoter. Initially, transfections were performed in COS-7 cells at an siRNA concentration of 50nM. Luciferase readouts were taken at 24h post-transfection. At this high concentration of siRNA, no knockdown of greater than 30% was observed against any off-target sequence for any of the three lead siRNAs. Activity against the on-target sequence was demonstrated with a relative reduction in Renilla luciferase activity of approximately 80%. IC50 curves were also generated for each siRNA against the on-target sequence and controlled with the off-target sequences identified above. For each lead siRNA, on- target IC50's in this reporter assay were of similar order of magnitude (10-50pM) to the IC50's obtained against the endogenous target in H441 (Example 3.3) indicating that for ND8356, ND8357 and ND8396, potency against the on-target sequence was at least 1000-5000 fold higher than for any of the predicted off-target sequences.

Example 3.8: Genotoxicity profiling

Cytotoxicity determination: Cytotoxicity was determined by using a cell counter for the assessment of culture cell number.

It is well known that testing cytotoxic concentrations *in vitro* may induce genotoxic effects such as micronucleus formation. Therefore, we considered increased numbers of cells containing micronuclei appearing at cell counts of around 50% or less (compared to the concurrent negative control) to be cytotoxicity-related if no dose-dependent increase in micronucleated cells could already be observed at concentrations showing moderate toxicity at most. The analysis of a concentration showing at least 50% reduction in cell count is required by the guidelines regulating *in vitro* mammalian cell assays (OECD and ICH guidelines for the conduction of chromosome aberration testing). In addition, OECD protocols require testing of non-toxic compounds to include at least one precipitating concentration (as long as this doesn't exceed 10 mM or 5 mg/ml, whichever is lower). Since the *in vitro* micronucleus test aims to predict the outcome of the regulatory assays, i.e. *in vitro* chromosomal aberration test, the protocol for the *in vitro* micronucleus test was designed to meet the requirements for these tests.

Test system: TK6 cells are Ebstein-Barr-Virus transfected and immortalized cells (human lymphoblastoid origin derived from the spleen). Determination of the clastogenic and/or aneugenic potential in the micronucleus test *in vitro* with TK6 cells with/without S9-liver homogenate (2%) from male rats (Aroclor 1254-pretreated). Treatment time: 20 hr (-S9), 3 hr (+S9). Sampling time: 24 hr after the start of 3-hour treatment, 48 hr after the start of 20-hour treatment. For each substance at least three concentrations (2 cultures per concentration) and 2000 cells per concentration were analyzed.

The micronucleus inducing effect for a tested concentration was considered positive if the frequency of micronucleated cells was

- >= 2% and showed at least a doubling of the concurrent solvent control value, OR
- < 2% and showed at least a 3-fold increase over the concurrent solvent control value

To conclude an experiment to be positive, dose-effect relationship and cytotoxicity have to be taken into account.

Summary: Lead therapeutic sequences ND8396, ND8356, ND8357 neither induced increased numbers of cells containing micronuclei after 20-hour treatment without

metabolic activation, nor after 3-hour treatment with or without S9. No cytotoxic concentration could be analyzed up to the testing limit of 5 mg/ml.

Example 3.9 In vitro functional efficacy in H441: ND8396

In order to demonstrate *in vitro* functional efficacy of lead siRNA against alphaENaC H441 cells were transfected with siRNA and prepared for Ussings chamber analysis of ion transport. For transfection, H441 cells were plated into T25 flasks at 2 x 10^6 cells per flask in culture medium supplemented with 200nM Dexamethasone. Cells in each flask were transfected with either ND8396 or a non targeting control siRNA at 30nM siRNA and 4mL/mL Lipofectamine 2000 in a total volume of 5mL (serum free medium). One day after transfection, cells were plated onto 1cm^2 Snapwell inserts at confluency (2 x 10^5 cells per insert) to minimise the time required for differentiation and formation of tight junctions and supplied with medium in both the apical and the basolateral chambers. After one additional day of culture the apical medium was removed and the basolateral medium replaced, thus taking the cells to air-liquid interface (ALI) culture. Cells were maintained at ALI for a further six days prior to ion transport analysis.

For functional analysis in Ussings chambers, the Snapwell inserts were mounted in Vertical Diffusion Chambers (Costar) and were bathed with continuously gassed Ringer solution (5% CO_2 in O_2; pH 7.4) maintained at 37°C containing: 120mM NaCl, 25mM $NaHCO_3$, 3.3mM KH_2PO_4, 0.8mM K_2HPO_4, 1.2mM $CaCl_2$, 1.2mM $MgCl_2$, and 10mM glucose. The solution osmolarity was determined within the range of 280 and 300 mosmol/kgH_2O. Cells were voltage clamped to 0 mV (model EVC4000; WPI). Transepithelial resistance (R_T) was measured by applying a 1 or 2-mV pulse at 30-s intervals, or using the initial potential difference across the cells and the initial current measured, and then calculating R_T by Ohm's law. Data were recorded using a PowerLab workstation (ADInstruments). Following siRNA treatment the basal characteristics of the cells and the amiloride-sensitive short circuit current (I_{SC} following application of 10 μM amiloride; apical side only) were recorded. ENaC channel activity in each culture was determined by the amiloride-sensitive I_{SC} in each case.

Following assay, cells on the individual inserts were lysed for RNA analysis. A knockdown of 75% at the RNA level at the time of assay (ND8396 as compared to non-

targeting control) was correlated with a functional knockdown of the amiloride sensitive current of approximately 30% (ND8396 as compared to non-targeting control).

Nucleic acid sequences are represented below using standard nomenclature, and specifically the abbreviations of Table A.

Table A: Abbreviations of nucleotide monomers used in nucleic acid sequence representation. It will be understood that these monomers, when present in an oligonucleotide, are mutually linked by 5'-3'-phosphodiester bonds.

Abbreviation[a]	**Nucleotide(s)**
A	adenosine-5'-phosphate
C	cytidine-5'-phosphate
G	guanosine-5'-phosphate
T	2'-deoxy-thymidine-5'-phosphate
U	uridine-5'-phosphate
c	*2'-O*-methylcytidine-5'-phosphate
u	*2'-O*-methyluridine-5'-phosphate
Ts	2'-deoxy-thymidine -5'-phosphorothioate

Table 1A: Selected siRNAs in initial screening set (human-rhesus ENaC alpha cross-reactive siRNAs). A total of 152 iRNA sequences were identified as an initial screening set, both with (sequence strands 1-304) and without (sequence strands 305-608) backbone modification. iRNA sequences were designed to be fully complementary to both the human and rhesus monkey alpha-ENaC sequences, according to the design criteria described in the examples section. The percentage residual expression of alpha-ENaC in two independent *single*-dose transfection experiments is shown (refer to examples section for methods used).

Duplex ID	Seq ID	Sense	Seq ID	Antisense	1st screen single dose @ 50 nM in H441; MV	SD	2nd screen @ 50 nM in H441	SD
ND8285	1	AGccGuAGcGuGGccuccuTsT	2	GGAGGCcACGCuACGGCUTsT	92%	4%	114%	15%
ND8286	3	ccGGGuAAuGGuGcAcGGGTsT	4	CCCGUGcACcAUuACCCGGTsT	60%	1%	84%	4%
ND8287	5	AuGcuAucGcGAcAGAAcATsT	6	UGUUCUGUCGCGAuAGcAUTsT	27%	2%	35%	3%
ND8288	7	uGcuAucGcGAcAGAAcAATsT	8	UUGUUCUGUCGCGAuAGcATsT	23%	1%	32%	4%
ND8289	9	GccGGuuuAuGuAuGcuccTsT	10	GGAGcAuAcAuAAACGGCCTsT	64%	2%	93%	8%
ND8290	11	GccGGuAGcGuGGccuccATsT	12	UGGAGGCcACGCuACGGCTsT	83%	2%	115%	5%
ND8291	13	ccGGAAAuuAAAGACGAGcTsT	14	GCUCCUCUUuAAUUUCCGGTsT	54%	2%	79%	10%
ND8292	15	ccGAAGGuucGAAGccGATsT	16	UCGGCUUCGGAACCUUCGGTsT	40%	1%	54%	8%
ND8293	17	GcAAuucGGccuGcuuuucTsT	18	GAAAAGcAGGCCGAAUUGCTsT	41%	2%	51%	4%
ND8294	19	GGcGAAuuAcGcucucAcuucTsT	20	GAAGUGAGAGuAAUUCGCCTsT	19%	1%	25%	5%
ND8295	21	GcGAAuuAcucucAcuucTsT	22	GGAAGUGAGAGuAAUUCGCTsT	19%	5%	20%	1%
ND8296	23	AAccAGGcGAAuuAcucucTsT	24	GAGAGuAAUUCGCCUGGUUTsT	92%	4%	115%	19%
ND8297	25	GGuAAuGGuGcAcGGGcAGTsT	26	CUGCCCGUGcACcAUuACCTsT	79%	2%	104%	14%
ND8298	27	cucAcGAuGGGcccucGGuGTsT	28	cACCGAGGGCcAUCGUGAGTsT	61%	3%	97%	14%
ND8299	29	GcucGAAGGuucGAAGcGcTsT	30	GCUUCGAACCUUCGGAGCTsT	16%	2%	19%	3%
ND8300	31	GcGAuAcuGGucucAGGcTsT	32	CCUGGAGACCAGUAUCGCTsT	50%	5%	55%	5%
ND8301	33	ccGAuAcuGGucucuccAGGcTsT	34	GCCUGGAGACCAGUAUCGGTsT	53%	2%	65%	6%
ND8302	35	uGcuGuuGcAccAuAcuuuTsT	36	AAAGuAUGGUGcAACAGcATsT	19%	1%	25%	3%
ND8303	37	AAcGGucuGucccuGAuGcTsT	38	GcAUcAGGGACAGACCGUUTsT	90%	3%	96%	11%
ND8304	39	uuAAcuuGcGGccuGGcGuTsT	40	ACGCcAGGCCGcAAGUuAATsT	97%	3%	101%	11%
ND8305	41	GcuGGuuAcucAcGAuGGcTsT	42	GCcAUCGUGAGuAACcAGCTsT	73%	2%	78%	7%

ND8306	43	uuAcuCAcGAuGGccucGTsT	44	CGAGGGCcAUCGUGAGuAATsT	91%	7%	93%	6%
ND8307	45	GAAGccGAuAcuGGucuccTsT	46	GGAGACcAGuAUCGGCUUCTsT	71%	3%	73%	6%
ND8308	47	GAuAcuGGucuccAGGccGTsT	48	CGGCCUGGAGACcAGuAUCTsT	86%	1%	90%	9%
ND8309	49	AuAcuGGucuccAGGccGATsT	50	UCGGCCUGGAGACcAGuAUTsT	71%	5%	70%	8%
ND8310	51	cAAcGGucuGucccuGAuGTsT	52	cAUcAGGGAcAGACCGUUGTsT	80%	2%	84%	9%
ND8311	53	uuuAAcuuGcGGccuGGcGTsT	54	CGCcAGGCCGcAAGUuAAATsT	95%	2%	107%	15%
ND8312	55	uAcuCAcGAuGGcccucGGTsT	56	CCGAGGGCcAUCGUGAGuATsT	44%	2%	97%	9%
ND8313	57	uuucGGAGAGuAcuucAGcTsT	58	GCUGAAGuACUCUCCGAAATsT	14%	2%	16%	2%
ND8314	59	GcAGAcGcucuuuGAccuGTsT	60	cAGGUcAAAGAGCGUCUGCTsT	55%	4%	58%	5%
ND8315	61	cuAcAucuucuAucccGcGGTsT	62	CCGCGGAuAGAAGAUGuAGTsT	20%	3%	26%	4%
ND8316	63	AGGcGAAuuAcucucAcuuTsT	64	AAGUGAGAGuAAUUCGCCUTsT	24%	1%	25%	2%
ND8317	65	ccGcuucAAccAGGucuccTsT	66	GGAGACCUGGUUGAAGCGGTsT	62%	5%	64%	4%
ND8318	67	cAAccGcAuGAAGAcGGccTsT	68	GGCCGUCUUcAUGCGGUUGTsT	54%	6%	54%	4%
ND8319	69	AuGAAGAcGGccuucuGGGTsT	70	CCcAGAAGGCCGUCUUcAUTsT	44%	4%	44%	6%
ND8320	71	AGcAcAAccGcAuGAAGAcTsT	72	GUCUUcAUGCGGUUGUGCUTsT	15%	1%	16%	1%
ND8321	73	ucGAGuuccAccGcuccuuATsT	74	uAGGAGCGGUGGAACUCGATsT	85%	5%	89%	13%
ND8322	75	cuGcuucuAccAGAcAuAcTsT	76	GuAUGUCUGGuAGAAGcAGTsT	46%	4%	44%	3%
ND8323	77	GAGGAGuGGuAccGcuucTsT	78	GAAGCGGuACcACUCCCUCTsT	60%	7%	56%	3%
ND8324	79	ccuuuAuGGAuGAuGGuGGTsT	80	CcACcAUcAUCCAuAAAGGTsT	83%	9%	82%	1%
ND8325	81	uGAGGGAGuGGuAccGcuuTsT	82	AAGCGGuACcACUCCCUcATsT	77%	6%	72%	2%
ND8326	83	ccuGcAAccAGGcGAAuuATsT	84	uAAUUCGCCUGGUUGcAGGTsT	41%	4%	44%	7%
ND8327	85	GGccuGGcGuGGAGAccucTsT	86	GAGGUCUCcACGCcAGGCCTsT	101%	5%	95%	4%
ND8328	87	uGcuuuucGGAGAGuAcuuTsT	88	AAGuACUCUCCGAAAGcATsT	36%	1%	29%	2%
ND8329	89	ccGuAGcGuGGccucccAGTsT	90	CUGGAGGCcACGCuACGGGTsT	52%	1%	51%	2%
ND8330	91	ccGuAGcGuGGccucccAGcTsT	92	GCUGGAGGCcACGCuACGGTsT	86%	9%	84%	3%
ND8331	93	ccAGGcGAAuuAcucucAcTsT	94	GUGAGAGuAAUUCGCCUGGTsT	15%	2%	13%	1%

ND8332	95	GAAAcuGcuAuAcuuucAATsT	96	UUGAAAGuAuAGcAGUUUCTsT	10%	1%	10%	1%
ND8333	97	GccCGGGuAAuGGuGcACGTsT	98	CGUGcACCAUuACCCGGGCTsT	83%	6%	82%	4%
ND8334	99	cccGGGuAAuGGuGcAcGGTsT	100	CCGUGcACcAUuACCCGGGTsT	56%	4%	71%	10%
ND8335	101	cGGGuAAuGGuGcAcGGcTsT	102	GCCCGUGcACcAUuACCCGTsT	42%	3%	91%	8%
ND8336	103	GGGuAAuGGuGcAcGGcATsT	104	UGCCCGUGcACcAUuACCCTsT	65%	5%	71%	7%
ND8337	105	uAAuGGuGcAcGGcAGGATsT	106	UCCUGCCCGUGcACcAUuATsT	46%	3%	46%	4%
ND8338	107	cuGGuuAcucAcGAuGGccTsT	108	GGCcAUCGUGAGuAACcAGTsT	74%	5%	79%	10%
ND8339	109	GuuAcucAcGAuGGccucuTsT	110	GAGGGCcAUCGUGAGuAACTsT	85%	6%	92%	8%
ND8340	111	uGucAcGAuGGucACccucTsT	112	GAGGGUGACcAUCGUGACATsT	85%	4%	74%	5%
ND8341	113	uGcucCGAAGGuuccGAAGTsT	114	CUUCGGAACCUUCGGAGCATsT	37%	2%	32%	3%
ND8342	115	ucCGAAGGuucCGAAGccGTsT	116	CGGCUUCGGAACCUUCGGATsT	60%	4%	47%	5%
ND8343	117	uucCGAAGccGAuAcuGGuTsT	118	ACcAGuAUCGGCUUCGGAATsT	15%	1%	13%	2%
ND8344	119	AGccGAuAcuGGucuccAGTsT	120	CUGGAGACcAGuAUCGGCUTsT	49%	3%	41%	3%
ND8345	121	cuuGGuAcuGCccucuGAAcTsT	122	GUUcAGAGGcAGuACcAAGTsT	55%	2%	47%	4%
ND8346	123	cucccGuAGcAcAcuAuAATsT	124	UuAuAGUGUGCuACGGGAGTsT	67%	3%	57%	5%
ND8347	125	ucccGuAGcAcAcuAuAAcTsT	126	GUuAuAGUGUGCuACGGGATsT	29%	1%	26%	3%
ND8348	127	uGcAccAuAGuuucuuGuATsT	128	uAcAAGAAAGuAUGGUGcATsT	17%	1%	15%	3%
ND8349	129	uuGcccGuuuAuGuAuGcuTsT	130	AGcAuACAuAuAAACGGGcAATsT	68%	2%	50%	4%
ND8350	131	uGcccGuuuAuGuAuGcucTsT	132	GAGcAuACAuAuAAACGGGcATsT	59%	8%	44%	6%
ND8351	133	GGAcccuAGAccucuGcAGTsT	134	CUGcAGAGGUCuAGGGUCCTsT	86%	11%	82%	2%
ND8352	135	ccuAGAccucuGcAGcccATsT	136	UGGGCUGcAGAGGUCuAGGTsT	69%	7%	79%	3%
ND8353	137	uGGcAuGAuGuAcuGGcAATsT	138	UUGCCAGuACAUcAUGCCATsT	58%	4%	52%	4%
ND8354	139	uAcuGGcAAuucGGccuGcTsT	140	GcAGGCCGAAUUGCCAGuATsT	101%	4%	100%	4%
ND8355	141	AAuucGGccuGcuuuucGGTsT	142	CCGAAAAGcAGGCCGAAUUTsT	49%	1%	43%	6%
ND8356	143	cuGcuuuucGGAGAGuAcuTsT	144	AGuACUCUCCGAAAAGcAGTsT	17%	3%	18%	1%
ND8357	145	uucGGAGAGuAcuucAGcuTsT	146	AGCUGAAGuACUCUCCGAATsT	13%	3%	16%	2%

ND8358	147	AGcAGAcGcuuuGAccuTsT	148	AGGUcAAAGAGCGUCUGCUTsT	73%	9%	71%	5%
ND8359	149	cuuGcAGcGccuGAGGGucTsT	150	GACCCUcAGGCGCUGcAAGTsT	57%	9%	64%	7%
ND8360	151	uGGcuuuAAcuuGcGGccuTsT	152	AGCCCGcAAGUuAAAGCcATsT	102%	12%	106%	10%
ND8361	153	GcuuuAAcuuGcGGccuGGTsT	154	CcAGGCCGcAAGUuAAAGCTsT	83%	5%	82%	8%
ND8362	155	uAAcuuGcGGccuGGcGuGTsT	156	cACGCcAGGCCGcAAGUuATsT	119%	2%	115%	6%
ND8363	157	AccuuuAccuucAAAGuATsT	158	uACUUUGAAGGGuAAAGGUTsT	17%	3%	13%	2%
ND8364	159	GGuAcucAcGAuGGccccuTsT	160	AGGGCcAUCGUGAGuAACCTsT	104%	9%	117%	17%
ND8365	161	cAcGAuGGccucGGuGAcTsT	162	GUcACCGAGGGCcAUCGUGTsT	140%	13%	100%	9%
ND8366	163	AGAuGcuAucGcGAcAGAATsT	164	UUCUGUCGCGAuAGcAUCUTsT	46%	2%	70%	6%
ND8367	165	AcGAuGGucACccucuGuTsT	166	AcAGGAGGGUGACcAUCGUTsT	85%	6%	128%	10%
ND8368	167	cucGGAAGGuucGAAGccTsT	168	GGCUUCGGAACCUUCGGAGTsT	12%	2%	18%	1%
ND8369	169	AAGGuucGAAGccGAuAcTsT	170	GuAUCGGCUUCGGAACCUUTsT	63%	7%	114%	19%
ND8370	171	GGuAcuGCcucuGAAcAcuTsT	172	AGUGUUcAGAGGcAGuACCTsT	36%	3%	71%	6%
ND8371	173	AGcuuuGAcAAGGAAcuuuTsT	174	AAAGUUCCUUGUcAAAGCUTsT	17%	1%	21%	1%
ND8372	175	uuuGAcAAGGAAcuuuccuTsT	176	AGGAAAGUUCCUUGUcAAATsT	16%	2%	26%	4%
ND8373	177	uGAcAAGGAAcuuuccuAATsT	178	UuAGGAAAGUUCCUUGUcATsT	12%	1%	22%	5%
ND8374	179	cccGuAGcAcAcuAuAAcATsT	180	UGUuAuAGUGUGCuACGGGTsT	41%	2%	75%	3%
ND8375	181	cAcuAuAAcAucuGcuGGATsT	182	UCcAGcAGAUGUuAuAGUGTsT	17%	1%	26%	2%
ND8376	183	uuGcuGuuGcAcAuAcuuTsT	184	AAGuAUGGUGcAACAGcAATsT	45%	4%	69%	6%
ND8377	185	GuAcuGGcAAuucGGccuGTsT	186	cAGGCCGAAUUGCcAGuACTsT	60%	6%	120%	8%
ND8378	187	uucGGccuGcuuuucGGAGTsT	188	CUCCGAAAAGcAGGCCGAATsT	57%	5%	86%	11%
ND8379	189	ccuGcuuuucGGAGAGuAcTsT	190	GuACUCUCCGAAAAGcAGGTsT	43%	5%	50%	3%
ND8380	191	GcuuuucGGAGAGuAcuucTsT	192	GAAGuACUCUCCGAAAAGCTsT	16%	2%	24%	2%
ND8381	193	cuuuucGGAGAGuAcuucATsT	194	UGAAGuACUCUCCGAAAAGTsT	12%	1%	16%	3%
ND8382	195	cAAccucAAcucGGAcAAGTsT	196	CUUGUCCGAGUUGAGGUUGTsT	33%	2%	39%	3%
ND8383	197	cuAccAGAcAuAcucAucATsT	198	UGAUGAGuAUGUCUGGuAGTsT	13%	1%	23%	6%

ND8384	199	cuGucGAGGcuGccAGAGATsT	200	UCUCUGGcAGCCUCGAcAGTsT	11%	1%	18%	3%
ND8385	201	AAAcuGcuAuACuuucAAuTsT	202	AUUGAAAGuAuAGcAGUUUTsT	48%	8%	64%	11%
ND8386	203	GGcuuuAAcuuGcGGccuGTsT	204	cAGGCCGcAAGUuAAAGCCTsT	55%	7%	70%	8%
ND8387	205	cuuuAAcuuGcGGccuGGcTsT	206	GCcAGGCCGcAAGUuAAAGTsT	40%	11%	87%	14%
ND8388	207	AGGuGuGuAuucAcuccuGTsT	208	cAGGAGUGAAuAcAcACCUTsT	45%	3%	41%	5%
ND8389	209	AcGAuGGccucGGuGAcATsT	210	UGUcACCGAGGGCcAUCGUTsT	43%	2%	60%	9%
ND8390	211	cuGAAcAcucuGGuuuccTsT	212	GGGAAACcAGAGUGUUcAGTsT	33%	2%	48%	11%
ND8391	213	cuAuAAcAucuGcuGGAGuTsT	214	ACUCcAGcAGAUGUuAuAGTsT	16%	1%	17%	4%
ND8392	215	GcAccAuAcuuucuuGuAcTsT	216	GuAcAAGAAAGuAUGGUGCTsT	19%	1%	22%	4%
ND8393	217	uGucuAGccAucAucuuGTsT	218	cAGGAUGAUGGGCuAGAcATsT	69%	3%	92%	15%
ND8394	219	AGGAccuAGAccucuGcATsT	220	UGcAGAGGUCuAGGGUCCUTsT	94%	5%	86%	13%
ND8395	221	ccAccGcuccuAccGAGAGTsT	222	CUCUCGGuAGGAGCGGUGGTsT	55%	1%	65%	6%
ND8396	223	uAccGAGAGcucuucGAGuTsT	224	ACUCGAAGAGCUCUCGGuATsT	11%	1%	11%	1%
ND8397	225	AAcAuccuGucGAGGcuGcTsT	226	GcAGCCUCGAcAGGAUGUUTsT	90%	7%	72%	11%
ND8398	227	GAAccuuuAcccuucAAAGTsT	228	CUUUGAAGGGuAAAGGUUCTsT	22%	2%	25%	4%
ND8399	229	GGuuccGAAGccGAuAcuGTsT	230	cAGuAUCGGCUUCGGAACCTsT	93%	9%	89%	9%
ND8400	231	AAGccGAuAcuGGucuccATsT	232	UGGAGACcAGuAUCGGCUUTsT	35%	2%	42%	9%
ND8401	233	ucuAGcccAucAuccuGcuTsT	234	AGcAGGAUGAUGGGCuAGATsT	95%	8%	95%	14%
ND8402	235	cGGcGccAucccGccuGGuGTsT	236	cACcAGGCGGAUGGCGCCGTsT	81%	8%	89%	17%
ND8403	237	uuuucGGAGAGuAcuucAGTsT	238	CUGAAGuACUCUCCGAAAATsT	13%	1%	13%	1%
ND8404	239	GAGAGuAcuucAGcuAcccTsT	240	GGGuAGCUGAAGuACUCUCTsT	71%	3%	100%	10%
ND8405	241	GAcGcucuuuGAccuGuAcTsT	242	GuACAGGUcAAAGAGCGUCTsT	84%	5%	92%	13%
ND8406	243	uGuGuAuucAcuccuGcuuTsT	244	AAGcAGGAGUGAAuAcAcATsT	78%	2%	89%	8%
ND8407	245	AAcAAcAAcAGAAAuGGAGTsT	246	CUCcAUUUCUCUUGUUGUUTsT	66%	3%	88%	21%
ND8408	247	AuuGAAGGAuGuGcAGGGcTsT	248	GCCCUGcAcAUCCUUcAAUTsT	25%	1%	36%	6%
ND8409	249	ucucAGAGCcGcccAAAGuTsT	250	AGUUUGGGCGGCUCUGAGATsT	18%	1%	24%	2%

ND8410	251	AAACACAACCAAGGGUACATsT	252	UGUACCCUUGGUUGUGUUUTsT	21%	1%	35%	2%
ND8411	253	UACCCGUGCCCUCACAGAGTsT	254	CUCUGUGAGGGCACGGGUATsT	57%	2%	67%	4%
ND8412	255	UAGCACACUAUAACAUCUGTsT	256	CAGAUGUUAUAGUGUGCUATsT	30%	2%	41%	1%
ND8413	257	GGUGUGUAUUCACUCCUGCTsT	258	GCAGGAGUGAAUACACACCTsT	73%	1%	90%	9%
ND8414	259	CAUGAUCAAGGAGUGUGGCTsT	260	GCCACACUCCUUGAUCAUGTsT	65%	2%	67%	5%
ND8415	261	ACUCACGAUGGCCCUCGGUTsT	262	ACCGAGGGCCAUCGUGAGUTsT	96%	6%	95%	6%
ND8416	263	GGAGCUUUGACAAGGAACUTsT	264	AGUUCCUUGUCAAAGCUCCTsT	24%	1%	28%	4%
ND8417	265	AUACCCGUGCCCUCACAGATsT	266	UCUGUGAGGGCACGGGUAUTsT	54%	1%	62%	2%
ND8418	267	GGAGUGGCCAAAGUCAACATsT	268	UGUUGACUUUGGCCACUCCTsT	93%	2%	86%	11%
ND8419	269	AACUACAAAACCAAUUCUGTsT	270	CAGAAUUGGUUUUGUAGUUTsT	101%	5%	108%	19%
ND8420	271	UGCUGGAGUGUUGCUGUUGTsT	272	CAACAGCACAACACUCCAGCATsT	29%	1%	26%	1%
ND8421	273	AGGUCUCCUUGCAACCAGGCTsT	274	GCCUGGUUGCAGGAGACCUTsT	95%	10%	91%	17%
ND8422	275	CUUUGGCAUGAUGUACUGGTsT	276	CCAGUACAUCAUGCCAAAGTsT	86%	3%	84%	6%
ND8423	277	CAUCUGCACCCUCAUCCCTsT	278	GGGAUUGAGGGUGCAGAUGTsT	82%	11%	73%	4%
ND8424	279	CGACUGCACCAAGAUGGCTsT	280	GCCAUUCUUGGUGCAGUCGTsT	70%	8%	69%	7%
ND8425	281	AAAACACAACCAAGGGUACTsT	282	GUACCCUUGGUUGUGUUUUTsT	95%	6%	106%	12%
ND8426	283	CAUCUGCUGGAGUGUUGCUTsT	284	AGCAACACUCCAGCAGAUGTsT	30%	2%	37%	1%
ND8427	285	CCUACAUCUUCUAUCCGCGTsT	286	CGCGGAUAGAAGAUGUAGGTsT	42%	6%	30%	1%
ND8428	287	GCCUACAUCUUCUAUCCGCTsT	288	GCGGAUAGAAGAUGUAGGCTsT	65%	7%	54%	3%
ND8429	289	GAGUGGUACCGCUUCCACUTsT	290	AGUGGAAGCCGGUACCACUCTsT	95%	11%	86%	19%
ND8430	291	GGUACCGCUUCCACUACAUTsT	292	AUGUAGUGGAAGCGGUACCTsT	111%	19%	96%	14%
ND8431	293	GUCGUACCGCUUCCACUACTsT	294	GUAGUGGAAGCCGGUACCACTsT	98%	13%	52%	26%
ND8432	295	GAAUUACUCUCACUUCCACTsT	296	GUGGAAGUGAGAGUAAAUUCTsT	111%	21%	73%	27%
ND8433	297	AAUUACUCUCACUUCCACCTsT	298	GGUGGAAGUGAGAGUAAAUUTsT	109%	22%	105%	7%
ND8434	299	UACUCUCACUUCCACCACCTsT	300	GGUGGUGGAAGUGAGAGUATsT	106%	23%	95%	7%
ND8435	301	AGUGGUACCGCUUCCACUATsT	302	UAGUGGAAGCCGGUACCACUTsT	109%	18%	102%	9%

ND8436	303	GGCAAcuucAucuucGccTsT	304	GGCGAAGAUGAAGUUGCCCTsT	109%	18%	107%	14%
ND-8501	305	AGCCCGUAGCGUGGCCUCCTsT	306	GGAGGCCACGCUACGGGCUTsT	84%	14%	69%	3%
ND-8502	307	CCGGGUAAUGGUGCACGGGTsT	308	CCCGUGCACCAUUACCCGGTsT	41%	6%	30%	2%
ND-8503	309	AUGCUAUCGCGACAGAACATsT	310	UGUUCUGUCGCGAUAGCAUTsT	11%	2%	10%	2%
ND-8504	311	UGCUAUCGCGACAGAACAATsT	312	UUGUUCUGUCGCGAUAGCATsT	15%	2%	10%	0%
ND-8505	313	GCCCGUUUAUGUAUGCUCCTsT	314	GGAGCAUACAUAAACGGGCTsT	23%	3%	16%	1%
ND-8506	315	GCCCGUAGCGUGGCCUCCATsT	316	UGGAGGCCACGCUACGGGCTsT	32%	3%	22%	1%
ND-8507	317	CCGGAAAUUAAAGAGGAGCTsT	318	GCUCCUCUUUAAUUUCCGGTsT	35%	4%	24%	1%
ND-8508	319	CCGAAGGUUCCGAAGCCGATsT	320	UCGGCUUCGGAACCUUCGGTsT	19%	2%	13%	1%
ND-8509	321	GCAAUUCGGCCUGCUUUUCTsT	322	GAAAAGCAGGCCGAAUUGCTsT	12%	1%	8%	1%
ND-8510	323	GGCGAAUUACUCUCACUUCTsT	324	GAAGUGAGAGUAAUUCGCCTsT	21%	2%	18%	1%
ND-8511	325	GCGAAUUACUCUCACUUCCTsT	326	GGAAGUGAGAGUAAUUCGCTsT	12%	2%	8%	1%
ND-8512	327	AACCAGGCGAAUUACUCUCTsT	328	GAGAGUAAUUCGCCUGGUUTsT	99%	11%	79%	5%
ND-8513	329	GGUAAUGGUGCACGGGCAGTsT	330	CUGCCCGUGCACCAUUACCTsT	61%	6%	42%	4%
ND-8514	331	CUCACGAUGGCCCUCGGUGTsT	332	CACCGAGGGCCAUCGUGAGTsT	94%	11%	70%	4%
ND-8515	333	GCUCCGAAGGUUCCGAAGCTsT	334	GCUUCGGAACCUUCGGAGCTsT	18%	2%	17%	2%
ND-8516	335	GCCGAUACUGGUCUCCAGGTsT	336	CCUGGAGACCAGUAUCGGCTsT	14%	1%	12%	1%
ND-8517	337	CCGAUACUGGUCUCCAGGCTsT	338	GCCUGGAGACCAGUAUCGGTsT	42%	5%	33%	2%
ND-8518	339	UGCUGUUGCACCAUACUUUTsT	340	AAAGUAUGGUGCAACAGCATsT	10%	1%	9%	0%
ND-8519	341	AACGGUCUGUCCCUGAUGCTsT	342	GCAUCAGGGACAGACCGUUTsT	60%	7%	52%	8%
ND-8520	343	UUAACUUGCGGCCUGGCGUTsT	344	ACGCCAGGCCGCAAGUUAATsT	82%	25%	77%	18%
ND-8521	345	GCUGGUUACUCACGAUGGCTsT	346	GCCAUCGUGAGUAACCAGCTsT	36%	4%	34%	7%
ND-8522	347	UUACUCACGAUGGCCCUCGTsT	348	CGAGGGCCAUCGUGAGUAATsT	105%	21%	113%	21%
ND-8523	349	GAAGCCGAUACUGGUCUCCTsT	350	GGAGACCAGUAUCGGCUUCTsT	24%	2%	18%	2%
ND-8524	351	GAUACUGGUCUCCAGGCCGTsT	352	CGGCCUGGAGACCAGUAUCTsT	30%	5%	25%	3%
ND-8525	353	AUACUGGUCUCCAGGCCGATsT	354	UCGGCCUGGAGACCAGUAUTsT	12%	1%	11%	2%

ND-8526	355	CAACGGUCUGUCCCUGAUGTsT	356	CAUCAGGGACAGACCGUUGTsT	24%	7%	24%	2%
ND-8527	357	UUUAACUUGCGGCCUGGCCGTsT	358	CGCCAGGCCGCAAGUUAAATsT	122%	6%	107%	9%
ND-8528	359	UACUCACGAUGGCCCUCGGTsT	360	CCGAGGGCCAUCGUGAGUATsT	78%	6%	84%	7%
ND-8529	361	UUUCGGAGAGUACUUCAGCTsT	362	GCUGAAGUACUCUCCGAAATsT	87%	18%	80%	17%
ND-8530	363	GCAGACGCCUCUUUGACCUGTsT	364	CAGGUCAAAGAGCGUCUGCTsT	14%	2%	13%	0%
ND-8531	365	CUACAUCUUCUAUCCGCGGTsT	366	CCGCGGAUAGAAGAUGUAGTsT	20%	4%	18%	3%
ND-8532	367	AGGCGAAUUACUCUCACUUTsT	368	AAGUGAGAGAUAAUUCGCCUTsT	25%	5%	18%	1%
ND-8533	369	CCGCUUCAACCAGGUCUCCTsT	370	GGAGACCUGGUUGAAGCGGTsT	30%	11%	22%	2%
ND-8534	371	CAACCGCAUGAAGACGGCCTsT	372	GGCCGUCUUCAUGCGGUUGTsT	33%	4%	23%	1%
ND-8535	373	AUGAAGACGGCCUUCUGGGTsT	374	CCCAGAAGGCCGUCUUCAUTsT	114%	12%	84%	15%
ND-8536	375	AGCACAACCGCAUGAAGACTsT	376	GUCUUCAUGCGGUUGUGCUTsT	18%	1%	16%	3%
ND-8537	377	UCGAGUUCCACCGCUCCUATsT	378	UAGGAGCGGUGGAACUCGATsT	25%	0%	26%	3%
ND-8538	379	CUGCUUCUACCAGACAUACTsT	380	GUAUGUCUGGUAGAAGCAGTsT	12%	1%	13%	2%
ND-8539	381	GAGGAGUGGUACCGCUUCTsT	382	GAAGCGGUACCACUCCCUCTsT	43%	1%	47%	14%
ND-8540	383	CCUUUAUGGAUGAUGGUGGTsT	384	CCACCAUCAUCCAUAAAGGTsT	61%	5%	60%	8%
ND-8541	385	UGAGGGAGUGGGUACCGCUUTsT	386	AAGCGGUACCACUCCCUCATsT	36%	5%	35%	5%
ND-8542	387	CCUGCAACCAGGCGAAUUATsT	388	UAAUUCGCCUGGUUGCAGGTsT	19%	2%	16%	1%
ND-8543	389	GGCCUGGCGUGGAGACCUCTsT	390	GAGGUCUCCACGCCAGGCCTsT	28%	7%	20%	2%
ND-8544	391	UGCUUUUCGGAGAGUACUUTsT	392	AAGUACUCUCCGAAAAGCATsT	22%	5%	17%	1%
ND-8545	393	CCCGUAGCGUGGCCUCCAGTsT	394	CUGGAGGCCACGCUACGGGTsT	25%	3%	22%	2%
ND-8546	395	CCGUAGCGUGGCCUCCAGCTsT	396	GCUGGAGGCCACGCUACGGTsT	62%	5%	57%	9%
ND-8547	397	CCAGGCGAAUUACUCUCACTsT	398	GUGAGAGUAAUUCGCCUGGTsT	23%	11%	16%	2%
ND-8548	399	GAAACUGCUAUACUUUCAAATsT	400	UUGAAAGUAUAGCAGUUUCTsT	9%	3%	3%	0%
ND-8549	401	GCCCGGGUAAUGGUCACGTsT	402	CGUGCACCAUUACCCGGGCTsT	87%	9%	92%	14%
ND-8550	403	CCCGGGUAAUGGUGCACGGTsT	404	CCGUGCACCAUUACCCGGGTsT	19%	12%	14%	1%
ND-8551	405	CGGGUAAUGGUGCACGGGCTsT	406	GCCCGUGCACCAUUACCCGTsT	68%	11%	73%	3%

ND-8552	407	GGGUAAUGGUGCACGGGCATsT	408	UGCCCGUGCACCAUUACCCTsT	30%	6%	33%	2%
ND-8553	409	UAAUGGUGCACGGGCAGGATsT	410	UCCUGCCCGUGCACCAUUATsT	29%	3%	31%	1%
ND-8554	411	CUGGUUACUCACGAUGGCCTsT	412	GGCCAUCGUGAGUAACCAGTsT	74%	15%	66%	8%
ND-8555	413	GUUACUCACGAUGGCCCUCTsT	414	GAGGGCCAUCGUGAGUAACTsT	91%	21%	88%	10%
ND-8556	415	UGUCACGAUGGUCACCCUCTsT	416	GAGGGUGACCAUCGUGACATsT	72%	4%	76%	12%
ND-8557	417	UGCUCCGAAGGUUCCGAAGTsT	418	CUUCGGAACCUUCGGAGCATsT	51%	2%	59%	18%
ND-8558	419	UCCGAAGGUUCCGAAGCCGTsT	420	CGGCUUUCGGAACCUUCGGATsT	109%	11%	77%	13%
ND-8559	421	UUCCGAAGCCGAUACUGGUTsT	422	ACCAGUAUCGGCUUCGGAATsT	46%	20%	33%	6%
ND-8560	423	AGCCGAUACUGGUCUCCAGTsT	424	CUGGAGACCAGUAUCGGCUTsT	15%	6%	10%	1%
ND-8561	425	CUUGGUACUCCCUCUGAACTsT	426	GUUCAGAGGCAGUACCAAGTsT	16%	3%	12%	3%
ND-8562	427	CUCCCGUAGCACACUAUAATsT	428	UUAUAGUGUGCUACGGGAGTsT	14%	6%	10%	1%
ND-8563	429	UCCCGUAGCACACUAUAACTsT	430	GUUAUAGUGUGCUACGGGATsT	43%	11%	36%	4%
ND-8564	431	UGCACCAUACUUUCUUGUATsT	432	UACAAGAAAGUAUGGUGCATsT	17%	6%	13%	3%
ND-8565	433	UUGCCCGUUUAUGUAUGCUTsT	434	AGCAUACAUAAACGGGCAATsT	84%	2%	103%	12%
ND-8566	435	UGCCCGUUUAUGUAUGCUCTsT	436	GAGCAUACAUAAACGGGCATsT	69%	25%	93%	4%
ND-8567	437	GGACCCUAGACCUCUGCAGTsT	438	CUGCAGAGGUCUAGGGUCCTsT	29%	8%	33%	2%
ND-8568	439	CCUAGACCUCUGCAGCCCATsT	440	UGGGCUGCAGAGGUCUAGGTsT	18%	2%	19%	1%
ND-8569	441	UGGCAUGAUGUACUGGCAATsT	442	UUGCCAGUACAUCAUGCCATsT	19%	3%	20%	5%
ND-8570	443	UACUGGCAAUUCGGCCUGCTsT	444	GCAGGCCGAAUUGCCAGUATsT	86%	15%	83%	16%
ND-8571	445	AAUUCGGCCUGCUUUUCGGTsT	446	CCGAAAAGCAGGCCGAAUUTsT	19%	3%	24%	4%
ND-8572	447	CUGCUUUUCGGAGAGUACUTsT	448	AGUACUCUCCGAAAAGCAGTsT	8%	2%	12%	2%
ND-8573	449	UUCGGAGAGUACUUCAGCUTsT	450	AGCUGAAGUACUCUCCGAATsT	27%	3%	40%	5%
ND-8574	451	AGCAGACGCUCUUUGACCUTsT	452	AGGUCAAAGAGCGUCUGCUTsT	15%	0%	19%	4%
ND-8575	453	CUUGCAGCGCCUGAGGGUCTsT	454	GACCCUCAGGCGCUGCAAGTsT	35%	1%	40%	4%
ND-8576	455	UGGCUUUAACUUGCGGCCUTsT	456	AGGCCGCAAGUUAAAGCCATsT	47%	3%	53%	8%
ND-8577	457	GCUUUAACUUGCGGCCUGGTsT	458	CCAGGCCGCAAGUUAAAGCTsT	20%	2%	25%	5%

ND-8578	459	UAACUUGCGGCCUGGCGUGTsT	460	CACGCCAGGCCGCAAGUUATsT	75%	7%	82%	4%
ND-8579	461	ACCUUUACCCUUCAAAGUATsT	462	UACUUUGAAGGGUAAAGGUTsT	14%	2%	17%	3%
ND-8580	463	GGUUACUCACGAUGGCCCUTsT	464	AGGGCCAUCGUGAGUAACCTsT	63%	5%	70%	11%
ND-8581	465	CACGAUGGCCCUCGGUGACTsT	466	GUCACCGAGGGCCAUCGUGTsT	56%	2%	50%	5%
ND-8582	467	AGAUGCUAUCGCGACAGAATsT	468	UUCUGUCGCGAUAGCAUCUTsT	18%	1%	18%	1%
ND-8583	469	ACGAUGGUCACCCUCCUGUTsT	470	ACAGGAGGGUGACCAUCGUTsT	48%	3%	52%	6%
ND-8584	471	CUCCGAAGGUUCCGAAGCCTsT	472	GGCUUCGGAACCUUCGGAGTsT	18%	2%	20%	5%
ND-8585	473	AAGGUUCCGAAGCCGAUACTsT	474	GUAUCGGCUUCGGAACCUUTsT	26%	2%	28%	1%
ND-8586	475	GGUACUGCCUCUGAACACUTsT	476	AGUGUUCAGAGGCAGUACCTsT	12%	1%	12%	1%
ND-8587	477	AGCUUUGACAAGGAACUUUTsT	478	AAAGUUCCUUGUCAAAGCUTsT	17%	2%	18%	2%
ND-8588	479	UUUGACAAGGAACUUUCCUTsT	480	AGGAAAGUUUCCUUGUCAAATsT	78%	5%	73%	2%
ND-8589	481	UGACAAGGAACUUUCCUAATsT	482	UUAGGAAAGUUCCUUGUCATsT	14%	1%	16%	1%
ND-8590	483	CCCGUAGCACACUAUAACATsT	484	UGUUAUAGUGUGCUACGGGTsT	9%	1%	11%	2%
ND-8591	485	CACUAUAACAUCUGCUGGATsT	486	UCCAGCAGAUGUUAUAGUGTsT	18%	2%	20%	2%
ND-8592	487	UUGCUGUUGCACCAUACUUTsT	488	AAGUAUGGUGCAACAGCAATsT	23%	2%	25%	8%
ND-8593	489	GUACUGGCAAUUCGGCCUGTsT	490	CAGGCCGAAUUGCCAGUACTsT	66%	3%	62%	4%
ND-8594	491	UUCGGCCUGCUUUUCGGAGTsT	492	CUCCGAAAAGCAGGCCGAATsT	97%	7%	86%	8%
ND-8595	493	CCUGCUUUUCCGAGAGUACTsT	494	GUACUCUCCGAAAAGCAGGTsT	11%	2%	14%	3%
ND-8596	495	GCUUUUCGGAGAGUACUUCTsT	496	GAAGUACUCUCCGAAAAGCTsT	12%	1%	17%	2%
ND-8597	497	CUUUUCGGAGAGUACUUCATsT	498	UGAAGUACUCUCCGAAAAGTsT	11%	1%	14%	2%
ND-8598	499	CAACCUCAACUCGGACAAGTsT	500	CUUGUCCGAGUUGAGGUUGTsT	15%	2%	16%	2%
ND-8599	501	CUACCAGACAUACUCAUCATsT	502	UGAUGAGUAUGUCUGGUAGTsT	17%	1%	18%	2%
ND-8600	503	CUGUCGAGGCUGCCAGAGATsT	504	UCUCUGGCAGCCUCGACAGTsT	17%	0%	16%	1%
ND-8601	505	AAACUGCUAUACUUUCAAUTsT	506	AUUGAAAGUAUAGCAGUUUTsT	28%	1%	26%	1%
ND-8602	507	GGCUUUAACUUGCGGCCUGTsT	508	CAGGCCGCAAGUUAAAGCCTsT	21%	2%	18%	1%
ND-8603	509	CUUUAACUUGCGGCCUGGCTsT	510	GCCAGGCCGCAAGUUAAAGTsT	81%	2%	69%	6%

ND-8604	511	AGGUGUGUAUUCACUCCUGTsT	512	CAGGAGUGAAUACACACCUTsT	47%	4%	40%	1%
ND-8605	513	ACGAUGGCCUCCGGUGACATsT	514	UGUCACCGAGGGCCAUCGUTsT	40%	6%	35%	2%
ND-8606	515	CUGAACACUCUGGUUUCCCTsT	516	GGGAAACCAGAGUGUUCAGTsT	60%	2%	75%	4%
ND-8607	517	CUAUAACAUCUGCUGGAGUTsT	518	ACUCCAGCAGAUGUUAUAGTsT	17%	1%	24%	3%
ND-8608	519	GCACCAUACUUUCUUGUACTsT	520	GUACAAGAAAGUAUGGUGCTsT	10%	1%	15%	3%
ND-8609	521	UGUCUAGCCCAUCAUCCUGTsT	522	CAGGAUGAUGGGCUAGACATsT	62%	2%	75%	12%
ND-8610	523	AGGACCCUAGACCUCUGCATsT	524	UGCAGAGGUCUAGGGUCCUTsT	61%	5%	73%	10%
ND-8611	525	CCACCGCUCCUACCGAGAGTsT	526	CUCUCGGUAGGAGCGGUGGTsT	21%	2%	29%	5%
ND-8612	527	UACCGAGAGCUCUUCGAGUTsT	528	ACUCGAAGAGCUCUCGGUATsT	13%	1%	22%	3%
ND-8613	529	AACAUCCUGUCGAGGCUGCTsT	530	GCAGCCUCGACAGGAUGUUTsT	57%	2%	70%	4%
ND-8614	531	GAACCUUUACCCUUCAAAGTsT	532	CUUUGAAGGGUAAAGGUUCTsT	13%	3%	16%	2%
ND-8615	533	GGUUCCGAAGCCGAUACUGTsT	534	CAGUAUCGGCUUCGGAACCTsT	18%	1%	24%	2%
ND-8616	535	AAGCCGAUACUGGUCUCCATsT	536	UGGAGACCAGUAUCGGCUUTsT	19%	1%	25%	2%
ND-8617	537	UCUAGCCCAUCAUCCUGCUTsT	538	AGCAGGAUGAUGGGCUAGATsT	93%	3%	101%	3%
ND-8618	539	CGGCGCCAUCCGCCUGGUGTsT	540	CACCAGGCGGAUGGCGCCGTsT	85%	4%	99%	4%
ND-8619	541	UUUUCGGAGAGUACUUCAGTsT	542	CUGAAGUACUCUCCGAAAATsT	63%	2%	77%	3%
ND-8620	543	GAGAGUACUUCAGCUACCCTsT	544	GGGUAGCUGAAGUACUCUCTsT	26%	1%	30%	4%
ND-8621	545	GACGCUCUUUGACCUGUACTsT	546	GUACAGGUCAAAGACGCUCTsT	17%	2%	19%	3%
ND-8622	547	UGUGUAUUCACUCCUGCUUTsT	548	AAGCAGGAGUGAAUACACATsT	49%	3%	58%	11%
ND-8623	549	AACAACAAGAGAAUGGAGTsT	550	CUCCAUUUCUCUUGUUGUUTsT	74%	7%	70%	4%
ND-8624	551	AUUGAAGGAUGUGCAGGGCTsT	552	GCCCUGCACAUCCUUCAAUTsT	85%	6%	87%	12%
ND-8625	553	UCUCAGAGCCGCCCAAACUTsT	554	AGUUUGGGCGGCUCUGAGATsT	53%	3%	51%	6%
ND-8626	555	AAACACAACCAAGGGUACATsT	556	UGUACCCUUGGUUGUGUUUTsT	17%	2%	18%	2%
ND-8627	557	UACCCGUGCCCUCACAGAGTsT	558	CUCUGUGAGGGCACGGGUATsT	58%	3%	55%	3%
ND-8628	559	UAGCACACUAUAACAUCUGTsT	560	CAGAUGUUAUAGUGUGCUATsT	64%	3%	64%	15%
ND-8629	561	GGUGUGUAUUCACUCCUGCTsT	562	GCAGGAGUGAAUACACACCTsT	25%	3%	23%	2%

ND-8630	563	CAUGAUCAAGGAGUGUGGCTsT	564	GCCACACUCCUUGAUCAUGTsT	32%	2%	28%	2%
ND-8631	565	ACUCACGAUGGCCCUCGGUTsT	566	ACCGAGGCCAUCGUGAGUTsT	96%	1%	88%	4%
ND-8632	567	GGAGCUUUGACAAGGAACUTsT	568	AGUUCCUUGUCAAAGCUCCTsT	14%	1%	14%	2%
ND-8633	569	AUACCGUGCCCUCACAGATsT	570	UCUGUGAGGGCACGGGUAUTsT	21%	2%	16%	1%
ND-8634	571	GGAGUGGCCAAAGUCAACATsT	572	UGUUGACUUUGGCCACUCCTsT	21%	3%	16%	1%
ND-8635	573	AACUACAAAACCAAUUCUGTsT	574	CAGAAUUGGUUUUGUAGUUTsT	49%	5%	37%	3%
ND-8636	575	UGCUGGAGUGUUGCUGUUGTsT	576	CAACAGCAACACUCCAGCATsT	27%	3%	21%	2%
ND-8637	577	AGGUCUCCUGCAACCAGGCTsT	578	GCCUGGUUGCAGGAGACCUTsT	62%	8%	61%	4%
ND-8638	579	CUUUGGCAUGAUGUACUGGTsT	580	CCAGUACAUCAUGCCAAAGTsT	66%	6%	52%	8%
ND-8639	581	CAUCUGCACCCUCAAUCCCTsT	582	GGGAUUGAGGGUGCAGAUGTsT	50%	7%	40%	4%
ND-8640	583	CGACUGCACCAGAAUGGCTsT	584	GCCAUUCUUGGUGCAGUCGTsT	67%	6%	54%	5%
ND-8641	585	AAAACACAACCAGGGUACTsT	586	GUACCCUUGGUUGUGUUUUTsT	14%	2%	14%	1%
ND-8642	587	CAUCUGCUGGAGUGUUGCUTsT	588	AGCAACACUCCAGCAGAUGTsT	13%	2%	13%	1%
ND-8643	589	CCUACAUCUUCUAUCCGCGTsT	590	CGCGGAUAGAAGAUGUAGGTsT	15%	4%	13%	0%
ND-8644	591	GCCUACAUCUUCUAUCCGCTsT	592	GCGGAUAGAAGAUGUAGGCTsT	14%	3%	11%	1%
ND-8645	593	GAGUGGUACCGCUUCCACUTsT	594	AGUGGAAGCGGUACCACUCTsT	16%	0%	20%	1%
ND-8646	595	GGUACCGCUUCCACUACAUTsT	596	AUGUAGUGGAAGCGGUACCTsT	12%	0%	14%	1%
ND-8647	597	GUGGUACCGCUUCCACUACTsT	598	GUAGUGGAAGCGGUACCACTsT	42%	4%	44%	3%
ND-8648	599	GAAUUACUCUCACUUCCACTsT	600	GUGGAAGUGAGAGUAAUUCTsT	10%	1%	11%	3%
ND-8649	601	AAUUACUCUCACUUCCACCTsT	602	GGUGGAAGUGACAGUAAUUTsT	105%	10%	102%	8%
ND-8650	603	UACUCUCACUUCCACCACCTsT	604	GGUGGUGGAAGUGAGAGUATsT	55%	6%	54%	8%
ND-8651	605	AGUGGUACCGCUUCCACUATsT	606	UAGUGGAAGCGGUACCACUTsT	57%	6%	59%	12%
ND-8652	607	GGCCAACUUCAUCUUCGCCTsT	608	GGCGAAGAUGAAGUUGCCCTsT	47%	12%	36%	7%

Table 1B: Selected siRNAs in extended screening set ("human-only" siRNAs). A further 344 iRNA sequences were identified and were designed to be fully complementary to the human alpha-ENaC sequences, according to the design criteria described in the examples section. All siRNAs listed in this screening set were modified only with a phosphorothioate linkage at the 3'-end between nucleotides 20 and 21 of each strand. The percentage residual expression of alpha-ENaC in single-dose transfection assay is shown (refer to examples section for methods used).

Duplex ID	Seq ID	Sense	Seq ID	Antisense	1st screen single dose @ 50 nM in H441; MV	SD
ND-10445	609	CUGCGGCUAAGUCUCUUUUTsT	610	AAAAGAGACUUAGCCGCAGTsT	94%	8%
ND-10446	611	AUCGCGACAGAACAAUUACTsT	612	GUAAUUGUUCUGUCGCGAUTsT	13%	2%
ND-10447	613	UCGCGACAGAACAAUUACATsT	614	UGUAAUUGUUCUGUCGCGATsT	18%	1%
ND-10448	615	CCCGUUUAUGUAUGCUCCATsT	616	UGGAGCAUACAUAAACGGGTsT	41%	1%
ND-10449	617	CCCGGGUAAGUAAAGGCAGTsT	618	CUGCCUUUACUUACCCGGGTsT	23%	1%
ND-10450	619	GGUACCCGGAAAUUAAAGATsT	620	UCUUUAAUUUCCGGGUACCTsT	14%	2%
ND-10451	621	GCUAUCGCGACAGAACAAUTsT	622	AUUGUUCUGUCGCGAUAGCTsT	24%	2%
ND-10452	623	UAUCGCGACAGAACAAUUATsT	624	UAAUUGUUCUGUCGCGAUATsT	12%	1%
ND-10453	625	UGCGGCUAAGUCUCUUUUUTsT	626	AAAAAGAGACUUAGCCGCATsT	46%	3%
ND-10454	627	GGCGAUUAUGGCGACUGCATsT	628	UGCAGUCGCCAUAAUCGCCTsT	14%	0%

ND-10455	629	AUGUCUAGCCCAUCAUCCUTsT	630	AGGAUGAUGGGCUAGACAUTsT	12%	2%
ND-10456	631	CUACAGGUACCCGGAAAUUTsT	632	AAUUUCCGGGUACCUGUAGTsT	28%	2%
ND-10457	633	CCGUCGAGCCCGUAGCGUGTsT	634	CACGCUACGGGCUCGACGGTsT	27%	3%
ND-10458	635	CGCGACAGAACAAUUACACTsT	636	GUGUAAUUGUUCUGUCGCGTsT	39%	7%
ND-10459	637	AGGUACCCGGAAAUUAAAGTsT	638	CUUUAAUUUCCGGGUACCUTsT	30%	3%
ND-10460	639	CAUGCACGGGUUUCCUGCCTsT	640	GGCAGGAAACCCGUGCAUGTsT	95%	6%
ND-10461	641	AGCUUGCCGGGACAACAACCTsT	642	GGUUGUUGUCCCGCAAGCUTsT	94%	8%
ND-10462	643	ACUGCGGCUAAGUCUCUUUTsT	644	AAAGAGACUUAGCCGCAGUTsT	13%	2%
ND-10463	645	CAUCCCUUAGAACCCUGCUTsT	646	AGCAGGGUUCUAAGGGAUGTsT	18%	1%
ND-10464	647	ACCCGGGUAAGUAAAGGCATsT	648	UGCCUUUACUUACCCGGGUTsT	41%	1%
ND-10465	649	GAUUAUGGCGACUGCACCATsT	650	UGGUGCAGUCGCCAUAAUCTsT	23%	1%
ND-10466	651	CUCGGACAAGCUCGUCUUCTsT	652	GAAGACGAGCUUGUCCGAGTsT	14%	2%
ND-10467	653	GCGAUUAUGGCGACUGCACTsT	654	GUGCAGUCGCCAUAAUCGCTsT	24%	2%
ND-10468	655	AAUUACACCGUCAACAACATsT	656	UGUUGUUGACGGUGUAAUUTsT	12%	1%
ND-10469	657	AACUGCCGUUGAUGUGUGGTsT	658	CCACACAUCAACGGCAGUUTsT	46%	3%
ND-10470	659	AACUGCGGCUAAGUCUCUUTsT	660	AAGAGACUUAGCCGCAGUUTsT	14%	0%

ND-10471	661	CCGCUGAUAACCAGGACAAsT	662	UUGUCCUGGUUAUCAGCGGsT	12%	2%
ND-10472	663	AAGGGUACACGCAGGCAUGsT	664	CAUGCCUGCGUGUACCCUUsT	28%	2%
ND-10473	665	CCGGGUAAGUAAAGGCAGAsT	666	UCUGCCUUUACUUACCCGGsT	27%	3%
ND-10474	667	CCCAUACCAGGUCUCAUGGsT	668	CCAUGAGACCUGGUAUGGGsT	39%	7%
ND-10475	669	AUUAUGGCGACUGCACCAAsT	670	UUGGUGCAGUCGCCAUAAUsT	30%	3%
ND-10476	671	AUGCACGCGGUUUCCUGCCCsT	672	GGGCAGGAAACCCGUGCAUsT	95%	6%
ND-10477	673	CUAGCCCUCCACAGUCCACsT	674	GUGGACUGUGGAGGGCUAGsT	43%	7%
ND-10478	675	CAGGUACCCGGAAAUUAAAsT	676	UUUAAUUUCCGGGUACCUGsT	11%	1%
ND-10479	677	AAUACAGCUCCUUCACCACsT	678	GUGGUGAAGGAGCUGUAUUsT	30%	3%
ND-10480	679	CACGGGUUUCCUGCCCAGCsT	680	GCUGGGCAGGAAACCCGUGsT	19%	1%
ND-10481	681	GGACUGAAUCUUGCCCGUUsT	682	AACGGGCAAGAUUCAGUCCsT	14%	2%
ND-10482	683	CGUUUAUGUAUGCUCCAUGsT	684	CAUGGAGCAUACAUAAACGsT	15%	1%
ND-10483	685	GGGUACUGCUACUAUAAGCsT	686	GCUUAUAGUAGCAGUACCCsT	11%	0%
ND-10484	687	UCGGUGUUGUCUGUGGUGGsT	688	CCACCACAGACAACACCGAsT	65%	5%
ND-10485	689	AAACUGCCGUUGAUGUGUGsT	690	CACACAUCAACGGCAGUUUsT	73%	6%
ND-10486	691	GCGAAACUUGGAGCUUUGAsT	692	UCAAAGCUCCAAGUUUCGCsT	8%	1%

ND-10487	693	GGCCCGUCGAGCCCGUAGCUsT	694	GCUACGGGCUCGACGGGCCUsT	26%		3%
ND-10488	695	GCGACAGAACAAUUACACCUsT	696	GGUGUAAUUGUUCUGUCGCUsT	10%		2%
ND-10489	697	GCGACGGCUUAAGCCAGCCUsT	698	GGCUGGCUUAAGCCGUCGCUsT	50%		1%
ND-10490	699	GACCCGGGUAAGUAAAGGCUsT	700	GCCUUUACUUACCCGGGUCUsT	74%		1%
ND-10491	701	UUGAUCACUCCGCCUUCUCUsT	702	GAGAAGGCGGAGUGAUCAAUsT	80%		7%
ND-10492	703	UCUAGCCCUCCACAGUCCAUsT	704	UGGACUGUGGAGGGCUAGAUsT	69%		4%
ND-10493	705	GUUUCACCAAGUGCCGGAAUsT	706	UUCCGGCACUUGGUGAAACUsT	23%		3%
ND-10494	707	CUCAACUCGGACAAGCUCGUsT	708	CGAGCUUGUCCGAGUUGAGUsT	45%		6%
ND-10495	709	CAACUCGGACAAGCUCGUCUsT	710	GACGAGCUUGUCCGAGUUGUsT	23%		3%
ND-10496	711	ACCCGGAAAUUAAAGAGGAUsT	712	UCCUCUUUAAUUUCCGGGUUsT	13%		2%
ND-10497	713	CCCGGAAAUUAAAGAGGAGUsT	714	CUCCUCUUUAAUUUCCGGGUsT	19%		1%
ND-10498	715	CACCACUCUCGUGGCCGGCUsT	716	GCCGGCCACGAGAGUGGUGUsT	94%		11%
ND-10499	717	CGUCGAGCCCGUAGCGUGGUsT	718	CCACGCUACGGGCUCGACGUsT	13%		1%
ND-10500	719	GCUUGCGGGACAACAACCCUsT	720	GGGUUGUUGUCCCGCAAGCUsT	49%		2%
ND-10501	721	GAAUCAACAACGGUCUGUCUsT	722	GACAGACCGUUGUUGAUUCUsT	18%		2%

ND-10502	723	GGGCGAUUAUGGCGACUGCUsT	724	GCAGUCGCCAUAAUCGCCCUsT	8%	1%
ND-10503	725	CGAUUAUGGCGACUGCACCUsT	726	GGUGCAGUCGCCAUAAUCGUsT	17%	1%
ND-10504	727	UCUGCUGGUUACUCACGAUUsT	728	AUCGUGAGUAACCAGCAGAUsT	38%	4%
ND-10505	729	CUAUCGCGACAGAACAAUUUsT	730	AAUUGUUCUGUCGCGAUAGUsT	9%	1%
ND-10506	731	CAAUUACACCGUCAACAACUsT	732	GUUGUUGACGGUGUAAUUGUsT	11%	1%
ND-10507	733	ACCGUCAACAACAAGAGAAUsT	734	UUCUCUUGUUGUUGACGGUUsT	9%	1%
ND-10508	735	CUCCUCGGUGUUGUCUGUGUsT	736	CACAGACAACACCGAGGAGUsT	78%	5%
ND-10509	737	GGAGGUAGCCUCCCACCCUGUsT	738	CAGGGUGGAGGCUACCUCCUsT	18%	1%
ND-10510	739	GGAGAGGUUUCUCACACCAUsT	740	UGGUGUGAGAAACCUCUCCUsT	13%	1%
ND-10511	741	CUGCCGUUGAUGUGUGGAGUsT	742	CUCCACACAUCAACGGCAGUsT	19%	2%
ND-10512	743	UGCCGUUGAUGUGUGGAGGUsT	744	CCUCCACACAUCAACGGCAUsT	82%	4%
ND-10513	745	AGAUGGGUAAGGGCUCAGGUsT	746	CCUGAGCCCUUACCCAUCUUsT	24%	1%
ND-10514	747	AGAACAGUAGCUGAUGAAGUsT	748	CUUCAUCAGCUACUGUUCUUsT	15%	0%
ND-10515	749	GCGGCUAAGUCUCUUUUUCUsT	750	GAAAAAGAGACUUAGCCGCUsT	13%	1%
ND-10516	751	CCUAAGAAACCGCUGAUAAUsT	752	UUAUCAGCGGUUUCUUAGGUsT	6%	0%

ND-10517	753	GAAACCGCUGAUAACCAGGTsT	754	CCUGGUUAUCAGCGGUUUCTsT	13%	0%
ND-10518	755	AACCGCUGAUAACCAGGACTsT	756	GUCCUGGUUAUCAGCGGUUTsT	42%	2%
ND-10519	757	ACCGCUGAUAACCAGGACATsT	758	UGUCCUGGUUAUCAGCGGUTsT	11%	1%
ND-10520	759	CCAAGGGUACACGCAGGCATsT	760	UGCCUGCGUGUACCCUUGGTsT	19%	1%
ND-10521	761	CAAGGGUACACGCAGGCAUTsT	762	AUGCCUGCGUGUACCCUUGTsT	12%	0%
ND-10522	763	AGGGUACACGCAGGCAUGCTsT	764	GCAUGCCUGCGUGUACCCUTsT	23%	1%
ND-10523	765	GUACACGCAGGCAUGCACGTsT	766	CGUGCAUGCCUGCGUGUACTsT	27%	1%
ND-10524	767	AGGCAUGCACGGGUUUCCUTsT	768	AGGAAACCCGUGCAUGCCUTsT	14%	0%
ND-10525	769	GGCAUGCACGGGUUUCCUGTsT	770	CAGGAAACCCGUGCAUGCCTsT	18%	3%
ND-10526	771	ACGGGUUUCCUGCCCAGCGTsT	772	CGCUGGGCAGGAAACCCGUTsT	30%	1%
ND-10527	773	GAGCAGACCCGGGUAAGUATsT	774	UACUUACCCGGGUCUGCUCTsT	24%	2%
ND-10528	775	AGCAGACCCGGGUAAGUAATsT	776	UUACUUACCCGGGUCUGCUTsT	24%	2%
ND-10529	777	GGGUAAGUAAAGGCAGACCTsT	778	GGUCUGCCUUUACUUACCCTsT	39%	3%
ND-10530	779	AGCCUCAUACCCGUGCCCUTsT	780	AGGGCACGGGUAUGAGGCUTsT	82%	5%
ND-10531	781	GUGAACGCUUCUGCCACAUTsT	782	AUGUGGCAGAAGCGUUCACTsT	13%	1%

ND-10532	783	AAAUUGAUCACUCCGCCUUTsT	784	AAGGCGGAGUGAUCAAUUUTsT	18%	2%
ND-10533	785	AAUUGAUCACUCCGCCUUCTsT	786	GAAGGCGGAGUGAUCAAUUTsT	19%	0%
ND-10534	787	GCCUUGCGGUCAGGACUGTsT	788	CAGUCCCUGACCGCAAGGCTsT	12%	1%
ND-10535	789	CUUGCGGUCAGGGACUGAATsT	790	UUCAGUCCCUGACCGCAAGTsT	11%	0%
ND-10536	791	UUGCGGUCAGGGACUGAAUTsT	792	AUUCAGUCCCUGACCGCAATsT	12%	0%
ND-10537	793	AUGUAUGCUCCAUGUCUAGTsT	794	CUAGACAUGGAGCAUACAUTsT	21%	1%
ND-10538	795	AGCAAGUAGGCAGGAGCUCTsT	796	GAGCUCCUGCCUACUUGCUTsT	19%	1%
ND-10539	797	CAGCCCAUACCAGGUCUCATsT	798	UGAGACCUGGUAUGGGCUGTsT	27%	2%
ND-10540	799	CAGCCGUCGCGACCUGCGGTsT	800	CCGCAGGUCGCGACGGCUGTsT	44%	4%
ND-10541	801	GGGCCCGUCGAGCCCCGUAGTsT	802	CUACGGGCUCGACGGGCCCTsT	71%	6%
ND-10542	803	CGUAGCGUGGCCUCCAGCUTsT	804	AGCUGGAGGCCACGCUACGTsT	84%	9%
ND-10543	805	GGUGAGGGAGUGGUACCGCTsT	806	GCGGUACCACUCCCUCACCTsT	108%	8%
ND-10544	807	AAAGUACACACAGCAGGUGTsT	808	CACCUGCUGUGUGUACUUUTsT	140%	7%
ND-10545	809	CCAGGUUGACUUCUCCUCATsT	810	UGAGGAGAAGUCAACCUGGTsT	18%	2%
ND-10546	811	UGUUUCACCAAGUGCCGGATsT	812	UCCGGCACUUGGUGAAACATsT	31%	2%

ND-10547	813	UGCUGGUUACUCACGAUGGTsT	814	CCAUCGUGAGUAACCAGCATsT	144%	10%
ND-10548	815	UCCUCGGUGUUGUCUGUGGTsT	816	CCACAGACAACACCGAGGATsT	106%	14%
ND-10549	817	AGGUAGCCUCCACCCUGGCTsT	818	GCCAGGGUGGAGGCUACCUTsT	74%	15%
ND-10550	819	GCCGUUGAUGUGUGGAGGGTsT	820	CCCUCCCACACAUCAACGGCTsT	26%	4%
ND-10551	821	GAUGGGUAAGGGCUCAGCATsT	822	UCCUGAGCCCUUACCCAUCTsT	22%	1%
ND-10552	823	CCCAACUGCGGCUAAGUCUTsT	824	AGACUUAGCCGCAGUUGGGTsT	18%	2%
ND-10553	825	CCAAGCGAAACUUGGAGCUTsT	826	AGCUCCAAGUUUCGCUUGGTsT	16%	1%
ND-10554	827	GGGUACACGCAGGCAUGCATsT	828	UGCAUGCCUGCGUGUACCCTsT	19%	2%
ND-10555	829	UGCACGGGUUUCCUGCCCATsT	830	UGGGCAGGAAACCCGUGCATsT	28%	2%
ND-10556	831	CUCCUCUAGCCUCAUACCCTsT	832	GGGUAUGAGGCUAGAGGAGTsT	109%	8%
ND-10557	833	UCCUCUAGCCUCAUACCCGTsT	834	CGGGUAUGAGGCUAGAGGATsT	117%	7%
ND-10558	835	UCUAGCCUCAUACCCGUGCTsT	836	GCACGGGUAUGAGGCUAGATsT	128%	9%
ND-10559	837	UUCAUACCUCUACAUGUCUTsT	838	AGACAUGUAGAGGUAUGAATsT	52%	4%
ND-10560	839	UCUACAUGUCUGCUUGAGATsT	840	UCUCAAGCAGACAUGUAGATsT	15%	2%
ND-10561	841	AUAUUUCCUCAGCCUGAAATsT	842	UUUCAGGCUGAGGAAAUAUTsT	15%	2%

ND-10562	843	AACUCCUAUGCAUCCCUUATsT	844	UAAGGGAUGCAUAGGAGUUTsT	14%	1%
ND-10563	845	GCAUCCCUUAGAACCCUGCTsT	846	GCAGGGUUCUAAGGGAUGCTsT	20%	1%
ND-10564	847	UGAUCACUCCGCCUUCUCCTsT	848	GGAGAAGGCGGAGUGAUCATsT	67%	7%
ND-10565	849	UGUAAGUGCCUUGCGGUCATsT	850	UGACCGCAAGGCACUUACATsT	17%	2%
ND-10566	851	CCUUGCGGUCAGGGACUGATsT	852	UCAGUCCCUGACCGCAAGGTsT	14%	1%
ND-10567	853	AAUCUUGCCCGUUUAUGUATsT	854	UACAUAAACGGGCAACAUUTsT	13%	2%
ND-10568	855	CCGUUUAUGUAUGCUCCAUTsT	856	AUGGAGCAUACAUAAACGGTsT	19%	6%
ND-10569	857	UGUAUGCUCCAUGUCUAGCTsT	858	GCUAGACAUGGAGCAUACATsT	87%	13%
ND-10570	859	CAUGUCUAGCCCAUCAUCCTsT	860	GGAUGAUGGGCUAGACAUGTsT	33%	4%
ND-10571	861	AGUAGGCAGGAGCUCAAUATsT	862	UAUUGAGCUCCUGCCUACUTsT	11%	1%
ND-10572	863	CCUACAGGUACCCGGAAAUTsT	864	AUUUCCGGGUACCUGUAGGTsT	22%	3%
ND-10573	865	CCCGUCGAGCCCGUAGCGUTsT	866	ACGCUACGGGCUCGACGGGTsT	23%	1%
ND-10574	867	GCGGUGAGGGAGUGGUACCTsT	868	GGUACCACUCCCCUCACCGCTsT	30%	1%
ND-10575	869	UUAUGGCGACUGCACCAAGTsT	870	CUUGGUGCAGUCGCCAUAATsT	77%	6%
ND-10576	871	CUAUAAGCUCCAGGUUGACTsT	872	GUCAACCUGGAGCUUAUAGTsT	11%	1%

ND-10577	873	UAUAAGCUCCAGGUUGACUTsT	874	AGUCAACCUGGAGCUUAUATsT	42%	8%
ND-10578	875	AGGUUGACUUCUCCUCAGATsT	876	UCUGAGGAGAAGUCAACCUTsT	13%	3%
ND-10579	877	CUGGGCUGUUUCACCAAGUTsT	878	ACUUGGUGAAACAGCCCAGTsT	19%	6%
ND-10580	879	AACAAUUACACCGUCAACATsT	880	UGUUGACGGUGUAAUUGUUTsT	13%	1%
ND-10581	881	UGGGUAAGGGCUCAGGAAGTsT	882	CUUCCUGAGCCCUUACCCATsT	20%	3%
ND-10582	883	GGGUAAGGGCUCAGGAAGUTsT	884	ACUUCCUGAGCCCUUACCCTsT	22%	3%
ND-10583	885	CACCCAACUGCGGCUAAGUTsT	886	ACUUAGCCGCAGUUGGGUGTsT	22%	10%
ND-10584	887	ACCCAACUGCGGCUAAGUCTsT	888	GACUUAGCCGCAGUUGGGUTsT	22%	5%
ND-10585	889	CCAACUGCGGCUAAGUCUCTsT	890	GAGACUUAGCCGCAGUUGGTsT	14%	2%
ND-10586	891	CUUGGAUCAGCCAAGCGAATsT	892	UUCGCUUGGCUGAUCCAAGTsT	15%	1%
ND-10587	893	GCCAAGCGAAACUUGGAGCTsT	894	GCUCCAAGUUUCGCUUGGCTsT	17%	2%
ND-10588	895	UCCUAAGAAACCGCUGAUATsT	896	UAUCAGCGGUUUCUUAGGATsT	11%	2%
ND-10589	897	GCAUGCACGGGUUUCCUGCTsT	898	GCAGGAAACCCGUGCAUGCTsT	24%	8%
ND-10590	899	UGUUACUUAGGCAAUUCCCTsT	900	GGGAAUUGCCUAAGUAACATsT	48%	10%
ND-10591	901	CUAGGGCUAGAGCAGACCCTsT	902	GGGUCUGCUCUAGCCCUAGTsT	58%	10%

ND-10592	903	CUCUAGCCUCAUACCCGUGTsT	904	CACGGGUAUGAGGCUAGAGTsT	34%	5%
ND-10593	905	UUAGAACCCUGCUCAGACATsT	906	UGUCUGAGCAGGGUUCUAATsT	14%	1%
ND-10594	907	UGUGAACGCUUCUGCCACATsT	908	UGUGGCAGAAGCGUUCACATsT	15%	0%
ND-10595	909	AUUGAUCACUCCGCCUUCUTsT	910	AGAAGGCGGAGUGAUCAAUTsT	43%	1%
ND-10596	911	UCACUCCGCCUUCUCCUGGTsT	912	CCAGGAGAAGGCGGAGUGATsT	90%	5%
ND-10597	913	GCGGUCAGGGACUGAAUCUTsT	914	AGAUUCAGUCCCUGACCGCTsT	11%	0%
ND-10598	915	GGUCAGGGACUGAAUCUUGTsT	916	CAAGAUUCAGUCCCUGACCTsT	13%	1%
ND-10599	917	GUAUGCUCCAUGUCUAGCCTsT	918	GGCUAGACAUGGAGCAUACTsT	28%	3%
ND-10600	919	CCAUGUCUAGCCCAUCAUCTsT	920	GAUGAUGGGCUAGACAUGGTsT	12%	1%
ND-10601	921	GAUCGAGUUCCACCGCUCCTsT	922	GGAGCGGUGGAACUCGAUCTsT	17%	1%
ND-10602	923	GGACUCUAGCCCUCCACAGTsT	924	CUGUGGAGGGCUAGAGUCCTsT	41%	4%
ND-10603	925	UCACCACUCUCGUGGCCGGTsT	926	CCGGCCACGAGAGUGGGUGATsT	83%	3%
ND-10604	927	CAGCUUGCGGGACAACAACTsT	928	GUUGUUGUCCCGCAAGCUGTsT	21%	1%
ND-10605	929	CAUCUUCUAUCCGCGGCCCTsT	930	GGGCCGCGGAUAGAAGAUGTsT	26%	2%
ND-10606	931	AUAAGCUCCAGGUUGACUUTsT	932	AAGUCAACCUGGAGCUUAUTsT	15%	1%

ND-10607	933	CUGCUGGUUACUCACGAUGTsT	934	CAUCGUGAGUAACCAGCAGTsT	85%	8%
ND-10608	935	GAACAAUUACACCGUCAACTsT	936	GUUGACGGUGUAAUUGUUCTsT	13%	1%
ND-10609	937	AUUACACCGUCAACAACAATsT	938	UUGUUGUUGACGGUGUAAUTsT	12%	0%
ND-10610	939	CUGUGGUUCGGCUCCUCGGTsT	940	CCGAGGAGCCGAACCACAGTsT	53%	2%
ND-10611	941	GAAGUGCCUUGGCUCCAGCTsT	942	GCUGGAGCCAAGGCACUUCTsT	24%	3%
ND-10612	943	GAUCAGCCAAGCGAAACUUTsT	944	AAGUUUCGCUUGGCUGAUCTsT	12%	0%
ND-10613	945	AGAAACCGCUGAUAACCAGTsT	946	CUGGUUAUCAGCGGUUUCUTsT	12%	1%
ND-10614	947	UGAUAACCAGGACAAAACATsT	948	UGUUUUGUCCUGGUUAUCATsT	7%	1%
ND-10615	949	CACGCAGGCAUGCACGGGUTsT	950	ACCCGUGCAUGCCUGCGUGTsT	12%	0%
ND-10616	951	GCUCUCCAGUAGCACAGAUTsT	952	AUCUGUGCUACUGGAGAGCTsT	9%	1%
ND-10617	953	CAGACCCGGGUAAGUAAAGTsT	954	CUUUACUUACCCGGGUCUGTsT	55%	3%
ND-10618	955	AGACCCGGGUAAGUAAAGGTsT	956	CCUUUACUUACCCGGGUCUTsT	72%	8%
ND-10619	957	AUCACUCCGCCUUCUCCUGTsT	958	CAGGAGAAGGCGGAGUGAUTsT	63%	6%
ND-10620	959	CACUCCGCCUUCUCCUGGGTsT	960	CCCAGGAGAAGGCGGAGUGTsT	28%	1%
ND-10621	961	AACUAGACUGUAAGUGCCUTsT	962	AGGCACUUACAGUCUAGUUTsT	23%	1%

ND-10622	963	UAUGCUCCAUGUCUAGCCCTsT	964	GGGCUAGACAUGGAGCAUATsT	98%	2%
ND-10623	965	CCCGAUGUAUGGAAACUGCTsT	966	GCAGUUUCCAUACAUCGGGTsT	11%	1%
ND-10624	967	GUACUGCUACUAUAAGCUCTsT	968	GAGCUUAUAGUAGCAGUACTsT	19%	1%
ND-10625	969	AGCGUGACCAGCUACCAGCTsT	970	GCUGGUAGCUGGUCACGCUTsT	49%	2%
ND-10626	971	ACAAUUACACCGUCAACAATsT	972	UUGUUGACGGUGUAAUUGUTsT	8%	0%
ND-10627	973	AUGCUCCUCUGGUGGGAGGTsT	974	CCUCCCACCAGAGGAGCAUTsT	76%	5%
ND-10628	975	AACAGUAGCUGAUGAAGCUTsT	976	AGCUUCAUCAGCUACUGUUTsT	22%	1%
ND-10629	977	CUGACUCCCGAGGGCUAGGTsT	978	CCUAGCCCUCGGGAGUCAGTsT	34%	2%
ND-10630	979	GUGCAACCAGAACAAAUCGTsT	980	CGAUUUGUUCUGGUUGCACTsT	10%	1%
ND-10631	981	UGCAACCAGAACAAAUCGGTsT	982	CCGAUUUGUUCUGGUUGCATsT	48%	4%
ND-10632	983	CUUCAAAGUACACACAGCATsT	984	UGCUGUGUGUACUUUGAAGTsT	20%	1%
ND-10633	985	CAGCGUGACCAGCUACCAGTsT	986	CUGGUAGCUGGUCACGCUGTsT	35%	1%
ND-10634	987	AGAACAAUUACACCGUCAATsT	988	UUGACGGUGUAAUUGUUCUTsT	14%	0%
ND-10635	989	GAUAACCAGGACAAAACACTsT	990	GUGUUUUGUCCUGGUUAUCTsT	11%	1%
ND-10636	991	ACAACCAAGGGUACACGCATsT	992	UGCGUGUACCCUUGGUUGUTsT	17%	1%

ND-10637	993	CCCAGCGACGGCUUAAGCCTsT	994	GGCUUAAGCCGUCGCUGGGTsT	27%	2%
ND-10638	995	CUCCCGAGGGCUAGGGCUATsT	996	UAGCCCUAGCCCUCGGGAGTsT	23%	1%
ND-10639	997	UAGAACCCUGCUCAGACACTsT	998	GUGUCUGAGCAGGGUUCUATsT	35%	2%
ND-10640	999	CCUGGCUGUUUCACCAAGTsT	1000	CUUGGUGAAACAGCCCAGGTsT	14%	1%
ND-10641	1001	GGAUCAGCCAAGCGAAACUTsT	1002	AGUUUCGCUUGGCUGAUCCTsT	16%	3%
ND-10642	1003	AAGAAACCGCUGAUAACCATsT	1004	UGGUUAUCAGCGGUUUCUUTsT	17%	1%
ND-10643	1005	ACCAAGGGUACACGCAGGCTsT	1006	GCCUGCGUGUACCCUUGGUTsT	37%	4%
ND-10644	1007	GUAGCACAGAUGUCUGCUCTsT	1008	GAGCAGACAUCUGUGCUACTsT	13%	3%
ND-10645	1009	UUUCAUACCUCUACAUGUCTsT	1010	GACAUGUAGAGGUAUGAAATsT	88%	8%
ND-10646	1011	CCAACCAUCUGCCAGAGAATsT	1012	UUCUCUGGCAGAUGGUUGGTsT	16%	2%
ND-10647	1013	GUCAGGGACUGAAUCUUGCTsT	1014	GCAAGAUUCAGUCCCUGACTsT	16%	3%
ND-10648	1015	AGCAUGAUCAAGGAGUGUGTsT	1016	CACACUCCCUUGAUCAUGCUTsT	50%	7%
ND-10649	1017	GCAGCGUGACCAGCUACCATsT	1018	UGGUAGCUGGUCACGCUGCTsT	40%	6%
ND-10650	1019	CAGCUCUCUGCUGGUUACUTsT	1020	AGUAACCAGCAGAGAGCUGTsT	56%	5%
ND-10651	1021	GUUCGGCUCCUCGGUGUUGTsT	1022	CAACACCGAGGAGCCGAACTsT	68%	5%

ND-10652	1023	GCAGAUGCUCCUCUGGUGGTsT	1024	CCACCAGAGGAGCAUCUGCTsT	26%	5%
ND-10653	1025	AGGAAGUUGCUCCAAGAACTsT	1026	GUUCUUGGAGCAACUUCCUTsT	18%	2%
ND-10654	1027	AACGCUUCUGCCACAUCUUTsT	1028	AAGAUGUGGCAGAAGCGUUTsT	18%	1%
ND-10655	1029	CACCUGGGCUGUUUCACCATsT	1030	UGGUGAAACAGCCCAGGUGTsT	17%	2%
ND-10656	1031	AAGCCAUGCAGCGUGACCATsT	1032	UGGUCACGCUGCAUGGCUUTsT	27%	3%
ND-10657	1033	CGAGGGCUAGGGCUAGAGCTsT	1034	GCUCUAGCCCUAGCCCUCGTsT	30%	1%
ND-10658	1035	GGAAACCCUGGACAGACUUTsT	1036	AAGUCUGUCCAGGGUUUCCTsT	14%	1%
ND-10659	1037	GUAGCUGAUGAAGCUGCCCTsT	1038	GGGCAGCUUCAUCAGCUACTsT	19%	1%
ND-10660	1039	UCUUUUUCCCUUGGAUCAGTsT	1040	CUGAUCCAAGGGAAAAAGATsT	88%	4%
ND-10661	1041	CUCCAGUAGCACAGAUGUCTsT	1042	GACAUCUGUGCUACUGGAGTsT	10%	1%
ND-10662	1043	CCAAAAUUGAUCACUCCGCTsT	1044	GCGGAGUGAUCAAUUUUGGTsT	25%	3%
ND-10663	1045	CAGACCACCUGGCUGUUUTsT	1046	AAACAGCCCAGGUGGUCUGTsT	24%	2%
ND-10664	1047	CCCUUCCCAACUAGACUGUTsT	1048	ACAGUCUAGUUGGGAAGGGTsT	15%	2%
ND-10665	1049	CGCAGCCGUCGCGACCUGCTsT	1050	GCAGGUCGCGACGCGCUGCGTsT	45%	2%
ND-10666	1051	UUCUCACACCAAGGCAGAUTsT	1052	AUCUGCCUUGGUGUGAGAATsT	25%	2%

ND-10667	1053	CACCACCAUCCACGGCGCCCTsT	1054	GGCGCCGUGGAUGGUGGUGTsT		35%	3%			
ND-10668	1055	CCAUUACUUUUGUGAACGCTsT	1056	GCGUUCACAAAAGUAAUGGTsT		19%	4%	2nd screen single dose @ 50 nM in H441; MV	16%	2%
ND-10669	1057	CCAAGAACAGUAGCUGAUGTsT	1058	CAUCAGCUACUGUUCUUGGTsT		23%	4%		17%	1%
ND-10670	1059	AGGAGAGGUUUCUCACACCTsT	1060	GGUGUGAGAAACCUCUCCUTsT		18%	3%		24%	2%
ND-10671	1061	AUCAUCCUGCUUGGAGCAATsT	1062	UUGCUCCAAGCAGGAUGAUTsT		33%	3%		27%	4%
ND-10672	1063	GCAUCACAGAGCAGACGCUTsT	1064	AGCGCUCUGCUCUGUGAUGCTsT		29%	2%		61%	3%
ND-10673	1065	AGGAGGUAGCCUCCACCCUTsT	1066	AGGGUGGAGGCUACCUCCCUTsT		63%	6%			
ND-10674	1067	ACAACCGCAUGAAGACGGCTsT	1068	GCCGUCUUCAUGCGGUUGUTsT		94%	2%		18%	1%
ND-10675	1069	GCAUGAAGACGGCCUUCUGTsT	1070	CAGAAGGCCGUCUUCAUGCTsT		20%	2%		60%	4%
ND-10676	1071	GUCACGAUGGUCACCCUCCTsT	1072	GGAGGGUGACCAUCGUGACTsT		66%	5%		18%	1%
ND-10677	1073	CCCUGCUCAGACACCAUUATsT	1074	UAAUGGUGUCUGAGCAGGGTsT		15%	3%			

ND-10678	1075	UCACGAUGGUCACCCUCCUTsT	1076	AGGAGGGUGACCAUCGUGATsT	80%	6%	70%	4%
ND-10679	1077	UCAACCUCAACUCGGACAATsT	1078	UUGUCCGAGUUGAGGUUGATsT	20%	3%	21%	1%
ND-10680	1079	UGACCAGCUACCAGCUCUCTsT	1080	GAGAGCUGGUAGCUGGUCATsT	88%	22%	77%	5%
ND-10681	1081	GAUGGCCCUCGGUGACAUCTsT	1082	GAUGUCACCGAGGGCCAUCTsT	88%	18%	60%	4%
ND-10682	1083	GCUUUGACAAGGAACUUUCTsT	1084	GAAAGUUCCUUGUCAAAGCTsT	19%	7%	14%	2%
ND-10683	1085	CGAUACUGGUCUCCAGGCCTsT	1086	GGCCUGGAGACCAGUAUCGTsT	27%	5%	27%	2%
ND-10684	1087	UCUGGAUGUCUUCCAUGCCTsT	1088	GGCAUGGAAGACAUCCAGATsT	92%	13%	89%	3%
ND-10685	1089	CAGGACCCUAGACCUCUGCTsT	1090	GCAGAGGUCUAGGGUCCUGTsT	58%	14%	50%	2%
ND-10686	1091	GACCCUAGACCUCUGCAGCTsT	1092	GCUGCAGAGGUCUAGGGUCTsT	27%	2%		
ND-10687	1093	ACCCUAGACCUCUGCAGCCTsT	1094	GGCUGCAGAGGUCUAGGGUTsT	21%	1%		
ND-10688	1095	CAGCCCACGGCGCGAGGAGGTsT	1096	CCUCCUCCGCCGUGGGCUGTsT	55%	4%		
ND-10689	1097	CUCUUCGAGUUCUUCUGCATsT	1098	UGCAGAAGAACUCGAAGAGTsT	13%	3%		
ND-10690	1099	UUGGCAUGAUGUACUGGCATsT	1100	UGCCAGUACAUCAUGCCAATsT	16%	2%		

ND-10691	1101	GGCAUGAUGUACUGGCAAUTsT	1102	AUUGCCAGUACAUCAUGCCTsT	13%	1%
ND-10692	1103	UGUACUGGCAAUUCGGCCUTsT	1104	AGGCCGAAUUGCCAGUACATsT	45%	2%
ND-10693	1105	ACUGGCAAUUCGGCCUGCUTsT	1106	AGCAGGCCGAAUUGCCAGUTsT	38%	3%
ND-10694	1107	GGCAAUUCGGCCUGCUUUUTsT	1108	AAAAGCAGGCCGAAUUGCCTsT	10%	1%
ND-10695	1109	CAAUUCGGCCUGCUUUUCGTsT	1110	CGAAAAGCAGGCCGAAUUGTsT	12%	1%
ND-10696	1111	UCGGAGAGUACUUCAGCUATsT	1112	UAGCUGAAGUACUCUCCGATsT	12%	1%
ND-10697	1113	CAACAUCCUGUCGAGGCUGTsT	1114	CAGCCUCGACAGGAUGUUGTsT	35%	7%
ND-10698	1115	CAUCCUGUCGAGGCUGCCATsT	1116	UGGCAGCCUCGACAGGAUGTsT	26%	6%
ND-10699	1117	UCCUGCAACCAGGCGAAUUTsT	1118	AAUUCGCCUGGUUGCAGGATsT	28%	6%
ND-10700	1119	GGAAACUGCUAUACUUUCATsT	1120	UGAAAGUAUAGCAGUUUCCTsT	7%	2%
ND-10701	1121	ACGGUCUGUCCCUGAUGCUTsT	1122	AGCAUCAGGGACAGACCGUTsT	28%	7%
ND-10702	1123	GGUCUGUCCCUGAUGCUGCTsT	1124	GCAGCAUCAGGGACAGACCTsT	33%	2%
ND-10703	1125	GGCCCGGGUAAUGGUGCACTsT	1126	GUGCACCAUUACCCGGGCCTsT	47%	10%
ND-10704	1127	CAGGAUGAACCUGCCUUUATsT	1128	UAAAGGCAGGUUCAUCCUGTsT	59%	2%
ND-10705	1129	GAUGAACCUGCCUUUAUGGTsT	1130	CCAUAAAGGCAGGUUCAUCTsT	77%	7%
ND-10706	1131	GGUGGCUUUAACUUGCGGCTsT	1132	GCCGCAAGUUAAAGCCACCTsT	47%	8%

ND-10707	1133	GUGGCUUUAACUUGCGGCCTsT	1134	GGCCGCAAGUUAAAGCCACTsT	17%	2%
ND-10708	1135	UUGCGGCCUGGCGUGGAGATsT	1136	UCUCCACGCCAGGCCGCAATsT	52%	3%
ND-10709	1137	UGCGGCCUGGCGUGGAGACTsT	1138	GUCUCCACGCCAGGCCGCATsT	81%	4%
ND-10710	1139	GCGGCCUGGCGUGGAGACCTsT	1140	GGUCUCCACGCCAGGCCGCTsT	57%	4%
ND-10711	1141	CAGGUGUAUUCACUCCUTsT	1142	AGGAGUGAAUACACACCUGTsT	24%	2%
ND-10712	1143	GUGUAUUCACUCCUGCUUCTsT	1144	GAAGCAGGAGUGAAUACACTsT	20%	1%
ND-10713	1145	GGCCCUCGGUGACAUCCCATsT	1146	UGGGAUGUCACCGAGGGCCTsT	40%	3%
ND-10714	1147	GAUGCUAUCGCGACAGAACTsT	1148	GUUCUGUCGCGAUAGCAUCTsT	24%	2%
ND-10715	1149	ACUACAAAACCAAUUCUGATsT	1150	UCAGAAUUGGUUUUGUAGUTsT	19%	2%
ND-10716	1151	CAAUUCUGAGUCUCCCUCUTsT	1152	AGAGGGAGACUCAGAAUUGTsT	35%	3%
ND-10717	1153	CUCUGUCACGAUGGUCACCTsT	1154	GGUGACCAUCGUGACAGAGTsT	41%	4%
ND-10718	1155	CUGCUCCGAAGGUUCCGAATsT	1156	UUCGGAACCUUCGGAGCAGTsT	16%	3%
ND-10719	1157	AGGUUCCGAAGCCGAUACUTsT	1158	AGUAUCGGCUUCCGAACCUTsT	16%	2%
ND-10720	1159	GUUCCGAAGCCGAUACUGGTsT	1160	CCAGUAUCGGCUUCGGAACTsT	21%	2%
ND-10721	1161	CGAAGCCGAUACUGGUCUCTsT	1162	GAGACCAGUAUCGGCUUCGTsT	16%	1%
ND-10722	1163	AAGAUUGAAGGAUGUGCAGTsT	1164	CUGCACAUCCUUCAAUCUUTsT	25%	2%

ND-10723	1165	GAUUGAAGGAUGUGCAGGGTsT	1166	CCCUGCACAUCCUUCAAUCTsT	26%	1%
ND-10724	1167	UGCCUCUGAACACUCUGGUTsT	1168	ACCAGAGUGUUCAGAGGCATsT	45%	3%
ND-10725	1169	CCUCUGAACACUCUGGUUUTsT	1170	AAACCAGAGUGUUCAGAGGTsT	15%	2%
ND-10726	1171	GACAAGGAACUUUCCUAAGTsT	1172	CUUAGGAAAGUUCCUUGUCTsT	105%	14%
ND-10727	1173	CAGGACAAAACACAACCAATsT	1174	UUGGUUGUGUUUUGUCCUGTsT	32%	5%
ND-10728	1175	AACACAACCAAGGGUACACTsT	1176	GUGUACCCUUGGUUGUGUUTsT	60%	13%
ND-10729	1177	UUGAACUUGGGUGGGAAACTsT	1178	GUUUCCCACCCAAGUUCAATsT	23%	8%
ND-10730	1179	UGAACUUGGGUGGGAAACCTsT	1180	GGUUUCCCACCCAAGUUCATsT	18%	4%
ND-10731	1181	ACCCGUGCCCUCACAGAGCTsT	1182	GCUCUGUGAGGGCACGGGUTsT	19%	1%
ND-10732	1183	ACUAUAACAUCUGCUGGAGTsT	1184	CUCCAGCAGAUGUUAUAGUTsT	17%	5%
ND-10733	1185	AUCUGCUGGAGUUGCUGTsT	1186	CAGCAACACUCCAGCAGAUTsT	119%	20%
ND-10734	1187	CUGCUGGAGUUGCUGUUUTsT	1188	AACAGCAACACUCCAGCAGTsT	58%	13%
ND-10735	1189	CUAGCCCAUCAUCCUGCUUTsT	1190	AAGCAGGAUGAUGGGCUAGTsT	20%	6%
ND-10736	1191	CUCUGGAUGUCUUCCAUGCTsT	1192	GCAUGGAAGACAUCCAGAGTsT	28%	8%
ND-10737	1193	AGCAGGACCCUAGACCUCUTsT	1194	AGAGGUCUAGGGUCCUGCUTsT	36%	2%

ND-10738	1195	UUCGAGUUCUUCUGCAACATsT	1196	UGUUGCAGAAGAACUCGAATsT	13%	1%
ND-10739	1197	CACCAUCCAGGGCGCCAUCTsT	1198	GAUGGCGCCGUGGAUGGUGTsT	13%	2%
ND-10740	1199	CCACGGCGCCAUCCGCCUGTsT	1200	CAGGCGGAUGGCGCCGUGGTsT	44%	4%
ND-10741	1201	CAGCACAACCGCAUGAAGATsT	1202	UCUUCAUGCGGUUGUGCUGTsT	23%	3%
ND-10742	1203	CCUUUGGCAUGAUGUACUGTsT	1204	CAGUACAUCAUGCCAAAGGTsT	12%	1%
ND-10743	1205	AUCCUGUCGAGGCUGCCAGTsT	1206	CUGGCAGCCUCGACAGGAUTsT	14%	3%
ND-10744	1207	UCUCCUGCAACCAGGCGAATsT	1208	UUCGCCUGGUUGCAGGAGATsT	12%	1%
ND-10745	1209	UGCAACCAGGCGAAUUACUTsT	1210	AGUAAUUCGCCUGGUUGCATsT	45%	5%
ND-10746	1211	ACCUCCAUCAGCAUGAGGATsT	1212	UCCUCAUGCUGAUGGAGGUTsT	82%	7%
ND-10747	1213	GCGACUGCACCAAGAAUGGTsT	1214	CCAUUCUUGGUGCAGUCGCTsT	82%	19%
ND-10748	1215	ACCAAGAAUGGCAGUGAUGTsT	1216	CAUCACUGCCAUUCUUGGUTsT	54%	18%
ND-10749	1217	UGGUUACUCACGAUGGCCCTsT	1218	GGGCCAUCGUGAGUAACCATsT	45%	7%
ND-10750	1219	AGAAAUGGAGUGGCCAAAGTsT	1220	CUUUGGCCACUCCAUUUCUTsT	11%	3%
ND-10751	1221	GGAGCUGAACUACAAAACCTsT	1222	GGUUUUGUAGUUCAGCUCCTsT	15%	3%
ND-10752	1223	CCUCUGUCACGAUGGUCACTsT	1224	GUGACCAUCGUGACAGAGGTsT	18%	5%
ND-10753	1225	AGAUUGAAGGAUGUGCAGGTsT	1226	CCUGCACAUCCUUCAAUCUTsT	26%	3%
ND-10754	1227	GAGCUUUGACAAGGAACUUTsT	1228	AAGUUCCUUGUCAAAGCUCTsT	14%	2%

ND-10755	1229	CUUUGACAAGGAACUUUCCTsT	1230	GGAAAGUUCCUUGUCAAAGTsT	50%	8%
ND-10756	1231	UCAGACACCAUUACUUUUGTsT	1232	CAAAAGUAAUGGUGUCUGATsT	32%	4%
ND-10757	1233	AGCACACUAUAACAUCUGCTsT	1234	GCAGAUGUUAUAGUGUGCUTsT	11%	2%
ND-10758	1235	GCACAACCGCAUGAAGACGTsT	1236	CGUCUUCAUGCGGUUGUGCTsT	34%	3%
ND-10759	1237	ACUGCUUCUACCAGACAUATsT	1238	UAUGUCUGGUAGAAGCAGUTsT	11%	1%
ND-10760	1239	GAAGACGGCCUUCUGGGCATsT	1240	UGCCCAGAAGGCCGUCUUCTsT	16%	2%
ND-10761	1241	AAGACGGCCUUCUGGGCAGTsT	1242	CUGCCCAGAAGGCCGUCUUTsT	58%	21%
ND-10762	1243	ACAUCAACCUCAACUCGGATsT	1244	UCCGAGUUGAGGUUGAUGUTsT	14%	3%
ND-10763	1245	UGGAAGGACUGGAAGAUCGTsT	1246	CGAUCUUCCAGUCCUUCCATsT	109%	29%
ND-10764	1247	ACAUCCUGUCGAGGCUGCCTsT	1248	GGCAGCCUCGACAGGAUGUTsT	101%	13%
ND-10765	1249	CAACCAGGCGAAUUACUCUTsT	1250	AGAGUAAUUCGCCUGGUUGTsT	19%	5%
ND-10766	1251	CAGGCGAAUUACUCUCACUTsT	1252	AGUGAGAGUAAUUCGCCUGTsT	24%	4%
ND-10767	1253	AGCAGAAUGACUUCAUUCCTsT	1254	GGAAUGAAGUCAUUCUGCUTsT	40%	8%
ND-10768	1255	AUGAUGGUGGCUUUAACUUTsT	1256	AAGUUAAAGCCACCAUCAUTsT	85%	8%
ND-10769	1257	AGAACCUUUACCCUUCAAATsT	1258	UUUGAAGGGUAAAGGUUCUTsT	22%	4%
ND-10770	1259	CCUUUACCCUUCAAAGUACTsT	1260	GUACUUUGAAGGGUAAAGGTsT	21%	4%

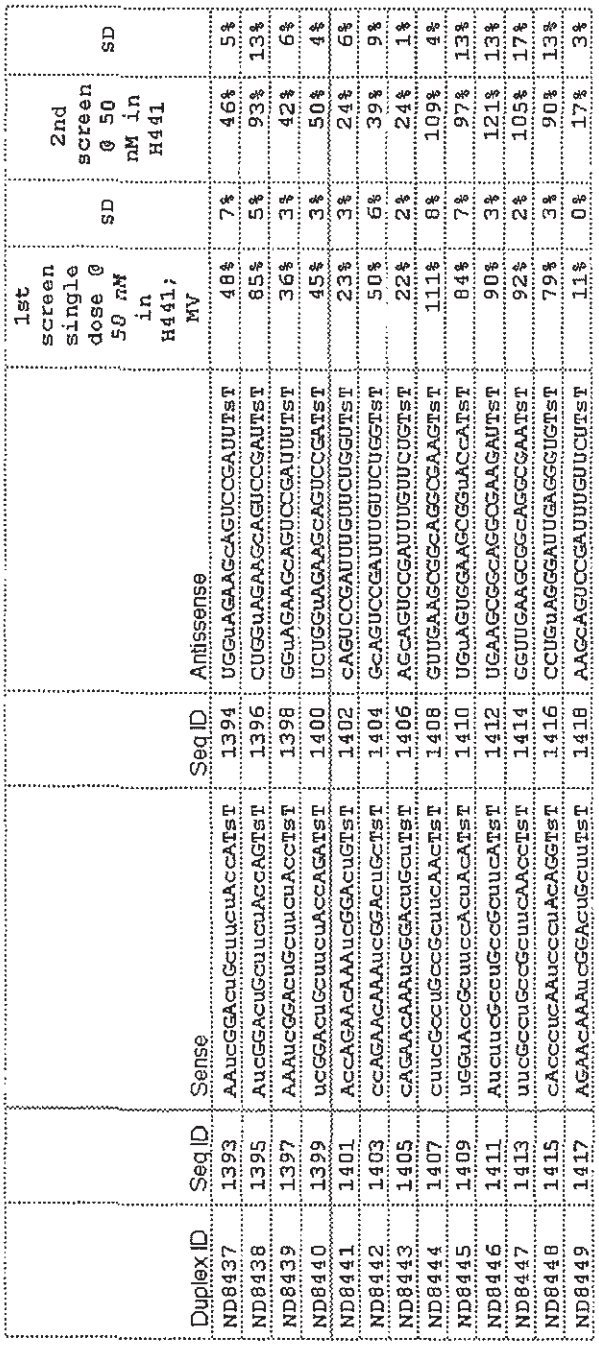
ND-10771	1261	GAGCCUGUGGUUCGGCUCCUsT	1262	GGAGCCGAACCACAGGCUCUsT	28%	1%
ND-10772	1263	UGGUACUGCCUCUGAACACUsT	1264	GUGUUCAGAGGCAGUACCAUsT	58%	4%
ND-10773	1265	CUCAUACCCGUGCCCUCACUsT	1266	GUGAGGGCACGGGUAUGAGUsT	15%	2%
ND-10774	1267	CCGUAGCACACUAUAACAUUsT	1268	AUGUUAUAGUGUGCUACGGUsT	24%	6%
ND-10775	1269	CGUAGCACACUAUAACAUCUsT	1270	GAUGUUAUAGUGUGCUACGUsT	21%	4%
ND-10776	1271	GCAGGACCCUAGACCUCUGUsT	1272	CAGAGGUCUAGGGUCCUGCUsT	25%	4%
ND-10777	1273	GCCUGCUUUUCGGAGAGUAUsT	1274	UACUCUCCGAAAAGCAGGCUsT	18%	4%
ND-10778	1275	GGGCCCGGGUAAUGGUGCAUsT	1276	UGCACCAUUACCCGGGCCCUsT	16%	2%
ND-10779	1277	CAACAACAAGAGAAAUGGAUsT	1278	UCCAUUUCUCUUGUUGUUGUsT	17%	0%
ND-10780	1279	GCUGUUGCACCAUACUUUCUsT	1280	GAAAGUAUGGUGCAACAGCUsT	14%	1%
ND-10781	1281	CUACCGAGAGCUCUUCGAGUsT	1282	CUCGAAGAGCUCUCGGUAGUsT	24%	3%
ND-10782	1283	ACCUGCCUUUAUGGAUGAUUsT	1284	AUCAUCCAUAAAGGCAGGUUsT	115%	10%
ND-10783	1285	UUGACAAGGAACUUUCCUAUsT	1286	UAGGAAAGUUCCUUGUCAAUsT	16%	1%
ND-10784	1287	GCUGGAGUGUUGCUGUUGCUsT	1288	GCAACAGCAACACUCCAGCUsT	12%	1%
ND-10785	1289	UCGGUGACAUCCCAGGAAUUsT	1290	AUUCCUGGGAUGUCACCGAUsT	28%	1%
ND-10786	1291	GCUGCCCAGAAGUGCCUUGUsT	1292	CAAGGCACUUCUGGGCAGCUsT	15%	2%
ND-10787	1293	AGUACACACAGCAGGUGUGUsT	1294	CACACCUGCUGUGUGUACUUsT	12%	1%
ND-10788	1295	CAAGUGCCGGAAGCCAUGCUsT	1296	GCAUGGCUUCCGGCACUUGUsT	94%	2%

Table 1C: Selected siRNAs in *in vivo* rat surrogate set (human-rat cross-reactive siRNAs with highest specificity in rat). A screening set of 48 human and rat cross-reactive alpha-ENaC iRNA sequences were identified. The percentage residual expression of alpha-ENaC in two independent single-dose transfection experiments is shown (refer to examples section for methods used).

Duplex ID	Seq ID	Sense	Seq ID	Antisense	1st screen single dose @ 50 nM in H441; MV	SD	2nd screen @ 50 nM in H441	SD
ND-9201	1297	uGuGcAAccAGAAcAAAAucTsT	1298	GAUUUGUUCUGGUUGcAcATsT	8%	1%	8%	1%
ND-9202	1299	uuuAuGGAuGAuGGuGGcuTsT	1300	AGCcACcAUcAUCcAUCcAuAAATsT	80%	9%	82%	6%
ND-9203	1301	GccuuuAuGGAuGAuGAuGuGuTsT	1302	cACcAUcAUCcAUCcAuAAAGGCTsT	76%	8%	76%	2%
ND-9204	1303	cAcAAccGcAuGAAGAcGGTsT	1304	CCGUCUUCuAUGCGGUUGUGTsT	73%	18%	57%	3%
ND-9205	1305	AccGcAuGAAGAcGGccuuTsT	1306	AAGGCCGUCUUCuAUGCGGUTsT	35%	3%	37%	2%
ND-9206	1307	AGGAcuGGAAGAucGGcuuTsT	1308	AAGCCGAUCUUCcAGUCCUTsT	17%	3%	16%	3%
ND-9207	1309	GAAGGAcuGGAAGAucGGcTsT	1310	GCCGAUCUUCcAGUCCUUCTsT	96%	18%	81%	5%
ND-9208	1311	GGAcuGGAAGAucGGcuucTsT	1312	GAAGCCGAUCUUCcAGUCCTsT	58%	6%	57%	3%
ND-9209	1313	AGuuccAccGcuccuAccGTsT	1314	CGGuAGGAGCGGUGGAACUTsT	85%	8%	94%	4%
ND-9210	1315	GAcuGGAAGAucGGcuuccTsT	1316	GGAAGCCGAUCUUCcAGUCTsT	79%	5%	82%	2%
ND-9211	1317	cGcAuGAAGAcGGccuucuTsT	1318	AGAAGGCCGUCUUCcAUGCGTsT	50%	1%	51%	1%
ND-9212	1319	GccAGuGGAGccuGuCGuuTsT	1320	AACcAcAGGCUCcACUGGCTsT	26%	3%	23%	2%
ND-9213	1321	uGccuuuAuGGAuGAuGGuTsT	1322	ACcAUcAUCcAuAAAGGcATsT	77%	5%	76%	4%
ND-9214	1323	uccuGuccAAccuGGGcAGTsT	1324	CUGCCcAGGUUGGAcAGGATsT	74%	9%	83%	6%

ND-9215	1325	AGGGAGuGGuACcGcuucCTsT	1326	GGAAGCGGuACcACUCCCUTsT	79%	6%	89%	4%
ND-9216	1327	GGcuGuGGcuAcAucuucuTsT	1328	AGAAGAUGuAGGcACAGCCTsT	11%	1%	13%	1%
ND-9217	1329	GAAAuuAAAGAGGAGCuGGTsT	1330	CcAGCUCCUCUUUuAAUUUCTsT	84%	14%	78%	5%
ND-9218	1331	AcuGGAAGAucGGcuucAcATsT	1332	UGGAAGCCGAUCUUCcAGUTsT	50%	4%	55%	3%
ND-9219	1333	ccuGucCAAccuGGGcAGcTsT	1334	GCUGCCCAGGUUGGAcAGGTsT	78%	6%	85%	5%
ND-9220	1335	ccuGccuuuAuGGAuGAuGTsT	1336	cAUcAUCcAuAAAGGcAGGTsT	76%	5%	77%	9%
ND-9221	1337	AAccGcAuGAAcGGccuTsT	1338	AGGCCGUCUUcAUGCGGUUTsT	79%	8%	66%	3%
ND-9222	1339	uGuccAAccuGGGcAGccATsT	1340	UGGCUGCCcAGGUUGGAcATsT	70%	4%	57%	4%
ND-9223	1341	GucCAAccuGGGcAGccAGTsT	1342	CUGGCUGCCcAGGUUGGACTsT	95%	10%	76%	4%
ND-9224	1343	AAAuuAAAGAGGAGCuGGATsT	1344	UCcAGCUCCUCUUuAAUUUTsT	83%	6%	69%	2%
ND-9225	1345	GGAAGGAcuGGAAGAucGGTsT	1346	CCGAUCUUCcAGUCCUUCCTsT	41%	2%	30%	2%
ND-9226	1347	GuGAGGGAGuGGuAccGcuTsT	1348	AGCGGuACcACUCCUcACTsT	21%	1%	17%	0%
ND-9227	1349	AcuuucAAuGAcAAGAAcATsT	1350	UGUUCUUGUcAUUGAAAGUTsT	13%	1%	10%	0%
ND-9228	1351	ucAAuGAcAAGAAcAAcucTsT	1352	GAGUUGUUCUUGUcAUUGATsT	36%	2%	28%	0%
ND-9229	1353	cuuuAuGGAuGAuGGuGGcTsT	1354	GCCACcAUcAUCcAUAAAGTsT	24%	1%	20%	1%
ND-9230	1355	GCcuGGcGuGGAGAcucccTsT	1356	GGAGGUCUCcACGCcAGGCTsT				
ND-9231	1357	uGGcGuGGAGAccucccAucTsT	1358	GAUGGAGGUCUCcACGCcATsT	45%	4%	35%	2%
ND-9232	1359	GAGuuccAccGcucucAccTsT	1360	GGuAGGAGCGCGUGGAACUCTsT	89%	4%	86%	8%
ND-9233	1361	cAGAGcAGAAuGAcuucAuTsT	1362	AUGAAGUcAUUCUGCUCUGTsT	21%	1%	17%	0%
ND-9234	1363	uucAcuccuGcuuccAGGATsT	1364	UCCUGGAAGcAGGAGUGAATsT	85%	4%	74%	6%
ND-9235	1365	ucAcuccuGcuuccAGGAGTsT	1366	CUCCUGGAAGcAGGAGUGATsT				
ND-9236	1367	cuGuGcAAccAGAAcAAAuTsT	1368	AUUUGUUCUGGUUGcAcAGTsT	23%	1%	17%	2%
ND-9237	1369	cuGcAAcAAcAccAccAucTsT	1370	GAUGGUGGUGUUGUUGcAGTsT	34%	2%	27%	2%
ND-9238	1371	uGuGGcuGuGccuAcAucuTsT	1372	AGAUGuAGGcAcAGCcAcATsT	86%	4%	73%	10%
ND-9239	1373	uGGcuGuGGcuAcAucuucTsT	1374	GAAGAUGuAGGcAcAGCcATsT	68%	6%	53%	4%
ND-9240	1375	cuGucCAAccuGGGcAGccTsT	1376	GGCUGCCcAGGUUGGAcAGTsT	80%	5%	73%	9%
ND-9241	1377	cccuGcuGuccAcAGuGAcTsT	1378	GUcACUGUGGAcAGcAGGGTsT	83%	5%	71%	5%
ND-9242	1379	GcAGccAGuGGAGccuGuGTsT	1380	cAcAGGCUCcACUGGCUGCTsT	105%	9%	90%	5%
ND-9243	1381	uucAAuGAcAAGAAcAAcuTsT	1382	AGUUGUUCUUGUcAUUGAATsT	23%	3%	21%	1%
ND-9244	1383	cuGccuuuAuGGAuGAuGGTsT	1384	CcAUcAUCcAuAAAGGcAGTsT	74%	6%	64%	7%
ND-9245	1385	AAuGAcAAGAAcAAcuccATsT	1386	UGGAGUUGUUCUUGUcAUUTsT	21%	1%	21%	1%
ND-9246	1387	uGGGcAGccAGuGGAGccuTsT	1388	AGGCUCcACUGGCUGCCcATsT	83%	3%	73%	2%
ND-9247	1389	cuccuGuccAAccuGGGcATsT	1390	UGCCcAGGUUGGAcAGGAGTsT	86%	3%	84%	1%
ND-9248	1391	GGcGuGGAGAccucccAucATsT	1392	UGAUGGAGGUCUCcACGCCTsT	92%	4%	88%	3%

Table 1D: Selected siRNAs in *in vivo* guinea pig surrogate set (human-guinea pig cross-reactive siRNAs). A screening set of 63 human and guinea-pig cross-reactive alpha-ENaC iRNA sequences were identified and synthesised, both with (sequence strands 1393-1518) and without (sequence strands 1519-1644) backbone modification. The percentage residual expression of alpha-ENaC in two independent single-dose transfection experiments is shown (refer to examples section for methods used).



Duplex ID	Seq ID	Sense	Seq ID	Antisense	1st screen single dose @ 50 nM in H441; MV	SD	2nd screen @ 50 nM in H441	SD
ND8437	1393	AAucGGAcuGcuucuAccATsT	1394	UGGuAGAAGcAGUCCGAUUTsT	48%	7%	46%	5%
ND8438	1395	AucGGAcuGcuucuAccAGTsT	1396	CUGGuAGAAGcAGUCCGAUTsT	85%	5%	93%	13%
ND8439	1397	AAAucGGAcuGcuucuAccTsT	1398	GGuAGAAGcAGUCCGAUUUTsT	36%	3%	42%	6%
ND8440	1399	ucGGAcuGcuucuAccAGATsT	1400	UCUGGuAGAAGcAGUCCGATsT	45%	3%	50%	4%
ND8441	1401	AccACAAcAAAucGGAcuGTsT	1402	cAGUCCGAUUUGUUCUGGUTsT	23%	3%	24%	6%
ND8442	1403	ccAGAAcAAAucGGAcuGcTsT	1404	GcAGUCCGAUUUGUUCUGGTsT	50%	6%	39%	9%
ND8443	1405	cAGAAcAAAucGGAcuGcuTsT	1406	AGcAGUCCGAUUUGUUCUGTsT	22%	2%	24%	1%
ND8444	1407	cuucGccuGccGcuucAAcTsT	1408	GUUGAAGCGGcAGGCGAAGTsT	111%	8%	109%	4%
ND8445	1409	uGGuAccGcuucccAcuACATsT	1410	UGuAGUGGAAGCGGuACcATsT	84%	7%	97%	13%
ND8446	1411	AucuucGccuGccGcuucATsT	1412	UGAAGCGGcAGGCGAAGAUTsT	90%	3%	121%	13%
ND8447	1413	uucGccuGccGcuucAAccTsT	1414	GGUUGAAGCGGCAGGCGAATsT	92%	2%	105%	17%
ND8448	1415	cAcccucAAucccuAcAGGTsT	1416	CCUGuAGGGAUUGAGGGUGTsT	79%	3%	90%	13%
ND8449	1417	AGAAcAAAucGGAcuGcuuTsT	1418	AAGcAGUCCGAUUUGUUCUTsT	11%	0%	17%	3%

ND8450	1419	GAACAAAucGGAcuGcuucTsT	1420	GAAGcAGUCCGAUUUGUUCTsT	21%	1%	30%	5%
ND8451	1421	cGGAcuGCuucuAccAGAcTsT	1422	GUCUGGuAGAAGcAGUCCGTsT	24%	2%	32%	5%
ND8452	1423	AGccucAAcAucAAccucAUTsT	1424	UGAGGUUGAUGUUGAGGCUTsT	51%	3%	57%	4%
ND8453	1425	GccucAACAcAucAAccucAATsT	1426	UUGAGGUUGAUGUUGAGGCTsT	16%	1%	26%	3%
ND8454	1427	GucAGccucAAcAucAAccTsT	1428	GGUUGAUGUUGAGGCUGACTsT	62%	5%	68%	6%
ND8455	1429	ucAGccucAAcAucAAccuTsT	1430	AGGUUGAUGUUGAGGCUGATsT	77%	4%	87%	6%
ND8456	1431	cAGccucAAcAucAAccucTsT	1432	GAGGUUGAUGUUGAGGCUGTsT	34%	2%	51%	8%
ND8457	1433	GGAGcuGGAccGcAucAcATsT	1434	UGUGAUGCGGUCcAGCUCCTsT	26%	2%	17%	1%
ND8458	1435	GuAccGccuuccAcuAcAucTsT	1436	GAUGuAGUGGAAGCGGuACTsT	101%	9%	99%	11%
ND8459	1437	ccGcuuccAcuAcAucAAcTsT	1438	GUUGAUGuAGUGGAAGCGGTsT	85%	8%	80%	6%
ND8460	1439	cGcuuccAcuAcAucAAcATsT	1440	UGUUGAUGuAGUGGAAGCGTsT	56%	6%	48%	3%
ND8461	1441	uuccAcuAcAucAAcAuccTsT	1442	GGAUGUUGAUGuAGUGGAATsT	77%	5%	82%	7%
ND8462	1443	uGGGcAAcuucAucuucGcTsT	1444	GCGAAGAUGAAGUUGCCcATsT	21%	0%	36%	6%
ND8463	1445	GcAAcuucAucuucGccuGTsT	1446	cAGGCGAAGAUGAAGUUGCTsT	80%	4%	84%	13%
ND8464	1447	cAAcuucAucuucGccuGcTsT	1448	GcAGGCGAAGAUGAAGUUGTsT	101%	1%	102%	14%
ND8465	1449	AAcuucAucuucGccuGccTsT	1450	GGcAGGCGAAGAUGAAGUUTsT	100%	4%	95%	12%
ND8466	1451	AcuucAucuucGccuGccGTsT	1452	CGGcAGGCGAAGAUGAAGUTsT	51%	4%	49%	5%
ND8467	1453	cuucAucuucGccuGccGcTsT	1454	GCGGcAGGCGAAGAUGAAGTsT	95%	5%	89%	4%
ND8468	1455	ucAucuucGccuGccGccuTsT	1456	AAGCGGcAGGCGAAGAUGATsT	91%	4%	85%	6%
ND8469	1457	cAucuucGccuGccGcuucTsT	1458	GAAGCGGcAGGCGAAGAUGTsT	66%	4%	55%	4%
ND8470	1459	ucuucGccuGccGcuucAATsT	1460	UUGAAGCGGcAGGCGAAGATsT	97%	2%	99%	11%
ND8471	1461	cGccuGccGcuucAAccAGTsT	1462	CUGGUUGAAGCGGcAGGCGTsT	96%	4%	100%	7%
ND8472	1463	GccuGccGcuucAAccAGGTsT	1464	CCUGGUUGAAGCGGcAGGCTsT	90%	4%	82%	5%
ND8473	1465	AuuAcucucAcuuccAccATsT	1466	UGGUGGAAGUGAGAGuAAUTsT	81%	3%	72%	4%
ND8474	1467	uuAcucucAcuuccAccAcTsT	1468	GUGGUGGAAGUGAGAGuAATsT	72%	2%	76%	9%

ND8475	1469	AcucuCAcuuccAccAcccTsT	1470	GGGUGGUGGAAGUGAGAGUTsT	90%	3%	97%	4%
ND8476	1471	ucuGcAcccucAAucccuATsT	1472	uAGGGAUUGAGGGUGcAGATsT	61%	1%	63%	3%
ND8477	1473	cuGcAcccucAAucccuAcTsT	1474	GuAGGGAUUGAGGGUGcAGTsT	74%	3%	73%	1%
ND8478	1475	uGcAcccucAAucccuAcATsT	1476	UGuAGGGAUUGAGGGUGcATsT	98%	4%	85%	1%
ND8479	1477	AcccuAAucccuAcAGGuTsT	1478	ACCUGuAGGGAUUGAGGGUTsT	55%	5%	48%	3%
ND8480	1479	cccucAAucccuAcAGGuATsT	1480	uACCUGuAGGGAUUGAGGGTsT	20%	1%	14%	1%
ND8481	1481	ccucAAucccuAcAGGuAcTsT	1482	GuACCUGuAGGGAUUGAGGTsT	40%	2%	31%	3%
ND8482	1483	AAccAGAAcAAAAucGGAcuTsT	1484	AGUCCGAUUUGUUCUGGUUTsT	57%	2%	52%	0%
ND8483	1485	AAcAAAAucGGAcuGcuucuTsT	1486	AGAAGcAGUCCGAUUUGUUTsT	102%	5%	86%	12%
ND8484	1487	AcAAAAucGGAcuGcuucuATsT	1488	uAGAAGcAGUCCGAUUUGUTsT	40%	2%	28%	3%
ND8485	1489	cAAAucGGAcuGcuucuAcTsT	1490	GuAGAAGcAGUCCGAUUUGTsT	41%	4%	38%	2%
ND8486	1491	GcAcccucAAucccuAcAGTsT	1492	CUGuAGGGAUUGAGGGUGCTsT	91%	7%	94%	4%
ND8487	1493	ccucAAcAucAAccucAAcTsT	1494	GUUGAGGUUGAUGUUGAGGTsT	46%	2%	37%	3%
ND8488	1495	cucAAcAucAAccucAAcuTsT	1496	AGUUGAGGUUGAUGUUUGAGTsT	48%	2%	39%	3%
ND8489	1497	ucAAcAucAAccucAAcucTsT	1498	GAGUUGAGGUUGAUGUUGATsT	17%	1%	17%	1%
ND8490	1499	uAccGcuuccAcuAcAucATsT	1500	UGAUGuAGUGGAAGCGGuATsT	90%	5%	74%	8%
ND8491	1501	AccGcuuccAcuAcAucAATsT	1502	UUGAUGuAGUGGAAGCGGUTsT	103%	5%	91%	15%
ND8492	1503	GcuuccAcuAcAucAAcAuTsT	1504	AUGUUGAUGuAGUGGAAGCTsT	85%	5%	71%	10%
ND8493	1505	cuuccAcuAcAucAAcAucTsT	1506	GAUGUUGAUGuAGUGGAAGTsT	60%	5%	45%	3%
ND8494	1507	uccAcuAcAucAAcAuccuTsT	1508	AGGAUGUUGAUGuAGUGGATsT	33%	3%	41%	3%
ND8495	1509	ccAcuAcAucAAcAuccuGTsT	1510	cAGGAUGUUGAUGuAGUGGTsT	60%	5%	55%	2%
ND8496	1511	cuGGGcAAcuucAucuucGTsT	1512	CGAAGAUGAAGUUGCCcAGTsT	18%	0%	20%	0%
ND8497	1513	GGcAAcuucAucuucGccuTsT	1514	AGGCGAAGAUGAAGUUGCCTsT	76%	1%	77%	2%
ND8498	1515	uucAucuucGccuGccGcuTsT	1516	AGCGGcAGGCGAAGAUGAATsT	65%	4%	74%	12%
ND8499	1517	ucGccuGccGcuucAAccATsT	1518	UGGUUGAAGCGGcAGGCGATsT	86%	5%	77%	3%

ND-8653	1519	AAUCGGACUGCUUCUACCATsT	1520	UGGUAGAAGCAGUCCGAUUTsT	16%	2%	20%	3%
ND-8654	1521	AUCGGACUGCUUCUACCAGTsT	1522	CUGGUAGAAGCAGUCCGAUTsT	54%	8%	67%	11%
ND-8655	1523	AAAUCGGACUGCUUCUACCTsT	1524	GGUAGAAGCAGUCCGAUUUTsT	25%	4%	28%	2%
ND-8656	1525	UCGGACUGCUUCUACCAGATsT	1526	UCUGGUAGAAGCAGUCCGATsT	12%	2%	17%	1%
ND-8657	1527	ACCAGAACAAAUCGGACUGTsT	1528	CAGUCCGAUUUGUUCUGGUTsT	33%	3%	35%	1%
ND-8658	1529	CCAGAACAAAUCGGACUGCTsT	1530	GCAGUCCGAUUUGUUCUGGTsT	27%	3%	30%	2%
ND-8659	1531	CAGAACAAAUCGGACUGCUTsT	1532	AGCAGUCCGAUUUGUUCUGTsT	15%	1%	22%	3%
ND-8660	1533	CUUCGCCUGCCGCUUCAACTsT	1534	GUUGAAGCGGCAGGCGAAGTsT	69%	17%	75%	10%
ND-8661	1535	UGGUACCGCUUCCACUACATsT	1536	UGUAGUGGAAGCGGUACCATsT	16%	2%	20%	3%
ND-8662	1537	AUCUUCGCCUGCCGCUUCATsT	1538	UGAAGCGGCAGGCGAAGAUTsT	19%	2%	25%	4%
ND-8663	1539	UUCGCCUGCCGCUUCAACCTsT	1540	GGUUGAAGCGGCAGGCGAATsT	90%	4%	97%	10%
ND-8664	1541	CACCCUCAAUCCCUACAGGTsT	1542	CCUGUAGGGAUUGAGGGUGTsT	19%	2%	25%	3%
ND-8665	1543	AGAACAAAUCGGACUGCUUTsT	1544	AAGCAGUCCGAUUUGUUCUTsT	13%	1%	22%	2%
ND-8666	1545	GAACAAAUCGGACUGCUUCTsT	1546	GAAGCAGUCCGAUUUGUUCTsT	11%	2%	18%	2%
ND-8667	1547	CGGACUGCUUCUACCAGACTsT	1548	GUCUGGUAGAAGCAGUCCGTsT	13%	1%	16%	2%
ND-8668	1549	AGCCUCAACAUCAACCUCATsT	1550	UGAGGUUGAUGUUGAGGCUTsT	17%	4%	21%	3%
ND-8669	1551	GCCUCAACAUCAACCUCAATsT	1552	UUGAGGUUGAUGUUGAGGCTsT	13%	1%	21%	3%
ND-8670	1553	GUCAGCCUCAACAUCAACCTsT	1554	GGUUGAUGUUGAGGCUGACTsT	43%	11%	27%	3%
ND-8671	1555	UCAGCCUCAACAUCAACCUTsT	1556	AGGUUGAUGUUGAGGCUGATsT	90%	17%	53%	13%
ND-8672	1557	CAGCCUCAACAUCAACCUCTsT	1558	GAGGUUGAUGUUGAGGCUGTsT	17%	3%	11%	3%
ND-8673	1559	GGAGCUGGACCGCAUCACATsT	1560	UGUGAUGCGGUCCAGCUCCTsT	25%	3%	18%	3%
ND-8674	1561	GUACCGCUUCCACUACAUCTsT	1562	GAUGUAGUGGAAGCGGUACTsT	21%	4%	16%	4%
ND-8675	1563	CCGCUUCCACUACAUCAACTsT	1564	GUUGAUGUAGUGGAAGCGGTsT	25%	4%	19%	3%
ND-8676	1565	CGCUUCCACUACAUCAACATsT	1566	UGUUGAUGUAGUGGAAGCGTsT	16%	3%	14%	1%
ND-8677	1567	UUCCACUACAUCAACAUCCTsT	1568	GGAUGUUGAUGUAGUGGAATsT	110%	19%	97%	9%

ND-8678	1569	UGGGCAACUUCAUCUUCGCTsT	1570	GCGAAGAUGAAGUUGCCCATsT	50%	8%	40%	5%
ND-8679	1571	GCAACUUCAUCUUCGCCUGTsT	1572	CAGGCGAAGAUGAAGUUGCTsT	19%	3%	17%	2%
ND-8680	1573	CAACUUCAUCUUCGCCUGCTsT	1574	GCAGGCGAAGAUGAAGUUGTsT	25%	2%	23%	2%
ND-8681	1575	AACUUCAUCUUCGCCUGCCTsT	1576	GGCAGGCGAAGAUGAAGUUTsT	104%	7%	85%	10%
ND-8682	1577	ACUUCAUCUUCGCCUGCGTsT	1578	CGCAGGCGAAGAUGAAGUUTsT	91%	8%	63%	9%
ND-8683	1579	CUUCAUCUUCGCCUGCCGCTsT	1580	GCGGCAGGCGAAGAUGAAGTsT	88%	6%	58%	6%
ND-8684	1581	UCAUCUUCGCCUGCCGCUUTsT	1582	AAGCGGCAGGCGAAGAUGATsT	76%	3%	64%	4%
ND-8685	1583	CAUCUUCGCCUGCCGCUUCTsT	1584	GAAGCGGCAGGCGAAGAUGTsT	15%	1%	18%	3%
ND-8686	1585	UCUUCGCCUGCCGCUUCAATsT	1586	UUGAAGCGGCCAGGCGAAGATsT	109%	22%	31%	3%
ND-8687	1587	CGCCUGCCGCUUCAACCAGTsT	1588	CUGGUUGAAGCGGCAGGCGTsT	90%	21%	49%	2%
ND-8688	1589	GCCUGCCGCUUCAACCAGGTsT	1590	CCUGGUUGAAGCGGCAGGCTsT	43%	9%	24%	7%
ND-8689	1591	AUUACUCUCACUUCCACCATsT	1592	UGGUGGAAGUGAGAGUAAUTsT	27%	4%	19%	2%
ND-8690	1593	UUACUCUCACUUCCACCACTsT	1594	GUGGUGGAAGUGAGAGUAATsT	109%	7%	85%	8%
ND-8691	1595	ACUCUCACUUCCACCACCCTsT	1596	GGGUGGUGGAAGUGAGAGUTsT	93%	11%	87%	12%
ND-8692	1597	UCUGCACCCUCAAUCCCUATsT	1598	UAGGGAUUGAGGGUGCAGATsT	31%	12%	17%	2%
ND-8693	1599	CUGCACCCUCAAUCCCUACTsT	1600	GUAGGGAUUGAGGGUGCAGTsT	41%	25%	31%	4%
ND-8694	1601	UGCACCCUCAAUCCCUACATsT	1602	UGUAGGGAUUGAGGGUGCATsT	75%	25%	43%	3%
ND-8695	1603	ACCCUCAAUCCCUACAGGUTsT	1604	ACCUGUAGGGAUUGAGGGUTsT	65%	26%	25%	5%
ND-8696	1605	CCCUCAAUCCCUACAGGUATsT	1606	UACCUGUAGGGAUUGAGGGTsT	18%	2%	13%	1%
ND-8697	1607	CCUCAAUCCCUACAGGUACTsT	1608	GUACCUGUAGGGAUUGAGGTsT	16%	4%	13%	2%
ND-8698	1609	AACCAGAACAAAUCGGACUTsT	1610	AGUCCGAUUUGUUCUGGUUTsT	40%	2%	30%	2%
ND-8699	1611	AACAAAUCGGACUGCUUCUTsT	1612	AGAAGCAGUCCGAUUUGUUTsT	56%	4%	45%	3%
ND-8700	1613	ACAAAUCGGACUGCUUCUATsT	1614	UAGAAGCAGUCCGAUUUGUTsT	18%	3%	12%	1%
ND-8701	1615	CAAAUCGGACUGCUUCUACTsT	1616	GUAGAAGCAGUCCGAUUUGTsT	15%	2%	15%	4%
ND-8702	1617	GCACCCUCAAUCCCUACAGTsT	1618	CUGUAGGGAUUGAGGGUGCTsT	53%	4%	46%	20%

ND-8703	1619	CCUCAACAUCAACCUCAACTsT	1620	GUUGAGGUUGAUGUUGAGGTsT	25%	6%	26%	9%
ND-8704	1621	CUCAACAUCAACCUCAACUTsT	1622	AGUUGAGGUUGAUGUUGAGTsT	30%	8%	37%	26%
ND-8705	1623	UCAACAUCAACCUCAACUCTsT	1624	GAGUUGAGGUUGAUGUUGATsT	55%	1%	50%	10%
ND-8706	1625	UACCGCUUCCACUACAUCATsT	1626	UGAUGUAGUGGAAGCGGUATsT	36%	7%	31%	7%
ND-8707	1627	ACCGCUUCCACUACAUCAATsT	1628	UUGAUGUAGUGGAAGCGGUTsT	23%	5%	27%	10%
ND-8708	1629	GCUUCCACUACAUCAACAUTsT	1630	AUGUUGAUGUAGUGGAAGCTsT	16%	4%	24%	12%
ND-8709	1631	CUUCCACUACAUCAACAUCTsT	1632	GAUGUUGAUGUAGUGGAAGTsT	62%	3%	74%	27%
ND-8710	1633	UCCACUACAUCAACAUCCUTsT	1634	AGGAUGUUGAUGUAGUGGATsT	45%	8%	41%	1%
ND-8711	1635	CCACUACAUCAACAUCCUGTsT	1636	CAGGAUGUUGAUGUAGUGGTsT	23%	4%	27%	10%
ND-8712	1637	CUGGGCAACUUCAUCUUCGTsT	1638	CGAAGAUGAAGUUGCCCAGTsT	34%	4%	26%	5%
ND-8713	1639	GGCAACUUCAUCUUCGCCUTsT	1640	AGGCGAAGAUGAAGUUGCCTsT	30%	3%	23%	2%
ND-8714	1641	UUCAUCUUCGCCUGCCGCUTsT	1642	AGCGGCAGGCGAAGAUGAATsT	90%	14%	85%	14%
ND-8715	1643	UCGCCUGCCGCUUCAACCATsT	1644	UGGUUGAAGCGGCAGGCGATsT	23%	2%	20%	4%

Table 2A: Concentration at 50% inhibition (IC50) for exemplary iRNA agents of Table 1A

Duplex ID	IC50 [nM] 1st DRC in H441	IC50 [nM] 2nd DRC in H441
ND8294	0.1949	0.0468
ND8295	0.1011	0.0458
ND8299	0.5986	0.5638
ND8302	0.0144	0.0134
ND8313	0.0315	0.0124
ND8320	0.0796	0.0078
ND8331	0.0213	0.0158
ND8332	0.0205	0.0089
ND8343	0.0523	0.0293
ND8348	0.0156	0.0182
ND8356	0.0241	0.0099
ND8357	0.0054	0.0032
ND8363	0.1186	0.0337
ND8368	0.0487	0.1209
ND8371	0.0811	0.0911
ND8372	0.0584	0.0425
ND8373	0.0066	0.0165
ND8375	0.1176	0.1187
ND8380	0.6817	0.5747
ND8381	0.0037	0.0041
ND8383	0.0275	0.1257
ND8384	0.0357	0.0082
ND8391	0.0260	0.0349
ND8392	0.3831	0.4775
ND8396	0.0023	0.0052
ND8403	0.0808	0.0759

Table 2B: Concentration at 50% inhibition (IC50) and for exemplary iRNA agents of Table 1D

Duplex ID	IC50 [nM] 1st DRC in H441	IC50 [nM] 2nd DRC in H441
ND8441	0.6738	0.8080
ND8443	0.0346	0.0263
ND8449	0.0120	0.0067

ND8450	0.0257	0.0106
ND8451	0.1320	0.0931
ND8453	0.0079	0.0033
ND8489	0.1640	0.1593
ND8496	0.0387	0.0185

Table 2C: % Activity of the exemplary RNAi towards inhibition of alpha-ENaC gene expression in the assays described in Example 3

Duplex identifier	% alpha-ENaC expression in primary HBEC (% of control) 50nM siRNA	Cynomolgous alpha-ENaC expression (% of control) 45nM siRNA
Untransfected	77.2	n/a
Non-targetting Control	100	93.3
Negative Control (Non-cyno alpha-ENaC) ND8449	n/a	100
ND-8302	30.2	57
ND-8332	24.7	54.3
ND-8348	40.1	56.2
ND-8356	36.6	55.8
ND-8357	29.6	50.4
ND-8373	30.4	53.8
ND-8381	32.5	40.4
ND-8396	34.1	46.3
ND-8450	45.9	78.9
ND-8453	30.1	55.3

CLAIMS

1. A composition comprising an iRNA agent for inhibiting the expression of an alpha-ENaC gene, wherein the iRNA agent comprises a sense strand and an antisense strand, wherein:

   nucleotides 2-19 (5’ à 3’) of the antisense strand comprise the nucleobase sequence of nucleotides 2-19 of SEQ ID NO: 1298 (5’ à 3’) (AUUUGUUCUGGUUGCACA);

   the nucleotide at position 1 (5’ à 3’) of the antisense strand forms a base pair with the sense strand;

   the sense strand comprises the nucleobase sequence of nucleotides 1-18 of SEQ ID NO: 1297 (5’ à 3’) (UGUGCAACCAGAACAAAU); and

   the antisense strand, the sense strand, or both strands comprise at least one modified nucleotide and/or at least one phosphate linker modification.

2. The composition of claim 1, wherein the antisense strand of the iRNA agent and the sense strand of the iRNA agent are each 19 to 23 nucleotides in length.

3. The composition of claim 1, wherein the iRNA agent comprises one or more 2′-modified nucleotides and/or one or more phosphorothioates.

4. The composition of claim 3, wherein the one or more 2′-modified nucleotides are independently selected from the group consisting of: 2'-deoxy, 2'-deoxy-2'-fluoro, 2'-O-methyl, 2'-O-methoxyethyl (2'-O-MOE), 2'-O-aminopropyl (2'-O-AP), 2'-O-dimethylaminoethyl (2'-O-DMAOE), 2'-O-dimethylarninopropyl (2'-O-DMAP), 2'-O-dimethylaminoethyloxyethyl (2'-O-DMAEOE), and 2'-O-N-methylacetamido (2'-O-NMA).

5. The composition of claim 3, wherein the sense strand and/or the antisense strand comprises a 3' overhang.

6. The composition of claim 3, wherein the sense strand and/or the antisense strand comprises a 5' overhang.

7. The composition of claim 3, wherein the iRNA agent comprises at least one blunt end.

8. The composition of claim 3, further comprising a buffer, diluent, penetration enhancer, carrier compound, and/or pharmaceutically acceptable carrier or excipient.

9. The composition of claim 3, wherein the composition is presented in unit dosage form.

10. The composition of claim 1, wherein the iRNA agent is ligated to one or more diagnostic compound, reporter group, cross-linking agent, nuclease-resistance conferring moiety, natural or unusual nucleobase, lipophilic molecule, cholesterol, lipid, lectin, steroid, uvaol, hecogenin, diosgenin, terpene, triterpene, sarsasapogenin, Friedelin, epifriedelanol-derivatized lithocholic acid, vitamin, carbohydrate, dextran, pullulan, chitin, chitosan, synthetic carbohydrate, Oligo Lactate 15-mer, natural polymer, low- or medium-molecular weight polymer, inulin, cyclodextrin, hyaluronic acid, protein, protein-binding agent, integrin-targeting molecule, epithelial receptor ligand, polycationic, peptide, polyamine, peptide mimic, and/or transferrin.

11. The composition of claim 3, further comprising one or more known agents effective in treatment of ENaC-related disorders.

12. The composition of claim 11, wherein the known agent is selected from the group consisting of: anti-inflammatory drug, bronchodilatory drug, antihistamine, anti-tussive drug, antibiotic, DNase drug substance, epithelial sodium channel blocker.

13. The composition of claim 3, wherein the iRNA agent comprises:
an antisense strand comprising a nucleotide sequence of nucleotides 1-19 of SEQ ID NO: 1298 and a sense strand comprising a nucleotide sequence of nucleotides 1-19 of SEQ ID NO: 1297.